AN ABSTRACT OF THE THESIS OF

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Craterellus tubaeformis is a small to medium-sized forest mushroom that is fairly common in the Douglas-fir/western hemlock forests of the Pacific Northwestern United States and is most often associated with decayed coarse woody debris. In this study, the mycorrhizae of *Craterellus tubaeformis* in western Oregon is identified by DNA analysis using polymerase chain reaction (PCR) amplification and restriction fragment length polymorphism (RFLP) typing, and the mantle morphology is described. Host associations with western hemlock, Douglas-fir, and Sitka spruce are identified using the same molecular techniques, with *Craterellus tubaeformis* most commonly associated with western hemlock. Differences in genetic sequences and host associations between western North America, eastern North America, and Europe are presented, and the possibility that variants of *Craterellus tubaeformis* from the different geographies might deserve their own species epithets is discussed.

The dependency of *Craterellus tubaeformis* on late seral stands and abundance of coarse woody debris was quantified by surveying 64 plots in the Coast and Cascade ranges of western Oregon. Logistic regression showed that the odds of *Craterellus tubaeformis* occurrence increased with stand age and coarse woody debris (CWD) volumes, however it is often found in younger stands. The likelihood of *Craterellus tubaeformis* occurrence in a stand was highly correlated to the presence of western hemlock. Linear regression analysis showed no significant relationships between stand age, CWD volume, slope, elevation, or aspect on *Craterellus tubaeformis* biomass productivity, though well-decayed CWD was the substrate for 88% of the collected biomass. The presence of *Hydnum spp.* was found to be a highly significant indicator for the presence of *Craterellus tubaeformis*.

Ecology of *Craterellus tubaeformis* in Western Oregon

by

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Ecology of *Craterellus tubaeformis* in Western Oregon: An Introduction

INTRODUCTION

Basis for research

Craterellus tubaeformis (Fries) Quélet (Basidiomycota, Cantharellales, Cantharellaceae) is a small to medium-sized forest mushroom that is fairly common in the *Tsuga heterophylla* (western hemlock) zone (Franklin and Dyrness 1973) of the Pacific Northwestern United States. Synonyms have included *Cantharellus tubaeformis* Fr. and *Cantharellus infundibuliformis* Fr. (Petersen 1979). It was listed for management under the Northwest Forest Plan Record of Decision (ROD) (U.S.D.A. et al., 1994) based on evidence that it required late seral stands with an abundance of well-decayed ('legacy') coarse woody debris (CWD), and also because of anticipated harvest pressure (W. Denison, pers. comm.). *Cr. tubaeformis* is suspected to be mycorrhizal, meaning that it derives its energy from symbiotic relationships with the roots of host trees rather than from decomposing forest floor detritus.

In the January 2001 amended ROD (U.S.D.A. et al., 2001) it was redesignated as a Category D Survey and Manage species. The goal for Category D Survey and Manage species is to "Identify and manage high priority sites to provide for a reasonable assurance of species persistence." Prerequisite to identifying high priority sites is an understanding of the key habitat features that are significant to reasonable assurance of *Craterellus tubaeformis* persistence.

Some organisms are thought to be effective indicators of ecosystem health. Notable examples are the northern spotted owl (*Strix occidentalis caurina*) (Forsman et al., 1984) and Pacific Northwest salmon (*Oncorhynchus spp.*) (Cone 1995). These are organisms whose health and population status is thought to be a reflection of the health of the ecosystems they live in. Fungal species such as *Craterellus tubaeformis* that may be

dependant on certain aspects of an ecosystem, such as late seral structure, volume of CWD, and stand diversity could also be useful indicators of ecosystem health.

Considering that *Craterellus tubaeformis* is an often encountered and well known mushroom, there are surprisingly few published papers on its ecology, trophic status, or habitat requirements. The first objective of this research was to conclusively determine whether *Cr. tubaeformis* in western Oregon is mycorrhizal, and if so to describe the mycorrhizal morphology and identify primary hosts. The second objective was to examine the assumptions underlying its listing in the ROD by testing the influences of stand age and the volume of well-decayed CWD on the probability of occurrence and the biomass productivity of *Cr. tubaeformis*.

Generic-level taxonomy

The confusion about generic and specific taxonomy of *Craterellus tubaeformis* predates its formal description by Fries in 1821 (Scopoli 1772, Bulliard 1791) and is detailed by Corner (1966), Donk (1969), Petersen (1979), and Dahlman et al. (2000). *Cr. tubaeformis* has the stature and hollow stipe that are characteristic of *Craterellus* and the hyphal clamp connections characteristic of *Cantharellus*. Fries' (1821) placement of it in the genus *Cantharellus* was based primarily on its coloration and decurrent lamellar hymenium. Fries (1821) did not mention the presence of clamp connections, and when he described *Cantharellus tubaeformis* the genus *Craterellus* had yet to be proposed (Persoon 1825). In his proposal of the new genus, Persoon (1825) described the hymenophore of members of genus *Craterellus* as "...nec distincte venosus" and did not suggest the inclusion of *Cantharellus tubaeformis*.

Cantharellus sect. *Leptocantharellus* was proposed by C. H. Peck (1887) to accommodate "thin-chanterelles", those fungi with the stature and hollow stipe of *Craterellus* and the lamellae and clamp connections of *Cantharellus*. Quélet (1888) was the first (and only, for over a century) to suggest that *Cantharellus tubaeformis* should be moved to the genus *Craterellus*. His proposal was not widely accepted, perhaps in part because he also suggested moving *Cantharellus cibarius* to *Craterellus* in the same publication. It wasn't until 1997 that phylogenetic analyses by Feibelman et al. (1997)

indicated that *Cantharellus tubaeformis* was more closely related to members of the genus *Craterellus* than to other members of *Cantharellus*. They noted that the morphological characteristics of shape and texture were more congruent with phylogenetic placements than the presence or absence of clamp connections. Further phylogenetic analyses of relationships among homobasidiomycetes (Pine et al., 1999) and among *Cantharellaceae* (Dahlman et al., 2000) agreed with the findings of Feibelman et al. (1997). Dahlman et al. (2000) confirmed the placement of *Cantharellus tubaeformis* in *Craterellus*, affirming Quélet's 1888 opinion.

Species-level taxonomy

Fries described *Cantharellus tubaeformis* in 1821 and *Cantharellus infundibuliformis* in 1838, and there has been debate ever since over whether they really were two separate species. Fries (1838) made fine distinctions based on spore color, hymenial coloration, spore width, and stipe consistency, which many have since concluded are within the range of intraspecific variation (Donk 1969, Petersen 1979). Konrad (1929) was the first mycologist to suggest that Fries described the same organism under two different names, and that *C. tubaeformis* was the correct and precedent name. Donk (1969) and Petersen (1979) proposed synonomizing many historic names with *Cantharellus tubaeformis* (*Cantharellus infundibuliformis*, *Merulius tubaeformis*, *M. infundibuliformis*, *M. villosus, Agaricus infundibuliformis*, and *Helvella tubaeformis*), though Petersen (1979) offered the interesting caveat that *C. tubaeformis* and *C. infundibuliformis* are "quite distinct" in Scandinavia. Petersen (1979) continues, "...in the moist forests of western North America, these two taxa are not distinct or easily separable, and do not even form poles of a single taxonomic continuum." Dahlman et al. (2000) formally proposed the synonymy of *C. infundibuliformis* with *C. tubaeformis*.

Geographic variation

According to phylogenetic analyses by Feibelman (1994) and Dahlman et al. (2000), the variant of *Craterellus tubaeformis* in eastern North America seems more closely related to the European variant than to the Pacific Northwestern variant. Published descriptions (Fries 1821 and 1838, Corner 1966, Donk 1969, Petersen 1979) report no striking morphological differences between *Cr. tubaeformis* collections from these geographic

regions beyond what might be expected from within-species variability. The apparent difference in host association may be an artifact of the different host species available for colonization in the different regions or it may be that the regional variants of *Cr. tubaeformis* have evolved to fill specific niches, congruent with Harley and Smith's (1983) concept of "ecological specificity." Bills et al. (1986) report *Cr. tubaeformis* in monoculture red spruce in eastern North America, but not in mixed hardwood stands (*Acer, Betula, Fagus, Fraxinus, Ilex, Prunus, Quercus,* and *Sorbus spp.*). Many of these hardwood genera are the same ones reported as mycorrhizal associates of *Cr. tubaeformis* in Europe. Few data have been published on the host associations of *Cr. tubaeformis* in eastern North America, so the full range of possible hosts and habitats in that region is unknown.

This research will not address all of the unknown aspects of *Craterellus tubaeformis* ecology, but will hopefully represent a step toward understanding this one organism. Its importance to the overall ecological functioning of western Oregon coniferous forests may be great or small, but as Aldo Leopold said, "The first rule of intelligent tinkering is to keep all of the pieces."

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Mycorrhizae and Host Associations of *Craterellus tubaeformis* in Western Oregon

ABSTRACT

In this paper the mycorrhizae of *Craterellus tubaeformis* in western Oregon is identified by DNA analysis using polymerase chain reaction (PCR) amplification and restriction fragment length polymorphism (RFLP) typing, and the mantle morphology is described. Host associations with western hemlock, Douglas-fir, and Sitka spruce are identified using the same molecular techniques, with *Cr. tubaeformis* most commonly associated with western hemlock. Differences in genetic sequences and host associations between western North America, eastern North America, and Europe are presented, and the possibility that variants of *Cr. tubaeformis* from the different geographies might deserve their own species epithets is discussed.

INTRODUCTION

Craterellus tubaeformis (Fries) Quélet (Basidiomycota, Cantharellales, Cantharellaceae) is a small to medium-sized forest mushroom in the Pacific Northwest that often grows on or near well-decayed coarse woody debris (CWD) (Fogel et al., 1973, Smith and Morse 1947). Synonyms include *Cantharellus tubaeformis* Fr. and *Cantharellus infundibuliformis* Fr. (Dahlman et al., 2000). In this paper the mycorrhizae of *Cr. tubaeformis* in western Oregon are identified by RFLP analysis, their mantle morphology is described, and several host associates are identified.

Kårén et al. (1997) identified *Craterellus tubaeformis* mycorrhizae in Sweden by molecular analysis, but the RFLP patterns they obtained differed from those of Oregon basidiomata. Their study was conducted in *Pinus sylvestris* L. and *Picea abies* (L.) Karst. stands, species not naturally found in western Oregon. *Cr. tubaeformis* in Scandinavia also do not seem to associate with CWD as strongly as they do in western Oregon (Persson 1997, Gru Gulden and Eric Danell, pers. comms.).

Many papers have assumed that *Cr. tubaeformis* was mycorrhizal without benefit of DNA analysis, and have described mycorrhizal associations based on stand composition. In Europe, reported associates have included *Fagus sylvatica* L. (Peyronel 1922, Kalmár 1950, Becker 1956, Gorova 1980, Tyler 1985, Hansen and Knudsen 1997, Persson 1997), *Picea abies* (Romell 1938, Becker 1956, Kraft 1978, Gorova 1980, Wästerlund and Ingelög 1981, Hansen and Knudsen 1997), *Pinus sylvestris* (Kreisel 1957, Wästerlund and Ingelög 1981, Agerer 1985, Högberg et al., 1999), *Picea sitchensis* (Bong.) Carr. (Alexander and Watling 1987), and *Abies alba* Miller and *Quercus spp.* (Becker 1956).

In the Pacific Northwestern United States and western Canada, *Tsuga heterophylla* (Raf.) Sarg. (western hemlock) has been a suspected mycorrhizal symbiont (Kropp 1981, Kropp and Trappe 1982), and *Tsuga mertensiana* (Bong.) Carr. (mountain hemlock) has also been suggested (Kropp and Trappe 1982). Trappe (1962) and Molina et al. (1992) speculated that *Craterellus tubaeformis* might have a very broad host range. In West Virginia *Cr. tubaeformis* has been reported in stands of monoculture *Picea rubens* Sarg. (Bills et al., 1986).

MATERIALS AND METHODS

Process overview

DNA was extracted from the root tips and amplified with fungus-specific PCR primers. The amplified DNA was subjected to restriction enzymes and the resultant RFLP patterns were compared to those of *Craterellus tubaeformis* basidiomata. If the RFLP pattern from a mycorrhizal tip matched that of *Cr. tubaeformis* basidiomata, *Cr. tubaeformis* is evidenced as mycorrhizal (Gardes et al., 1990, Egger and Fortin 1990). Host species of the *Cr. tubaeformis* mycorrhizae were also determined by the same process with different PCR primers specific to plants. No DNA probes are required for this process.

Root sample collection

Root samples were collected from stands in the Oregon Coast Ranges and the Cascade Range from beneath *Craterellus tubaeformis* basidiomata. Most source stands were of the *Tsuga heterophylla* (Raf.) Sarg. type as described by Franklin and Dyrness (1973),

however some collections were made in *Pseudotsuga menziesii* (Mirb.) Franco (Douglas-fir) or *Picea sitchensis* (Bong.) Carr. (Sitka spruce) stands with little or no western hemlock component.

Samples were collected by excavating approximately one liter of soil from beneath *Craterellus tubaeformis* colonies. The soil collections were washed with an elutriator (Eberhart et al., 1996) resulting in clean, intact root systems. Subsamples (~100 ml) of the tips of root systems were assayed for mycorrhizae with a stereo microscope. Mycorrhizas from each subsample were sorted by morphotype, briefly described, and vouchered in CTAB buffer (Gardes and Bruns 1996). A total of 312 vouchers were prepared for DNA extraction.

DNA extraction

DNA was extracted by the methods described by Gardes and Bruns (1993 and 1996). Ten to 15 mg of each mycorrhiza type were removed for DNA analysis. Each mycorrhiza was placed in a 1.5 ml Eppendorf tube with 300 µl of CTAB buffer. Samples were subjected to three 5 minute cycles of freezing with dry ice and incubating at 65° C using a VWR Standard Heat block to break cell walls. Samples were then ground with highspeed sterile micropestles, incubated for another hour at 65° C to further degrade cell walls, mixed with chloroform, vortexed (Cole-Parmer Touch Mixer), and centrifuged at 13,000 RPM for 15 minutes (Haereus Instruments Biofuge Pico microfuge). After centrifuging, the supernatant was carefully drawn off and transferred to new tubes and the residue discarded. The supernatant (containing both plant and fungal DNA) was precipitated with cold isopropanol, vortexed, and centrifuged again for 15 minutes. The supernatant was again drawn off, placed in new tubes, and rinsed in cold (-20° C) ethanol overnight. After the ethanol rinse, samples were centrifuged again to pellet the DNA in the bottom of the tube. Most of the ethanol was poured off and the residual ethanol removed by 30 minutes in a vacuum dryer (Savant Universal). The DNA extract was suspended in TE buffer (Gardes and Bruns 1996) and stored at -20° C until ready for PCR amplification.

Dilutions and PCR amplification

The PCR process and ingredients generally followed the protocols established by White et al. (1990), and Gardes and Bruns (1996). Ten µl of each sample of DNA extract (suspended in TE buffer) were placed into a new .5 ml Eppendorf tube and diluted to 1:10 with 90 µl of double filtered and autoclaved dH_20 ('PCR water'). A PCR 'cocktail' was then mixed in an autoclaved 1.5 ml Eppendorf tube. The PCR cocktail consisted of PCR water (56 parts), dNTP (nucleotide triphosphates; 20 parts), polymerase buffer (20 parts), TAQ polymerase (1 part), and two primers specific to the ITS/5.8S region of fungal nuclear rDNA (primers ITS1F and ITS4, 2 parts each). The ITS/5.8S region of the nuclear rDNA is useful in species-level identifications (White et al., 1990, Gardes and Bruns 1993, Erland et al., 1994, Cullings and Vogler 1998), and the PCR process isolates and multiplies that specific section of the DNA. Another primer often coupled with ITS1F is ITS4B. ITS4B is specific to many basidiomycetes but does not amplify *Craterellus tubaeformis* DNA.

Eppendorf tubes containing 12.5 µl of each DNA dilution and 12.5 µl of the PCR cocktail were vortexed, centrifuged briefly, and placed in the PCR thermal cycling machine (MJ Research PTC-100). One tube of known good DNA extract dilution was also mixed with the same PCR cocktail and run in the PCR machine as a positive control in the PCR process and one tube of dH_20 was run as a negative control. The PCR cycling program used for this study heated the PCR cocktail/DNA dilution mixes to 94° C for 30 seconds, then repeated the following temperature cycles 35 times: 93° C for 35 seconds, 55° C for 53 seconds, and 72° C for 30 seconds. Success of the amplification was checked by running 5 µl samples of the PCR product through a 2.5% DNA grade agarose gel in an electrophoresis bath.

Electrophoresis

The electrophoresis procedures generally followed Sambrook et al. (1989). Gels were created by pouring 25 ml of molten 2.5% DNA grade agarose onto a 8.3 x 10.2 cm glass plate, with wells formed along one edge of the gel by a 24 tooth comb (Owl Scientific Plastics). The first well in each gel was reserved for Lambda marker (Promega) to provide a reference pattern of various base pair sizes. Every fifth well was marked with

dye (Promega Loading Dye) to prevent sample loading errors. Wells were topped off with TBE buffer (Sambrook et al., 1989) and the electrophoresis bath filled with TBE buffer such that the gel edges contacted the buffer on all sides. DC voltage was applied through the buffer (~100 volts potential) to pull the PCR product and Lambda marker through the gel from the cathode toward the anode. As the DNA moved from the wells into the gel (as indicated by the progress of the marker dye), more TBE buffer was added to completely submerge the gel.

When the PCR product had advanced through the gel enough that band resolution was possible, the gel was removed from the gel rig and glass plate and submersed in an ethidium bromide bath for ten minutes on a Thermolyne Rotomixer at ~40 cycles per minute. Ethidium bromide infiltrates the DNA molecules and fluoresces under ultraviolet light (Sambrook et al., 1989). After the ethidium bath, the gel was moved to a dH_20 rinse for another ten minutes, also using the Thermolyne Rotomixer.

After rinsing, the gel was placed in a light cabinet (Alpha Innotech Co.) where it was exposed to UV light. The image of the gel was detected by a video camera mounted on top of the light cabinet and displayed on a computer screen by use of Alpha-Ease software (Alpha Innotech Co.). A distinct band of DNA appeared in each lane where the PCR amplification succeeded. PCR amplifications sometimes failed due to residual proteins in the extraction. In these cases, the PCR process was repeated at different dilution levels (1:100, 1:1000, etc.) (Gardes and Bruns 1996). By trying different dilutions, most samples were amplified successfully.

RFLP analysis

The RFLP process uses enzymes to cut the amplified DNA into pieces at specific points (Gardes and Bruns 1996). This results in groups of DNA fragment sizes which appear on an electrophoresis gel as one or more distinct bands. The band patterns generated by this method are useful for identifying species associations between samples. In some species, this process may even discriminate between individuals or subtypes (Dahlberg and Stenlid 1990), but this does not appear to be the case with western Oregon *Craterellus tubaeformis*. All *Cr. tubaeformis* sporocarps collected in western Oregon and used for

DNA reference had identical RFLP patterns (n=26). Subjecting the same samples of PCR product to several different RFLP enzymes provides cross-confirmation of results as different enzymes will produce different patterns from the same sample. If the patterns from several different enzymes match between mycorrhizae and basidiomata it is extremely likely that they are of the same species (Gardes and Bruns 1996). The enzymes used were HinF, DpnII, AluI, and HaeIII (New England Biolabs).

For RFLP analysis, each enzyme was mixed into a cocktail with its own specific buffers per manufacturers instructions. Seven ml of each enzyme cocktail was aliquoted into a separate group of clean tubes and 8 ml of the amplified PCR product then added. The reactions were vortexed, briefly centrifuged, and placed in a 37°C incubator for 3 hours. During incubation, the enzymes digest the DNA molecules in the PCR product, cutting the DNA into fragments at sites determined by specific sequences of nucleotides (for HinF, the sequence is 3'G'ANT C5'; for DpnII 3'GmeA'TC5'; for AluI 3'AG'CT5', and for HaeIII 3'GG'CC5') (McClelland et al., 1994). The resulting DNA fragment lengths are unique to the interaction between the RFLP enzymes and the DNA of the subject species (Rogers et al., 1989, Gardes et al., 1990, Gardes and Bruns 1996).

After incubation, samples were run on an agarose gel in a process similar to that described in the PCR section above. The differences when running the RFLP gel are that a thinner 1%/2% RFLP-grade agarose gel (Sambrook et al., 1989) is used and the runout time is increased to about 2 hours. The smallest fragments were allowed to run almost to the edge of the gel (80-90 mm), allowing the different size DNA fragments to separate and increase resolution. A 100 base pair (bp) ladder solution (Promega) was loaded into the first well (instead of the Lambda marker used with PCR gels), permitting close estimation of the fragment sizes of the target samples. The smaller fragments of DNA are pulled through the gel faster that the larger fragments, separating into distinct band patterns. After electrophoresis was complete, the gel was stained in ethidium bromide, rinsed, and exposed with ultraviolet light as described above. The resultant RFLP band patterns were compared to those of *Craterellus tubaeformis* sporocarps for matches by use of Alpha-Imager fragment length scoring software.

Host identification

If the mycorrhizal symbiont on a root tip was identified as *Craterellus tubaeformis*, the DNA extract from that root tip would again be subjected to the same PCR/RFLP process as above, except that PCR primers 28C and 28KJ are used. These primers amplify a part of the 28S LSU rDNA gene useful in the identification of many plants, including most Pacific Northwest conifers (Cullings 1992). This allows positive identification of the tree species of a mycorrhizal root with the same DNA extract used for the fungal identification.

RESULTS

RFLP pattern matching

RFLP band pattern matches were discovered between *Craterellus tubaeformis* basidiomata tissue and the mycorrhizae of 25 root tips, indicating *Cr. tubaeformis* as the mycobiont. Positive matches between mycorrhizae and *Cr. tubaeformis* basidiomata were confirmed with four RFLP restriction enzymes: HinF, AluI, DpnII, and HaeIII. The RFLP band sizes (in bp) with these enzymes were: HinF 105, 135, 440; AluI 160, 380; DpnII 255, 470; and HaeIII 760. There is no restriction site for the HaeIII enzyme, so this single 760 bp band represents the size of the entire ITS/5.8S region of *Cr. tubaeformis* rDNA. The mycorrhizal host of every *Cr. tubaeformis*-colonized root tip collected on the strip plots was western hemlock.

Description of Craterellus tubaeformis/Tsuga heterophylla mycorrhizae

By stereomicroscopy, *Tsuga heterophylla* root tips colonized by *Craterellus tubaeformis* are peach orange (apices often lighter colored) to yellow-brown in age, with a loosely felty texture and short, emanating cottony, hyaline hyphae. The mycorrhizae are monopodial pinnate; 4.1 (1.9-23.8) mm long; main axis .6 (0.5-0.7) mm wide; tips straight, 2.7 (1.4-3.8) x .5 (0.4-0.6) mm; apices .3 (.2-.4) mm wide. *Cr. tubaeformis* mycorrhizae frequently share root systems with *Cenococcum geophilum* Fries.

Under a compound microscope, the outer mantle is a felty prosenchyma without ornamentation, gelatinous or other matrix materials; hyphal cells are 89 (76-108) x 4.2

(2.7-5.4) μm, hyaline, smooth, with clear contents; oil-like droplets common; septa common, with clamp connections; hyphal branches common, junction angles 30° to 90°; Hartig net present, contact anastomoses rare. The inner mantle is a noninterlocking, irregular to net-like synenchyma; cells 16.8 (6.3-28.0) x 8.4 (3.5-12.6) μm; cells hyaline with clear oil-like contents; septa common, clamp connections lacking. Emanating hyphae are common, hyaline, cottony, with clamp connections, without ornamentation, appearing as loose extensions of the outer mantle, the cells 95 (43-144) x 2.9 (1.8-5.4) μm. Cystidia were not observed. *Craterellus tubaeformis* mycorrhizae tested negative to KOH and Melzer's reagent. The mycorrhizae is further illustrated in Trappe et al. (2000).

Craterellus tubaeformis host associations

Craterellus tubaeformis was documented by field observation in 69 of 92 mixed western hemlock/Douglas-fir stands studied, even when the western hemlock component was minimal. All *Cr. tubaeformis* host rootlets that amplified successfully from those stands were western hemlock; none were Douglas-fir. These results initially seemed to indicate that in the Pacific Northwest *Cr. tubaeformis* might be mycorrhizal with western hemlock but not with Douglas-fir.

Craterellus tubaeformis has been reported in Douglas-fir/*Libocedrus decurrens* Torr. (incense cedar) stands in southern Oregon entirely lacking western hemlock (Dan Luoma, pers. comm.) Field surveying of 25 monoculture Douglas-fir stands in western Oregon revealed populations of *Cr. tubaeformis* in two of them, and its mycorrhizal association with Douglas-fir was confirmed by PCR/RFLP analysis. The overall odds of locating *Cr. tubaeformis* in a stand with western hemlock was 6.25 times greater than in a stand without western hemlock (chi-square, p=.017). Association of *Cr. tubaeformis* with Douglas-fir when western hemlock is available has yet to be demonstrated. These data suggest that *Cr. tubaeformis* in western Oregon can colonize Douglas-fir but it may only do so in the absence of western hemlock, and occurrence of *Cr. tubaeformis* in Douglas-fir stands lacking western hemlock is infrequent.

Alexander and Watling (1987) report *Craterellus tubaeformis* in monoculture Sitka spruce plantations in Scotland. They speculated that because Sitka spruce was introduced to Europe by seed rather than by transplants, the mycorrhizal flora (including *Cr. tubaeformis*) of Sitka spruce in Scotland may have migrated from native relict *Betula* or *Pinus* stands. In contrast to the usual substrate of *Cr. tubaeformis* in western Oregon (CWD), they report that their collections were "...mostly on mineral soil."

On the Oregon coast Sitka spruce is usually interspersed with western hemlock, but some pure Sitka spruce stands occur in exposed coastal locations (Franklin and Dyrness 1973, Roche and Haddock 1987). Nineteen Sitka spruce stands between Florence and Lincoln City were surveyed for the presence of *Craterellus tubaeformis* during January and February 2001. Seven of these stands had no western hemlock component and in these stands *Cr. tubaeformis* was not observed. The other 12 stands were mixed hemlock/Sitka spruce, and *Cr. tubaeformis* were documented in eight of them. *Cr. tubaeformis* mycorrhizae was confirmed on several Sitka spruce roots at one site, a ~50 m diameter pocket of pure spruce in an otherwise mixed spruce/hemlock stand near Carter Lake, Oregon.

These data show that *Craterellus tubaeformis* can form mycorrhizae with Sitka spruce, but in the course of this study were never observed in a Sitka spruce stand without some western hemlock nearby. This contrasts with Douglas-fir, where the only confirmed *Cr. tubaeformis* mycorrhizae on Douglas-fir roots occurred in pure Douglas-fir stands. It is possible that the western hemlock provides a launching point for colonization of spruce by *Cr. tubaeformis*, and that the pure Sitka spruce stand may lack some element conducive to *Cr. tubaeformis* establishment. The sample size of pure Sitka spruce stands is small however, and more research on *Cr. tubaeformis* in the spruce forests of the Pacific Northwest coast is needed.

DISCUSSION

Speciation

According to phylogenetic analyses by Feibelman (1994) and Dahlman et al. (2000), the variant of *Craterellus tubaeformis* in eastern North America seems more closely related to the European variant than to the Pacific Northwestern variant. Published descriptions (Fries 1821 and 1838, Corner 1966, Donk 1969, Petersen 1979) report no striking morphological differences between *Cr. tubaeformis* collections from these geographic regions beyond what might be expected from within-species variability. The apparent difference in host association may be an artifact of the different host species available for colonization in the different regions or it may be that the regional variants of *Cr. tubaeformis* have evolved to fill specific niches, congruent with Harley and Smith's (1983) concept of "ecological specificity." Bills et al. (1986) report *Cr. tubaeformis* in monoculture red spruce in eastern North America, but not in mixed hardwood stands (*Acer, Betula, Fagus, Fraxinus, Ilex, Prunus, Quercus,* and *Sorbus spp.*). Many of these hardwood genera are the same ones reported as mycorrhizal associates of *Cr. tubaeformis* in Europe. Few data have been published on the host associations of *Cr. tubaeformis* in eastern North America, so the full range of possible hosts and habitats in that region is unknown.

Kårén et al. (1997) used RFLP with the same PCR primers and some of the same restriction enzymes to identify *Craterellus tubaeformis* mycorrhizae on roots in Sweden. The DNA fragment sizes they reported are different from those produced by basidiomata and mycorrhizae in Oregon. Using the HinF enzyme, they produced fragment sizes of 148, 158, and 307 bp, and with the Mbo1 enzyme (Mbo1 is an isoschizomer of DpnII; McClelland et al., 1994) their fragment sizes were 188, 199, and 255 bp. From Oregon basidiomata the fragment sizes from the HinF enzyme were 105, 135, and 440 bp, and with the DpnII enzyme 255 and 470.

Feibelman et al. (1994) found an 80 bp difference in the length of the ITS region between a German and Californian *Craterellus tubaeformis*. Sequencing of the ITS region of the rDNA from *Cr. tubaeformis* performed by the author found the Oregon variant has an

ITS region 90 bp longer than that of samples from Sweden. Comparison of the sequences shows that the 65% of this difference is a result of three insertion events. The sequences have been submitted to GenBank (GenBank accession numbers AF385632 and AF385633). These insertions could explain the differences in RFLP fragment sizes in the ITS region between European and North American specimens, but they may not translate to any distinguishable morphological differences.

Whether the Pacific Northwestern variant of *Craterellus tubaeformis* deserves its own species designation remains a murky question, as discussed by Petersen and Hughes (1999), "...requisite in this process is a hiatus in gene exchange...allow(ing) each group to accumulate genetic differences..." If the variants of *Cr. tubaeformis* are genetically isolated to the extent that they have evolved different host compatibility matrices, they would probably qualify as distinctly different species with their own epithets (Mayr 1970, Carson 1985, Brasier 1997). If *Cr. tubaeformis* is broadly compatible with a wide range of hosts and is morphologically indistinguishable between western North America and Europe, it is arguable whether differences in the ITS region justify a new species designation. More data on host and habitat associations are needed, and a comprehensive phylogenetic analysis of the *Cr. tubaeformis* complex is prerequisite to reaching conclusions on the speciation of geographic *Cr. tubaeformis* variants.

Summary and conclusions

The western Oregon variety of *Craterellus tubaeformis* is mycorrhizal and requires western hemlock to thrive. While *Cr. tubaeformis* can occasionally be found in monoculture Douglas-fir stands, these situations are relatively rare. *Cr. tubaeformis* can form mycorrhizae with Sitka spruce but has never been observed in Sitka spruce stands without a western hemlock component. The substrate and host associations of *Cr. tubaeformis* are different between the Pacific Northwestern United States, the eastern United States, and Europe. More research is needed before conclusions about differences in host associations and specificity can be drawn. Currently molecular data is incomplete, but suggests that there are differences in the genotypes between regions.

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Importance of Stand Age and Coarse Woody Debris to *Craterellus tubaeformis* Occurrence and Productivity in the *Tsuga heterophylla* Zone of the Western Oregon Cascade and Coast Ranges

ABSTRACT

Craterellus tubaeformis is a small to medium-sized forest mushroom that is fairly common in the Douglas-fir/western hemlock forests of the Pacific Northwestern United States, and thought to be associated with decayed coarse woody debris (CWD) and late seral forests. This study examined the habitat requirements of *Cr. tubaeformis* by surveying 64 plots in the Coast and Cascade ranges of western Oregon. Logistic regression showed that the odds of *Cr. tubaeformis* occurrence increased with stand age and CWD volumes, although it is often found in younger stands. The likelihood of *Cr. tubaeformis* occurrence in a stand was highly correlated to the presence of western hemlock. Linear regression analysis showed no significant relationships between stand age, CWD volume, slope, elevation, or aspect on *Cr. tubaeformis* biomass productivity, although well-decayed CWD was the substrate for 88% of the collected biomass. The presence of *Hydnum spp.* was a significant indicator for *Cr. tubaeformis*.

INTRODUCTION

Craterellus tubaeformis (Fries) Quélet (Basidiomycota, Cantharellales, Cantharellaceae) is a small to medium-sized forest mushroom that is fairly common in the *Tsuga heterophylla* zone (Franklin and Dyrness 1973) of the Pacific Northwestern United States. Synonyms include *Cantharellus tubaeformis* Fr. and *Cantharellus infundibuliformis* Fr. (Petersen 1979). It was listed for management under the Northwest Forest Plan Record of Decision (ROD) (U.S.D.A. et al., 1994) based on evidence that it required late seral stands with an abundance of well-decayed ('legacy') coarse woody debris (CWD), and also because of anticipated harvest pressure (Bill Denison, pers. comm.). In the January 2001 amended ROD (U.S.D.A. et al., 2001) it was redesignated as a Category D Survey and Manage species. The goal for Category D Survey and Manage species is to "Identify and manage high priority sites to provide for a reasonable assurance of species persistence." Prerequisite to identifying high priority sites is an

understanding of the key habitat features that contribute to persistence. Fungal species such as *Cr. tubaeformis* that might be dependant on certain aspects of an ecosystem, such as seral stage, volume of CWD, and tree biodiversity could serve as useful indicators of ecosystem health. The objective of this research was to examine the assumptions underlying its listing in the ROD by testing the influences of stand age and the volume of well-decayed CWD on the probability of occurrence and the biomass productivity of *Cr. tubaeformis*.

MATERIALS AND METHODS

Experimental design overview

The experiment was designed as a 2^2 factorial with stand age (years) and coarse woody debris (CWD) volume (m^3 m^{-2}) as explanatory variables. Sixty-four stands in the Oregon Coast and western Cascade Ranges were selected for survey plots, balancing the site characteristics of early and late seral stages with high and low CWD volumes. One 700 m^2 strip plot was established in each selected stand, and the plots were surveyed for the presence of *Craterellus tubaeformis* 6 times; every 3 to 4 weeks from September 1999 to April 2000, and once during February-March 2001. The occurrence and biomass of *Cr. tubaeformis* was recorded along with substrate and other habitat data.

Coarse woody debris classification

Coarse woody debris (pieces >10 cm in diameter) were classified by the system proposed in Fogel et al. (1973). The system rates CWD by its level of decay on a scale of 1 to 5. Class 1 CWD is freshly fallen wood with twigs and bark intact, class 2 has mostly intact bark but fine limbs are gone, and in class 3 the bark is sloughing off and the sapwood is softening, but the heartwood is not permeated by roots. In class 4 CWD the bark is mostly gone, the sapwood is punky, and the heartwood is being invaded by roots. Branch stubs can be pulled from class 4 CWD but not from class 3 CWD (Sollins 1982). In class 5 CWD the heartwood and sapwood are punky, cubical-rotted, and permeated by roots, and the entire structure is settling. Over 88% of *Craterellus tubaeformis* collections in this study used class 4 or 5 CWD as substrate; less than 1% used class 1-3 CWD. Thus,

in this paper the term 'CWD' will refer to decay classes 4 and 5 unless otherwise specified.

Stand selection

Thirty-two stands in the Oregon Coast ranges and 32 stands in the western Oregon Cascade ranges were chosen for study. Within each of those regions stands were selected to create a balanced mix of the explanatory variables, e.g. an equal number of early and late seral stands combined with high and low levels of CWD. Mean CWD levels differed between the Coast and Cascade ranges; in the Coast Range the mean level of CWD was .034 m^3 m^{-2} and in the Cascade ranges it was .060 m^3 m^{-2}. In the Coast Range 16 stands were below the mean and 16 stands above, and in the Cascade Ranges 14 stands were below the mean and 18 stands above. In each of the Coast and Cascade ranges 16 stands were late seral and 16 were early seral. The mean age of early seral stands was 48 years (30-97), and of late seral stands 358 years (128-650). This mix of stands with varying combinations of seral stages and CWD levels was designed to facilitate separation of their explanatory effects. All stands were below 1000 meters in elevation for winter accessibility.

In the Oregon Coast ranges, research stands were located at Honey Grove (N44°23', W123°34'), on Flat Mountain (N44°27', W123°28'), in the Corvallis watershed (N44°31', W123°31'), on the north slope of Marys Peak (N44°32', W123°32'), and in the Van Duzer Scenic Corridor (N45°03', W123°45'). In the Oregon Cascade ranges, stands were located near Detroit (N44°44', W122°05'), on Moose Mountain (N44°24', W122°26'), off Gordon Road (N44°23', W122°22'), at Soda Creek (N44°25', W122°16'), in the H.J. Andrews Experimental Forest (N44°14', W122°14'), at Slide Creek (N44°04', W122°13'), and at Hidden Lake (N44°01', W122°14').

Strip plot layout

One strip plot 70 meters long and 10 meters wide was established in each selected stand to create a uniform area for linear regression analysis of productivity data. Strip plots were placed by a constrained randomized bearing that avoided influential factors such as streams, roads, cliffs, or deep windthrow. This 700 m^2 sample area was 87% effective at

representing the presence of *Craterellus tubaeformis* (in only 12 of 92 site visits where *Cr. tubaeformis* was located somewhere in the stand was it not represented on the plot). Data on the elevation, slope, and aspect were recorded upon plot layout.

Volume of coarse woody debris

Line intercept sampling (van Wagner 1968, Harmon and Sexton 1996) was used to quantify CWD volume per unit area, measured in cubic meters of CWD per square meter of forest floor ($m^3\ m^{-2}$). A 200 meter CWD sampling transect was established in each stand, and each piece of CWD crossed by the transect was inventoried by decay class and diameter. Transects were set up as either a 200 meter line, two perpendicular 100 meter lines, or as an equilateral triangle with 67 meter sides, depending on the size and geographic constraints of the stand. The formula used to calculate CWD volume (V) was:

$$V = \pi^2 \Sigma (d^2/8L)$$

where d^2 is the squared diameter of each piece of CWD (m) intersected by the transect and L is the transect length (m).

Seral stage

Seral stage was determined by stand structure and the age of the predominant cohort. Stand ages ranged from 30 to 650 years and were determined either by documented stand histories or increment boring. When a borer was used, the predominant cohort of trees was sampled. If the bole radius of a sampled tree was greater than the 45 cm length of the increment borer the age was extrapolated from the rings at the pith end of the core.

Seral stage was used to categorize stands in the selection process but stand age was used as the nominal variable in the data analysis. The oldest 'early seral' stand was a 97 year-old stand predominated by even-age *Tsuga heterophylla* (Raf.) Sarg. (western hemlock). The youngest 'late seral' stand had a 128 year-old predominant cohort of *Pseudotsuga menziesii* (Mirb.) Franco. (Douglas-fir) and western hemlock, with occasional larger trees and a mixed species mid-story.

Elimination of stands lacking western hemlock

Craterellus tubaeformis will occasionally form mycorrhizae with Douglas-fir but it is rare in stands without any western hemlock component. During the entire course of this study, only one *Cr. tubaeformis* basidiocarp was recorded among the seven stands lacking western hemlock. Fisher's Exact Test indicated that the odds of finding *Cr. tubaeformis* in a stand without hemlock is significantly lower than for those when western hemlock is present (p=.012), and as a result all seven stands lacking western hemlock were excluded from data analysis. All seven of these stands were in the Coast Ranges and had low levels of CWD; four of them were late seral stands and three of them were early seral.

Correlation and interaction

Correlation between stand age and volume of CWD was effectively minimized in the plot selection process. Late seral stands with high CWD levels generally had slightly more CWD than the early seral stands with high CWD levels. Even after elimination of those stands without any western hemlock, correlation between stand age and volume of CWD was not significant (Pearson's correlation analysis p=.133, R^2=.0405). The interaction term of stand age and CWD volume also was not significant (p=.549).

Craterellus tubaeformis productivity

All *Craterellus tubaeformis* basidiomata within strip plot boundaries were collected at each survey iteration. *Cr. tubaeformis* normally occurs in colonies, a colony being defined as a group of basidiomata sharing the same immediate substrate. Each colony on a plot was treated as a separate collection. Surveying consisted of looking at every square meter in the plot for *Cr. tubaeformis*. When a *Cr. tubaeformis* colony was observed, the substrate, distance from and decay class of the nearest CWD were recorded. Collections were mapped, dried, weighed, vouchered, and accessioned in the Oregon State University herbarium.

Despite efforts to select stands below 1000 meter elevations, some were inaccessible for surveying due to snow in January and/or February of 2000. Three stands had suffered extreme disturbance from thinning operations in the interval between the year 2000

surveys and year 2001 survey. For the analysis of biomass productivity, mean biomass for each strip plot was calculated by dividing the total biomass collected by the number of successful survey iterations.

Probability of Craterellus tubaeformis occurrence

If *Craterellus tubaeformis* was recorded in a stand at any time during data collection, the stand was scored as '*Cr. tubaeformis* present'. Presence/absence data for probability analysis was not restricted to the confines of the productivity strip plots, though only rarely was *Cr. tubaeformis* recorded in a stand but not on the plot. Logistic regression was used to analyze probabilities of *Cr. tubaeformis* occurrence and is robust to null data points (Allison 1999), e.g. if *Cr. tubaeformis* is found just once at a site then it does not matter whether that site is accessible for future survey iterations. Conversely, if *Cr. tubaeformis* is absent in 5 successful survey iterations, it is unlikely to be present in the one occasion that the site was inaccessible.

Stand descriptions

The late seral stands with high CWD levels were of the western hemlock type as described by Franklin and Dyrness (1973), with overstories predominated by mature to old-growth western hemlock, Douglas-fir, and occasionally *Thuja plicata* Donn. (western red cedar). These stands were usually located on mesic, moderately sloped or planar landscapes and in most cases had experienced no management. The canopies were multi-storied and mostly closed but with occasional gaps. Mid-stories were usually composed of western hemlock, *Abies grandis* (Dougl.) Lindl. (grand fir), *Taxus brevifolia* Nutt. (western yew), and *Acer circinatum* Pursh (vine maple). The understories consisted of *Gaultheria shallon* Pursh (salal), *Berberis nervosa* Pursh (Oregon grape), *Polystichum munitum* (Kaulf.) Presl. (sword fern), *Vaccinium parvifolium* Smith (huckleberry), *Oxalis oregana* Nutt. (Oregon oxalis), and various mosses in moister sites. The forest floor was characterized by an abundance of class 4 and 5 CWD in the form of large fallen logs, snags and stumps, various sized chunks and subterranean aggregates.

The late seral stands with low CWD levels were also of the western hemlock type but quite different from those described above. The combination of late seral stage with low

levels of CWD is unusual, and locating an adequate number of replicate stands was difficult. These stands were usually on xeric, steeply sloped or convex landscapes, frequently with rocky soil types and relatively open (<60%) canopy. In some cases there was evidence of selective thinning, but in most cases the open canopies appeared to result from poor site conditions. In most of these stands, the overstory was predominated by mature to old-growth western hemlock and Douglas-fir, but 4 of 16 sites were too xeric to support western hemlock. Mid-story vegetation was sparse and composed of western hemlock, *Castanopsis chrysophylla* (Dougl.) A. DC. (chinquapin), grand fir, and *Rhododendron macrophyllum* G. Don (Pacific rhododendron). The understories were often dense and brushy as a result of the open canopy and variously consisted of salal and Oregon grape, often with sword fern, huckleberry, and *Rhus diversiloba* T. and G. (poison oak). The forest floor cover was mostly needle litter and fine woody debris (sticks of <10 cm diam.) with occasional large fallen logs and stumps. In the Cascade Range, 12 of 16 stands had southerly aspects, while in the Coast Ranges 11 of 16 stands had northerly aspects. The same conditions that result in the paucity of CWD (xeric sites, open canopies) could also affect mushroom productivity, although in the fall and winter when *Craterellus tubaeformis* fruits all sites likely receive adequate precipitation.

The early seral stands with high CWD levels were also of the western hemlock type, usually in the process of regenerating from clearcuts. They were mostly on U.S.D.A. Forest Service land, had an abundance of legacy CWD from the preceding stand, and often had evidence of post-harvest slashburning. In most cases, the canopy was completely closed and primarily consisted of young, even-age Douglas-fir with occasional western hemlock, though one stand was a Douglas-fir monoculture. Mid-stories were sparse and usually composed of western hemlock and grand fir. The understories were poorly developed due to canopy closure and consisted of salal, Oregon grape, and sword fern.

Most of the early seral stands with low CWD levels were regenerated western hemlock/Douglas-fir, but four of the 16 sites were monoculture Douglas-fir. Many of the sites were on private land and had most or all of the legacy CWD removed. In most cases, the canopy was completely closed and was predominated by young, even-aged

Douglas-fir. Mid-stories were sparse and usually composed of western hemlock and grand fir when present. Understory vegetation was minimal and low growing due to canopy closure and consisted of scattered salal, Oregon grape, and sword fern. The ground cover was mostly needle litter and fine woody debris with some mossy areas and invasive *Brachypodium sylvaticum* Huds. (Beauv.). Stumps were sometimes present but they were usually small and not well decayed.

Presence of Hydnum species

During the course of survey work it was observed that *Hydnum spp.* seemed to be a particularly effective indicator for *Craterellus tubaeformis*. The species of *Hydnum* (*umbilicatum* Peck vs. *repandum* L:Fr.) varied between sites (often both were present), so the expression *Hydnum spp.* is used. Data on the presence of *Hydnum spp.* was collected with the data for *Cr. tubaeformis* in the survey stands and in other areas visited. Because this study was not designed as a time-lag analysis only one datapoint of *Cr. tubaeformis*/*Hydnum spp.* coincidence was permitted for any given stand, even if they were repeatedly observed together.

RESULTS

Probability of Craterellus tubaeformis occurrence

Logistic regression provides an odds ratio for the likelihood of occurrence of *Craterellus tubaeformis*. Logarithmic transformation was required on both the stand age and volume of CWD explanatory variables to normalize residuals. After removing the stands lacking a western hemlock component from analysis, the model for the odds of the presence of *Cr. tubaeformis* is:

$$\text{Odds of } Cr.\ tubaeformis \text{ occurrence} = {}_e(-.3376 + .9382\{\ln\text{Age}\} + 1.0293\{\ln\text{Volume}\})$$

The table which follows shows the estimates for the intercept term and explanatory variables with their associated confidence limits and p-values at α=95% (n=57):

$ß_0$ (intercept)	Confidence	$ß_1$ ln(Age)	Confidence	$ß_2$ ln (CWD)	Confidence
-.3376	-4.044 to 3.369 p=.8583	**.9382**	.246 to 1.63 p=.0079	**1.0293**	.191 to 1.868 p=.0191

Model estimates with associated confidence limits and p-values

The probability of locating *Craterellus tubaeformis* is calculated from the odds:

Probability = Odds / 1 + Odds

The probability of locating *Craterellus tubaeformis* is expressed on a scale of 0 to 1 and may be interpreted as the "percent chance of" *Cr. tubaeformis* occurring in a Douglas-fir/western hemlock stand, given the stand's age and CWD volume. Figure 2.1 shows the probability of occurrence of *Cr. tubaeformis* with stand ages of 30 and 650 years with 95% confidence limits against the gradient of CWD volumes encountered in the field. The model is extrapolative and the high p-value and wide confidence limits at the intercept reflect the loss of certainty in stands with very little CWD. For example, the model predicts a 55.5% chance of locating *Cr. tubaeformis* in a 650 year-old stand with .0047 m^3 m^{-2} of CWD, however no late seral stands had such low volumes of CWD nor was *Cr. tubaeformis* ever located in a stand with less than .009 m^3 m^{-2} of CWD.

The mean volume of CWD in early seral stands was .0402 m^3 m^{-2} (.0047-.1406) and in late seral stands was .0548 m^3 m^{-2} (.0157-.3477). The stand with the lowest level of CWD recorded in this study (.0047 m^3 m^{-2}) was 53 years old with almost no decayed wood greater than 10 cm in diameter; the CWD groundcover was composed of smaller twigs and there were very few if any well-decayed stumps. The highest level of CWD recorded in this study (.3477 m^3 m^{-2}) was a 500 year-old stand with many large class 4 and 5 fallen boles, well decayed stumps, and abundant CWD aggregate in the organic soil horizon.

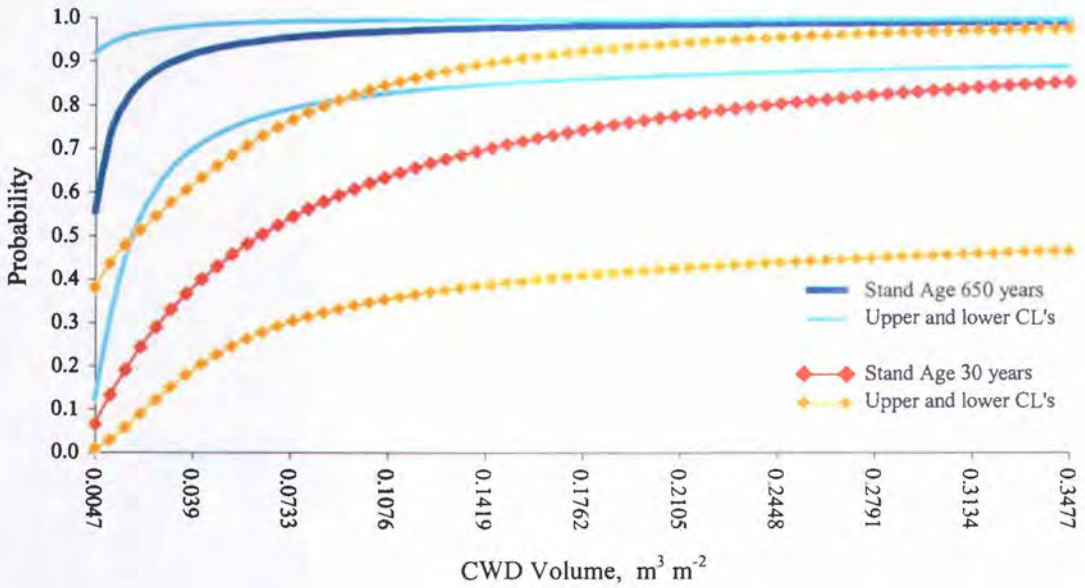

Figure 2.1. Probability of occurrence of *Craterellus tubaeformis* with stand ages of 30 and 650 years plotted against a gradient of CWD volumes, with 95% confidence limits.

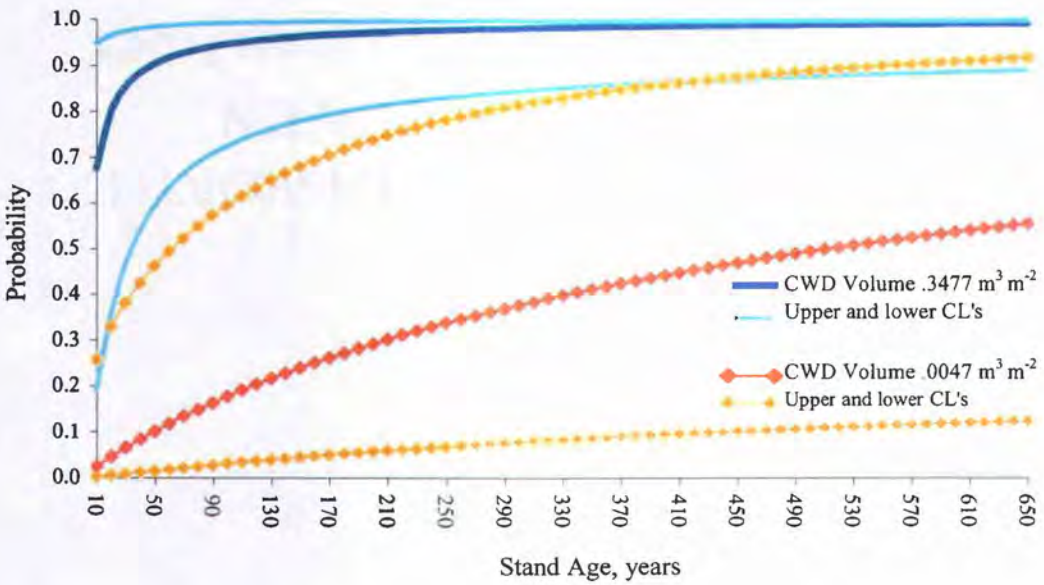

Figure 2.2. Probability of occurrence of *Craterellus tubaeformis* with CWD levels of .0047 and .3477 m^3 m^{-2} plotted against a gradient of stand ages, with 95% confidence limits.

Figure 2.2 shows the probability of occurrence of *Craterellus tubaeformis* with the two extreme levels of CWD encountered in this field work (.0047 and .3477 $m^3 m^{-2}$), along a gradient of stand ages. Again the model extrapolates, and it is noteworthy that in this study the late seral stand with the least amount of CWD was 374 years old and had .0157 $m^3 m^{-2}$ of CWD. Conversely, the early seral stand with the greatest amount of CWD was 48 years old and had .1406 $m^3 m^{-2}$ of CWD. Thus, some of the combinations of variables depicted in the graph are unlikely to be encountered in the field.

Productivity of Craterellus tubaeformis

Craterellus tubaeformis productivity was measured in mean grams of biomass, calculated by dividing the total dry biomass of *Cr. tubaeformis* collected within each 700 m^2 strip plot by the number of times that plot was surveyed (usually six times, minimum four times). Nineteen plots did not produce any *Cr. tubaeformis* and were excluded from analysis because their presence in the model violated the assumption of constant variance, and consequently had disproportionate influence on regressions. Among the remaining 38 stands, the average mean biomass was 1.82 g. (.03 to 12.20 g.). Logarithmic transformation was applied to the mean biomass, stand age, CWD volume, slope, and class 1-3 CWD data to normalize distribution of residuals. Linear regression was used to check significance of stand age, volume of CWD, slope, elevation, and abundance of class 1-3 CWD on *Cr. tubaeformis* biomass productivity, and Pearson's rank-order analysis was used to test correlation between explanatory variables. ANOVA was used to analyze the categorical aspect data.

Stand age was not significant to *Craterellus tubaeformis* productivity (p=.129). Eighteen of the 20 oldest stands produced *Cr. tubaeformis*, but 12 of those were at levels below the mean biomass of 1.82 g. Four of the eight highest-producing stands were less than 100 years old, and two of the most productive stands were 30 and 48 years old (mean biomass 5.08 and 5.18 g. respectively).

The volume of CWD on a plot was not significant to *Craterellus tubaeformis* productivity (p=.151). Eleven of the 12 stands with the highest levels of CWD had *Cr. tubaeformis* populations, but five of those 11 stands had biomass levels below the mean.

Five of the 12 stands with the lowest levels of CWD produced *Cr. tubaeformis*, but only one of them (at 3.06 g.) was above the mean biomass.

Slope was not significant to *Craterellus tubaeformis* productivity (p=.849), but it was closely correlated with stand age (p=.021). Ten of the 17 stands with slopes less than the mean (of 19.6%) were under 100 years old, and 15 of the 21 stands with slope greater than the mean were over 200 years old. This suggests that in the stands under study the older trees occur on steeper slopes.

Elevation initially does not appear to be significant in *Craterellus tubaeformis* productivity (p=.42), but after removing one high productivity outlier at 1000 meters a slight negative relationship becomes apparent (p=.064). Although the *Cr. tubaeformis* fruiting season begins earlier at higher elevations, at the lower elevations spending less time under frozen conditions could permit more basidiomata productivity. However, the influence of one outlier indicates that this data is weak and more study is required before confident statements can be made about the relationship between elevation and productivity.

Abundance of class 1-3 CWD was not significant to *Craterellus tubaeformis* productivity (p=.103). Less than one percent of *Cr. tubaeformis* field collections were associated with class 1-3 CWD. Interestingly, the abundance of class 1-3 CWD was not significantly correlated to the abundance of class 4 and 5 CWD (p=.48). One might expect such a relationship as the input of fresh woody debris represents the next cohort of decayed woody debris. A possible explanation is that CWD persists in decay classes 4 and 5 much longer than its tenure in classes 1-3 (Maser et al., 1989). This could result in the levels of class 1-3 CWD being more variable at any given sampling iteration because of seasonal effects and recent weather events.

No single aspect was significantly more or less productive than others to *Craterellus tubaeformis* productivity. North, west, and southwest aspects were the most productive but not significantly different from the mean (p=.357, .163, and .174 respectively). South and southeast facing slopes were the least productive but not significantly below the

mean (p=.577 and .774 respectively). In all cases sample sizes were small for effective statistical analysis. Sixteen stands were south-facing (S, SE, SW) and had a mean biomass productivity of 1.39 g. Twenty stands had north-facing aspects and mean biomass productivity of 2.05 g. Two stands faced due west (mean biomass 3.5 g.), and none faced due east.

Substrates of Craterellus tubaeformis

Figure 2.3 shows the substrate associations for *Craterellus tubaeformis* colonies, measured in dry weight biomass. A colony was considered associated with a piece of CWD if a substantial proportion (more that half) of the basidiomata were within 20 cm of the CWD. Buried chunks of CWD aggregate hidden under moss or needle litter were frequently encountered as a CWD substrate. Eighty-eight percent of the biomass in documented *Cr. tubaeformis* colonies was produced on or in very close proximity (>10 cm) to CWD. Almost all of these (98.9% of the biomass) were associated with class 4 and 5 CWD. Only one colony was documented on class 3 CWD, and none on class 1 or 2 CWD.

Stumps were not uncommon as a *Craterellus tubaeformis* substrate (4.4% of biomass). Stumps were categorized differently from other types of CWD as the heartwood was often decay class 4 or 5 while the bark would remain at decay class 3. *Cr. tubaeformis* was encountered growing from the soft heartwood at the top of stumps as well as out of the class 3 bark on the outside, sometimes more than a meter above ground level.

Mossy areas and needle covered ground where no subterranean CWD was detectable were somewhat less common as substrates (3.25 and 3.0% of total biomass, respectively). Most of these collections on these substrates were made in older stands with lower levels of CWD. Another substrate occasionally encountered (.35% of biomass) was the bark of living trees. This was observed two times, in both cases at the base of old-growth Douglas-fir trees (less than 20 cm above ground level) in mesic stands. Pulling off chunks of the bark under the basidiomata revealed that the lower region of the bark of the living tree was permeated with active fine roots.

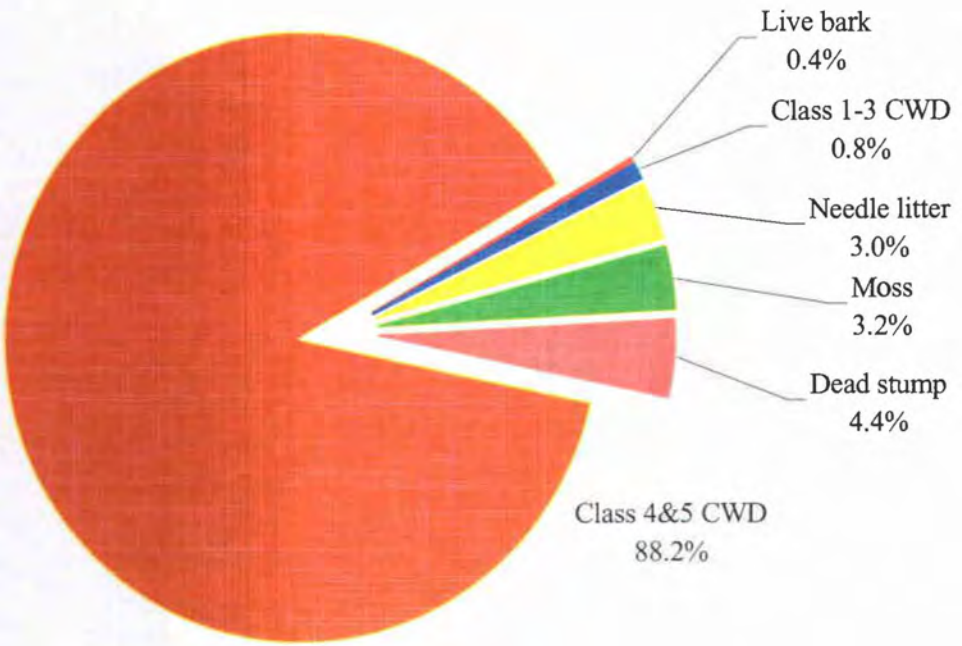

Figure 2.3. Substrate associations for *Craterellus tubaeformis* colonies as percentages of total dry weight biomass.

Hydnum species as an indicator

The presence of *Hydnum spp.* proved to be a positive indicator for the presence of *Craterellus tubaeformis*. Maximum likelihood analysis was used to check relationships between the occurrence of *Cr. tubaeformis* and *Hydnum spp.* in a stand during the fruiting season (October through April). Probabilities were calculated for finding *Cr. tubaeformis* in the presence of *Hydnum spp.* (.90, p=.0001) and in the absence of *Hydnum spp.* (.42, p=.269). The probability of finding *Hydnum spp.* in the presence of *Cr. tubaeformis* is .74 (p=.0001) and in the absence of *Cr. tubaeformis* .70 (p=.0005). Mycorrhizae from *Hydnum umbilicatum* was positively identified by RFLP in this study, and *Hydnum spp.* mycorrhizae were often found in the same root samples as *Cr. tubaeformis* mycorrhizae.

DISCUSSION

Coarse woody debris and western hemlock

Stand age has been shown to influence the likelihood of occurrence of *Craterellus tubaeformis*. In older stands, likelihood of occurrence is quite high even with minimal CWD volumes. It is also evident that *Cr. tubaeformis* can thrive in younger stands. Figure 2.2 suggests that the abundance of CWD is particularly significant to the likelihood of occurrence in stands less than 100 years old, as the probability of *Cr. tubaeformis* occurrence increases rapidly with increasing CWD volumes in this age range. Younger stands with an abundance of CWD are more likely to have *Cr. tubaeformis* than older stands with a paucity of CWD.

In this model, the confidence limits are widest in older stands with less CWD volume and in younger stands with more CWD volume. This reflects the distribution of stand ages and CWD volumes measured in the field, and as a result the model is most confident in those stand age and CWD volume combinations likely to be encountered.

The influence of CWD on likelihood of occurrence coupled with the tendency of *Craterellus tubaeformis* to use it as a substrate suggests that the abundance of CWD is particularly important. The close association of western hemlock was clearly

demonstrated as an important factor in the likelihood of occurrence of *Cr. tubaeformis*, and western hemlock is known to associate with CWD (Minore 1972, Kropp and Trappe 1982).

Does the close association of *Craterellus tubaeformis* with CWD in the Pacific Northwest result from its frequent association with western hemlock or vice-versa? Western hemlock commonly utilizes CWD as a seedbed, and as the trees grow larger their roots and mycorrhizae permeate the CWD (Maser and Trappe 1984). It is reasonable to presume that the roots of western hemlock also permeate mineral and organic soil elsewhere than in CWD, yet *Cr. tubaeformis* is not nearly as prevalent in those areas. This suggests a probable interaction between the abundance of western hemlock and CWD on the likelihood and productivity of *Cr. tubaeformis*.

Some mycorrhizal fungi produce lignase and cellulase enzymes (Trojanowski et al., 1984, Griffiths and Caldwell 1992, Durall et al., 1994, Bending and Read 1995, 1997). The saprobic capability of *Craterellus tubaeformis* is unknown but it seems a likely adaptation to its ecological niche in western Oregon. Kropp and Trappe (1982) hypothesized that because western hemlock usually regenerates on CWD in the understory, western hemlock-preferring mycorrhizae would have to compete with the already established mycorrhizal community. Being specifically adapted to a microhabitat such as CWD could provide the competitive advantage needed for *Cr. tubaeformis* to become established in an extant mycorrhizal community. The C:N ratio in Douglas-fir and western hemlock CWD ranges from 200:1 to 500:1 (Graham and Cromack 1982, Sollins et al., 1987), and most plants can only access nitrogen when the C:N ratio falls below about 25:1 (Russell 1988, Maser et al., 1989). Fungi that have saprobic capabilities can extract nitrogen from substrates with C:N ratios as high as 1800:1 (Maser et al., 1989). This could explain some of the ability of both *Cr. tubaeformis* and western hemlock to gain a foothold in the competitive understory of established forests.

Coarse woody debris provides a stable source of moisture throughout seasonal variations, thereby helping to support mycorrhizae during dry periods (Amaranthus et al., 1989, Maser et al., 1989, Amaranthus et al., 1994). Boddy (1983) showed that water could

comprise more than half of the mass of CWD under some circumstances. Most mushrooms have a very high water content; Pilz et al. (1998) reported that the mean moisture content of *Cantharellus formosus* Corner (Pacific golden chanterelle) was 89% (57-98%, n=80 basidiomata), but indicated that the specimens with very high water content were likely "past their prime" or collected on rainy days and may have had inflated moisture values via hydroscopy (David Pilz, pers. comm.). The mean water content of *Craterellus tubaeformis* is slightly higher at 93.4% but much less variable (90.0-94.1%, n=74 colonies). Coarse woody debris offers the reservoir of water necessary for substantial *Cr. tubaeformis* basidiomata production (a typical *Cr. tubaeformis* colony of ~3 g. dry weight would require over 45 g. of water to form), however substantial precipitation characterizes the *Cr. tubaeformis* fruiting season and soils are often saturated as well. The water content of CWD during the dry parts of the year may facilitate the acquisition and storage of nutrients needed for basidiomata formation, but it is unclear how this would affect *Cr. tubaeformis* differently than any other species.

Relationship with Hydnum species

Analysis shows that the likelihood of finding *Craterellus tubaeformis* is more than twice as great when *Hydnum spp.* are present (90% vs. 42%). The presence of *Cr. tubaeformis* is not as effective an indicator for *Hydnum spp.* (74% with *Cr. tubaeformis* vs. 70% without). Field observations suggest that their fruiting seasons are concurrent, and it could be that *Hydnum spp.* are slightly more common than *Cr. tubaeformis*. It is unclear whether they simply have similar climatic requirements to fruit or if a more complex interaction is involved such as seasonal variations in host nutrient cycling. Pine et al. (1999) showed that *Hydnum* is a sister group to *Cantharellaceae*, resolving to the same phylogenetic clade and sharing the synapomorphic characteristic of stichic nuclear division.

Geographic differences in substrate utilization

In Europe the habitat association of *Craterellus tubaeformis* with CWD is not nearly as prominent (Persson 1997, Gru Gulden and Eric Danell, pers. comms.). There it associates with both deciduous and coniferous trees, and there is much less CWD

available. Persson (1997) and Tyler (1985) report that *Cr. tubaeformis* in Swedish beech forests is restricted to areas with more acidic soil types. In the southeastern United States substrates of wet leaf mulch beneath mixed hardwoods and *Tsuga canadensis* (L.) Carr. (Ronald Petersen, pers. comm.) are reported, as well as associations with *Picea rubens* Sarg. (red spruce) (Bills et al., 1985). More research is needed to quantify the host range and habitat types of *Cr. tubaeformis* variants in different geographies. Dahlman et al. (2000) found that the western United States variant of *Cr. tubaeformis* falls into a different phylogenetic clade than the variants in the eastern United States and Europe. A comprehensive phylogenetic study of *Cr. tubaeformis* worldwide in needed to provide conclusive taxonomic data.

Management implications

A diverse array of mature stands with western hemlock and an abundance of all decay classes of CWD will provide optimum habitat for *Craterellus tubaeformis* in western Oregon and the Pacific Northwest. Protecting 'legacy' CWD and recruiting a new cohort of class 1 CWD is key to managing current and future habitat. CWD supply is a continuing cycle, and an ongoing infusion of CWD is required to maintain habitat over the long term.

Summary and conclusions

Craterellus tubaeformis does not strictly depend on late seral forests, being found in stands as young as 30 years old. However, the likelihood of encountering it increases with stand age and with the volume of CWD. The abundance of CWD is particularly important to the likelihood of *Cr. tubaeformis* occurrence in stands less than about 100 years old. In western Oregon, it is almost always found in close proximity to class 4 and 5 CWD, though it will occasionally occur on moss, needle litter, or on or near decaying stumps. Western hemlock is the primary but not only host symbiont. *Hydnum spp.* are excellent indicators for *Cr. tubaeformis* in western Oregon. These habitat and host associations may be different in the eastern United States and in Europe. The genetic characteristics of *Cr. tubaeformis* may also differ between these regions. Additional research is needed to understand these relationships, why they may differ between geographic regions, and how significant the genetic differences are.

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Ecology of *Craterellus tubaeformis* in Western Oregon: A Summary

SUMMARY

Mycorrhizal host associations

This research has shown that the *Craterellus tubaeformis* in western Oregon is mycorrhizal and associates primarily with *Tsuga heterophylla* (western hemlock). It has been found to also associate with *Pseudotsuga menziesii* (Douglas-fir) and *Picea sitchensis* (Sitka spruce) on occasion but these associations are relatively rare. The overall odds of locating *Cr. tubaeformis* in a stand with western hemlock was 6.25 times greater than in a stand without western hemlock (p=.017). Occurrence of *Cr. tubaeformis* in Douglas-fir stands lacking western hemlock is infrequent, and it might only colonize Douglas-fir in the absence of western hemlock. Conversely, *Cr. tubaeformis* was never observed in a Sitka spruce stand without a western hemlock component. It is possible that the western hemlock provides a launching point for colonization of spruce by *Cr. tubaeformis*, and that the pure Sitka spruce stand may lack some element conducive to *Cr. tubaeformis* establishment. More research on the occurrence of *Cr. tubaeformis* in forest stands lacking western hemlock is needed.

Likelihood of occurrence and biomass productivity

Stand age proved significant to the likelihood of *Craterellus tubaeformis* occurrence (p=.0079), but not to the biomass productivity among stands with *Cr. tubaeformis* (p=.129). Young stands were less likely to produce *Cr. tubaeformis*, but when they did they had the capacity to produce them in quantity.

Likewise, the abundance of CWD proved significant to the likelihood of *Craterellus tubaeformis* occurrence (p=.0191), but not to the biomass productivity among those stands with *Cr. tubaeformis* (p=.151). When *Cr. tubaeformis* was encountered in a stand, even a few pieces of CWD supported large colonies. Abundance of CWD has a greater influence on the probability of *Cr. tubaeformis* in stands less than 100 years old. Eighty-nine percent of the biomass in documented *Cr. tubaeformis* colonies were produced on or in very close proximity (>10 cm) to class 4 and 5 CWD.

Species concept

According to phylogenetic analyses by Feibelman (1994) and Dahlman et al. (2000), the variant of *Craterellus tubaeformis* in eastern North America seems more closely related to the European variant than to the Pacific Northwestern variant. Host associations may be different in these various regions as well, however that could be an artifact of the different cohort of host species available. Few data have been published specifically on the ecology or host associations of *Cr. tubaeformis* in eastern North America and Europe, and more research on the phylogeny, host associations, and habitat requirements of *Cr. tubaeformis* in those regions is needed.

Hydnum species as an indicator

Hydnum spp. proved to be a positive indicator for *Craterellus tubaeformis*. During the fruiting season (late October through April), there was a 90% probability of finding *Cr. tubaeformis* in the presence of *Hydnum spp.* and only a 42% probability of finding it in the absence of *Hydnum spp.* There was a 74% probability of finding *Hydnum spp.* in the presence of *Cr. tubaeformis* and a 70% probability of finding *Hydnum spp.* in its absence. It is unclear whether they simply have similar climatic requirements for fruiting or if a more complex interaction is involved, such as seasonal variations in host nutrient cycling.

Future research opportunities

Some mycorrhizal fungi demonstrate saprobic enzyme activity in the form of lignase, cellulase, or peroxidase production (Trojanowski et al., 1984, Griffiths and Caldwell 1992, Durall et al., 1994, Bending and Read 1995, 1997). Bruns et al. (1998) hypothesize that many genera seem to have switched between mycorrhizal and saprobic lifestyles several times over the course of their evolution. Until recently, enzyme analysis has required tissue in culture on agar doped with polymeric dyes (Platt et al., 1985, Gold et al., 1988) or subjected to spot tests (Gramss et al., 1998), or isotope labeling of synthesized mycorrhizae (Trojanowski et al., 1984, Haselwandter et al., 1990, Durall et al., 1994). Growing cantharelloids in pure culture is difficult due to contamination by bacteria and other unidentified fungi within the sporocarp tissue (Fries 1978, Moore et al., 1989, Danell and Fries 1990, Danell et al., 1993). In any event Cairney and Burke (1998) report possible false positives on some types of agar assays. DNA probes for

transcribed genes coding for the production of lignases, cellulases, and peroxidases have been developed (Kimura et al., 1990, Gold and Alic 1993, Dahlbøge and Heldt-Hansen 1994, Tempelaars et al., 1994, Broda et al., 1995 and 1996, Cullen 1996) and offer very interesting opportunities for future research on the saprobic capacity of mycorrhizal fungi.

Mass spectrometry could also be used to determine if *Craterellus tubaeformis* produces saprobic enzymes. If an organism obtains its energy from decomposition it will have a high ratio of the heavier ^{13}C isotope to that of the lighter ^{12}C isotope. Plants will tend to favor the lighter ^{12}C when absorbing nutrients from the soil, leaving a higher proportion of ^{13}C in the soil. If a fungus obtains its energy from a living tree (e.g. is mycorrhizal), then it will in turn have a higher level of ^{12}C than ^{13}C (Hobbie 1999, Hobbie et al., 2001). Saprobic fungi obtaining their energy from decomposing litter on the forest floor will tend to have a higher level of ^{13}C. Preliminary analyses of *Cr. tubaeformis* have been mixed; signatures have fallen midway between mycorrhizal and saprobic controls. This may suggest that they may have some enzyme production capabilities in addition to their mycorrhizal trophism.

Summary and conclusions

The western Oregon variety of *Craterellus tubaeformis* is mycorrhizal, and requires western hemlock and an abundance of class 4 and 5 CWD to thrive. *Cr. tubaeformis* is more likely to be found in older stands than younger stands, but can thrive in younger stands with abundant CWD. *Cr. tubaeformis* will occasionally occur in monoculture Douglas-fir stands or growing on a non-CWD substrate, these situations are relatively rare. *Cr. tubaeformis* can form mycorrhizae with Sitka spruce, but has never been observed in Sitka spruce stands without a western hemlock component. The substrate and host associations of *Cr. tubaeformis* are different between the Pacific Northwestern United States, the eastern United States, and Europe. Additional research is needed before conclusions about differences in host associations and specificity can be drawn. Currently molecular data are incomplete, but suggests that there are differences in the genotypes between regions. *Hydnum spp.* are an excellent indicator for *Cr. tubaeformis*.

Craterellus tubaeformis is a common mushroom in the mixed-hemlock forests of the Pacific Northwest. This research has revealed several facets of its ecology, yet much remains unknown. Why is western hemlock the host of choice when Douglas-fir is abundant and available as a source of nutrient energy? Is the supply of water the reason for the strong association with CWD, is it simply a result of its association with western hemlock, or is it something else, such as pH? What is the reason for the temporal and spatial connection between the fruiting of *Cr. tubaeformis* and *Hydnum spp.*? Is it similar habitat and climate requirements or is something more involved? If so much remains unknown about such a common mushroom, imagine how much we have yet to learn about lesser known organisms... and those organisms of whose existence we are not even yet aware.

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