

Rhizopogon Mycorrhizal Networks With Interior Douglas-
fir In Selectively Harvested And Non-Harvested Forests

by

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Abstract

Rhizopogon vesiculosus and *Rhizopogon vinicolor* are sister species of ectomycorrhizal fungi that associate exclusively with Douglas-fir (DF). My first objective was to determine whether a change in the number or relative abundance of *R. vesiculosus* and *R. vinicolor* tubercles and genotypes was related to a change in the percent of DF in a regenerating phase (<50 years-old). The number of *R. vesiculosus* tubercles correlated positively with an increasing proportion of DF in a regenerating phase, while the number of *R. vinicolor* tubercles was similar across all age structures. The number of *R. vesiculosus* genotypes did not correlate with age structure, whereas the number of *R. vinicolor* genotypes showed a negative relationship with an increasing proportion of DF in a regenerating phase. When numbers of *R. vesiculosus* tubercles and genotypes were expressed as a relative abundance of the two species, there was a positive correlation with an increasing proportion of DF in a regenerating phase for both genotypes and tubercles. Results suggest that the degree of DF regeneration or ecosystem factors related to DF regeneration affect the population dynamics of *R. vesiculosus* and *R. vinicolor* differently.

My second objective was to quantify and compare the extent of *Rhizopogon* mycorrhizal networks in selectively logged (25 years-old) and non-logged plots. The networks of the harvested plots, when including both *Rhizopogon* species, were as highly connected as the undisturbed plots. However, they differed in that they had two *Rhizopogon* species rather than one and had a higher node density. *R. vinicolor* networks of harvested and unharvested forests had very different link densities. When I accounted for density differences, networks of both treatments were not significantly different, apart from a greater vulnerability to fragmentation in harvested forests than in undisturbed forest. When *R. vesiculosus* was included in the analysis, both treatments had similar connectivity and limited vulnerability to fragmentation. Results suggest that when a forest transitions from a regenerating to a non-regenerating one, the *Rhizopogon* network will lose *R. vesiculosus* and will have increased link density. Nevertheless, both the selectively harvested and non-harvested forests will have well connected and potentially functional *Rhizopogon* networks.

Preface

Chapter 2 of the thesis was wholly published in the journal *Mycorrhizae*, February 2016 Volume 26 Issue 2 pp 169-175 “Dominance of a *Rhizopogon* sister species corresponds to forest age structure”. Chapter 2 was written in collaboration with Carrie Van Dorp (data, ideas and writing) Kevin J Beiler (data, ideas and editing) and Daniel Durall (funding, support, ideas and revisions).

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Abbreviations and Glossary:

CMN – Common Mycorrhizal Network, a term referring to the network formed by mycorrhizal fungi and plant symbionts. The term refers to a shared network of multiple species of both fungi and plants.

DF – Douglas-fir, *Psuedotsuga menziesii* without discrimination between the coastal (ver. *menziesii*) or the interior (var. *glauca*) subspecies.

IDF – Interior Douglas-fir, *Psuedotsuga menziesii* var. *glauca* (Mirb.) Franco.

MN – Mycorrhizal Network, a term referring to a network formed by ectomycorrhizal fungi and plant symbionts. In this thesis, the MN is used to describe the network formed by DF trees and *Rhizopogon vesiculosus* and *R. vinicolor* fungi.

Genet – samples which have the same multi-locus genotype are considered to be the same individual, we use the world genet to identify the genetically distinct individuals.

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1. Introduction

1.1 Ectomycorrhizal Fungi

The fascinating and complicated symbiosis of plants and fungi, termed mycorrhizae, has been a subject of considerable study in the last century. There are many different combinations of associations within the symbiosis, which involve different scales of individuals and species as well as different levels of specificity. Within the bounty of scientific exploration, there is clear evidence that, in many cases, mycorrhizal fungi are critical for the health and survival of the plants with which they are associated. The potential positive effect of mycorrhizae may be related to mediating overstory competition between seedlings and mature trees via mycorrhizal fungi (Booth & Hoeksema 2010) or by influencing nitrogen availability (Ho & Trappe 1980; Plassard et al. 1991) or mediating water stresses (Dosskey et al. 1991). The potential for networking between plants, mediated by mycorrhizae, is a key area for study to understand ecosystem complexity; however, species specificity, genet size and genet persistence are extremely variable, so it is appropriate to focus our attention on what has been observed with closely related ectomycorrhizal fungal species.

1.2 *Rhizopogon* fungi

Rhizopogon is a genus within the Basidiomycota that forms ectomycorrhizal associations with Pinaceae hosts. All members of the genus produce truffle fruiting bodies, which are important in the diets of small mammal species (Johnson 1996). *Rhizopogon* species have been identified as critical factors in tree survival and health since the 1920's when exotic conifer species, which were introduced into Australian plantations, had poor growth until a *Rhizopogon* sp. was introduced (Molina et al. 1994).

1.3 *Rhizopogon vinicolor* and *R. vesiculosus*

Rhizopogon vinicolor (now recognized as two species, *R. vinicolor* and *R. vesiculosus*) was noted as one of the dominant ectomycorrhizal fungi of *P. menziesii* in the 1970's (Molina et al. 1994). *R. vinicolor* and *R. vesiculosus* form durable tuberculate ectomycorrhizae and have relatively long rhizomorphs. These durable rhizomorphs extend several decameters in the soil (Kretzer et al. 2003b), and allow the fungus to interact with the roots of many trees. Agerer et al. (2001) classify ECM with this growth

habit 'long distance exploration types'. Other ectomycorrhizal species, such as *Laccaria amethystina*, which are a short distance exploration type, do not form extensive networks (Hortal et al. 2012). Tubercles consist of a rind-covered cluster of mycorrhizal roots, where the rind is made of compact layers of fungal hyphae. Each DF root inside the rind develops a fungal mantle and a Hartig net. The function of the tubercles is not entirely understood, though some studies have shown the presence of nitrogen fixing bacteria within *Rhizopogon vinicolor* sporocarps (Li and Castellano 1987); however, in one study, the bacteria abundance in and on tubercles was low and the nitrogen fixing enzyme could not be detected (Kretzer et al. 2009).

The *Rhizopogon* species, *R. vinicolor* and *R. vesiculosus*, examined in this research, are within the subgenus *Villosuli* (Kretzer et al. 2003b), and known to associate only with *Pseudotsuga menziesii* spp. They were originally identified as separate species based on the presence of distinctive vesicles in the fresh fruiting body of *R. vesiculosus* (Smith 1964). Nevertheless, in studies sampling mycorrhizas, the two species were not distinguished until the ITS regions for the type specimens were sequenced (Kretzer et al. 2003a). Because of the difficulty in morphologically differentiating between *R. vesiculosus* and *R. vinicolor* (Louma et al. 2011), they continue to be grouped in descriptive ectomycorrhizal community studies (Twieg et al. 2007). Recent studies, focusing on the spatial patterns of individual *R. vesiculosus* and *R. vinicolor* genotypes, have uncovered differences in the autecological traits of these species. *R. vesiculosus* genotypes have been found to span greater distances than those of *R. vinicolor* in both coastal and interior DF forests (Kretzer et al. 2005; Dunham et al. 2013; Beiler et al. 2015). Dunham et al. (2013) performed population genetic analysis of an Oregon population and found that *R. vesiculosus* genotypes are closely related to their neighbors, whereas *R. vinicolor* appears to disperse away from related individuals. Beiler et al. (2012) mapped species interaction at fine spatial scales (20-cm cubes over a 1x2-m area) and determined that the species partitioned vertically in the soil profile; *R. vesiculosus* genotypes were always lower in the soil horizon than *R. vinicolor* when both species were present at the same sampling point.

Research comparing *R. vesiculosus* and *R. vinicolor* has been conducted in ecologically and geographically separated forests. The coastal DF (*Pseudotsuga menziesii* var. *menziesii*) forest at Mary's Peak, Oregon, experiences a relatively high annual precipitation, has a dense tree and shrub understory, and consists primarily of 40- to 80-year-old trees, which established following a stand-replacing disturbance (Kretzer et al. 2005). In contrast, the interior DF (*Pseudotsuga menziesii* var. *glauca*) forest, studied by Beiler et al. (2010, 2012, 2015), is subject to strong seasonal moisture deficits and gap-phase regeneration following natural mortality or disturbance, which results in a patchy, mixed-cohort stand structure (LeMay et al. 2009). Despite these different ecological conditions and different DF subspecies, there was a consistent pattern: both species of *Rhizopogon* were frequently encountered, and *R. vesiculosus* genotypes reached greater spatial extents and formed mycorrhizas in greater abundance than *R. vinicolor*.

1.4 Ecological networks

There has been a recent emphasis to develop understanding of system complexity using network theory. Reductionist scientific processes cannot always be used to examine relationships, whereas network theory can be used to describe and then evaluate relationships and influences of parts of the network. These theories have been used to describe and understand the internet, social networks, cellular pathways, and have also been applied to mycorrhizal fungal networks (Bray 2003; Southworth et al. 2005; Beiler et al. 2010).

The architecture of a network is determined by the links between the nodes. For example, some networks are connected regularly, in a logical pattern related to geographic proximity. Networks can also be connected randomly, where any can be connected to any other node. They can also be scale free, where a small number of nodes are highly connected (hubs), and traverse from one end of the network to the other requiring relatively few nodes. The architecture of a network can give insight as to how the network was formed in the first place and its potential resilience to stress (Bray 2003). Scale free networks are common in biological systems, and have a particular robustness to errors and high link failure rates as a result of the small world

arrangement of redundant linkages of scale free networks. Albert et al. (2000) used a modeling procedure studying communication networks to show that scale free networks are particularly vulnerable to targeted removal of a few highly connected nodes.

The degree distribution is the probability distribution of the degrees within a network. Nodes which have a high degree compared to the distribution of degrees in the network are said to have high centrality. The network can be classified as scale free if some of the nodes have a high centrality and high degree compared to the average centrality and degree of the nodes in the network. The clustering coefficient of a node is calculated by comparing the density of links of a node compared to the density of neighbouring nodes (Nooy et al. 2005).

Networks can be compared to each other based upon a measure of the overall network centralization; which can be done by calculating the variation of the node degrees in the network as compared to the maximum number of possible variation. A second network-level comparison can be made using the network clustering. The network clustering coefficient is the average clustering coefficient among all nodes in the network. A higher clustering coefficient indicates that the network has a very 'small world' connectedness (most nodes can be connected to each other by a small number of link steps). Networks with high clustering coefficients are considered to be very robust against disturbance (loss of nodes and links) (Bray 2003, Beiler et al. 2010).

1.5 Mycorrhizal Networks

Network theory can be applied to mycorrhizal networks using the trees as the nodes and fungi as the links or using the fungi as the nodes linked to other fungi by the tree roots (Southworth et al. 2005). In the case of the mycorrhizal network of *R. vinicolor* and *R. vesiculosus* it is appropriate to use the trees as nodes connected by fungal links (Beiler et al. 2010). The trees (nodes) are connected by fungal genets (links) which are found to have formed tubercles with their roots and the roots of other trees. The number of these connecting links is the node's degree.

1.6 Microsatellite Identification in *Rhizopogon* sp. and *Pseudotsuga Menziesii*.

The use of microsatellite markers for the identification of specific individuals within a population takes advantage of simple sequence repeats (SSR), generally found in the non-coding portions of a genome. These short segments of the genome can be highly polymorphic, and differ between individuals, because of at least two mutational processes. Firstly, single base pair mutations (deletions, additions) within the repeated region or the flanking regions can cause changes in fragment length, as per the infinite alleles model of mutation. Secondly, discrete repeat units of the simple sequence repeats can be omitted or added, which is attributed to slippage of the polymerases during DNA replication, this is known as the stepwise mutation model (Auckland et al. 2002). Taking advantage of the resulting polymorphisms, by measuring the length of several SSRs one can compare samples of DNA to distinguish between individuals, and match samples to a 'library' of known samples (Zietkiewicz et al. 1994).

Use of microsatellite markers for the identification of ectomycorrhizal fungal genets and individual trees was pioneered in a study by Saari et al. (2005). In this study, soil cores were taken in a Scots pine forest, and both ectomycorrhizal fungus and pine root DNA samples were amplified using microsatellite primers of different loci, which allowed the DNA fingerprints of the ectomycorrhizal root to be linked with that of individual trees (Saari et al. 2005). The authors used this information to describe the level of community development on the individual trees, but did not determine individual genets of the fungus in order to describe the mycorrhizal network. A set of primers for the identification of individual genets of *R. vesiculosus* and *R. vinicolor* was developed by Kretzer. With these markers, they differentiated the maximum number of genets using a minimum of five microsatellite markers ($PI < 0.004$). (Kretzer et al. 2000, 2003a).

Primer sets targeting specific microsatellite loci have been developed for Douglas-fir trees by Slavov et al. (2004). Three of these highly polymorphic SSR loci were used to distinguish between different Douglas-fir individuals in an interior Douglas-fir forest near Kamloops, British Columbia (Beiler et al. 2010). There is some concern that due to somatic mutation, microsatellite markers in needle and root tissue may not

be consistent in long lived organisms like Douglas-fir trees. (Somatic mutation is mutation of a cell within an organism which is passed to replicated cells, but does not change the genetics of offspring). A study of *Pinus strobus* microsatellite stability of embryonic tissue found that the loci were conserved, though the study was only testing change over two years in young trees (Cloutier et al. 2003). Because of the potential for somatic mutation of the tissue of long lived species like Douglas-fir, Beiler sampled known root, cambium and needle tissue and selected primers for which the loci were conserved (Beiler, Personal Communication).

The two *Rhizopogon* species were selected for mycorrhizal network research because they are frequently encountered on DF roots, and have been shown to colonize the roots of more than one DF tree and are dominant fungi as stands age (Twieg et al. 2007). Hence they are suitable for field sampling of mycorrhizal networks to study the connections between individual organisms. This system is also ideal for this research because the tuberculate ectomycorrhizae are obviously morphologically distinct from other DF ectomycorrhizae in field sampling.

1.7 Forestry Practices: Selective Logging of Interior Douglas Fir Zone

The interior Douglas-fir biogeoclimatic zone includes the low to mid elevation land in the southern regions of the Interior Plateau and the southern Rocky Mountain trench. The continental climate of the region has warm, dry summers, which makes for a long growing season with limited moisture. The mean annual precipitation is about 400mm and the mean annual temperature 5.7°C (1981-2009 Climate BC). The ecosystem is shaped by many different types of disturbances, including historically mixed severity fire and insect damage, and more recently timber harvest and human-caused fires (Klenner et al. 2008).

A host of variations on selective harvest have been used in the history of logging for Douglas-fir in the interior of British Columbia. Initially, only the best logs were removed (high-grading) for their commercial value. In the 1950's, a limit of diameter cutting was mandated, which resulted in retaining 40% of the stand volume. This mandated removal of 60% of the forest, where the tallest, straightest and best trees were removed. This was the practice through the 1970's when faller selection was

subsequently mandated. Faller selection practice includes more site specific cutting guidelines that require retention of some trees of all age/size classes. Currently harvesters use variable retention depending upon the site. They usually include selecting a disproportional number of older trees as opposed to younger ones (Vyse et al. 1991: Nyland 2001).

The Interior Douglas-fir forest region includes over 1 million ha of forest, so a variety of silviculture techniques are used due to the variability of insect issues, grades, and accessibility. Uneven aged IDF stands are particularly susceptible to western spruce budworm and tussock moth infestations (Alfaro & MacLauchlan 1992). If the risk of insect damage is greater than risks of erosion and windfall associated with patch-cutting, patch-cutting to create even aged stands may be the optimal strategy. Similarly, where *Armillaria oostyae* fungus has infected and damaged roots, it is expected to spread to retained trees, so the removal of all age classes may be the preferred management technique (Morrison 2000). An experimental plot (Opax Mountain) to test the effect of different silvicultural techniques was set up in the Kamloops forest district in 1992. Various measures of forest health from this project have shown that there are multiple silviculture systems which can be biologically justified for this forest type (Klenner & Vyse 1997). However, due to the sloped geography and shade tolerance exhibited by interior DF seedlings, selective cutting or variable retention techniques are considered to be the best harvest practice in most cases (Klenner et al. 2008: Day 1997: Guldin 1996). The harvested plots in our study were subject to a patch cutting variable retention technique where trees were removed to create small gaps about 25m wide and 40 to 60 m along the slope with some mature trees remaining. These trees responded to increased light with a flush of growth and grew to be much larger trees in the intervening 25 years between the tree harvest and the sampling for this study.

1.8 Forest Resilience and Mycorrhizal Networks

Forest disturbance is recognized as an important driver in landscape diversity and critical for persistence of many ecosystems in the Pacific North-west. However a forest's ability to re-develop following disturbance is not guaranteed. Forest resilience can be understood in terms of the ability of the system to adapt to change without

undergoing a major transformational shift. The definition of the transformational shift is important. Is it a shift in species composition, a shift in biodiversity or is it a capacity for carbon storage that shifts the ecosystem composition irretrievably?

For the purposes of this study, we are interested in the resilience of the community to support and sustain the species that were present before the disturbance. Interior Douglas-fir is a valuable timber species which has been selectively logged for valuable timber production. These selective logging practices have fragmented the forest with roads and introduced non-native herbaceous plants that have altered the biodiversity and species composition. Douglas-fir regeneration is limited in the IDF by drought stress, shading, nutrient stress, herbivory by animals or insects, and root rot. Connection to a mycorrhizal network could mediate these stresses to improve overall seedling survival and forest health. Bingham and Simard (2012) demonstrated MN mediation of drought stress, and Simard et al. (1997) have shown carbon transfer between trees and Deslippe and Simard (2011) demonstrate MN carbon transfer between shrubs which would mitigate shade stress. Teste et al. (2010) demonstrated carbon and nitrogen transfer through the MN, which could mitigate nutrient stress. Both Babikova et al. (2013) and Song et al. (2015) observed a change in leaf chemistry to repel herbivores when a neighbouring plant was connected in the MN and was subjected to foliar damage (simulated herbivory). Finally, Baleshta et al. (2005) demonstrated that the risk of DF root rot was also reduced when DF trees were in a mixed mycorrhizal network with paper birch.

Researchers have demonstrated that living legacy trees enhance the recovery of the total ecosystem at the landscape scale (Seidl et al. 2014). In the interior Douglas-fir, where selective logging leaves legacy trees with intact root systems, we suspect that the shade, moisture retention, and mycorrhizal inoculum to connect the seedlings to the network facilitate a quick recovery of DF forests from logging or single tree mortality.

1.9 Research objectives and predictions

My overall objective in this research was to determine if there were significant negative impacts of selective logging on the *R. vinicolor* and *R. vesiculosus* –DF network in interior DF forests.

My first objective (presented in Chapter 2) was to determine whether the abundance and proportion of *R. vesiculosus* and *R. vinicolor* tubercles and genotypes would be altered by a difference in DF regeneration. We predicted that a change in the number or relative abundance of *R. vesiculosus* and *R. vinicolor* tubercles and genotypes would not be related to a change in the percent of DF seedlings, saplings and/or trees <50 years old. We based this prediction on previous studies showing that *R. vesiculosus* consistently had more tubercles than *R. vinicolor*, *R. vinicolor* consistently had more genotypes than *R. vesiculosus*, and both species associated with roots from DF of all ages (Kretzer et al. 2005; Beiler et al. 2010; Dunham et al. 2013).

My second objective (presented in Chapter 3) was to determine the resilience of MNs after 25 years following removal of hubs. We predicted that the targeted removal of mature trees would have a significant lasting impact on the network; the mycorrhizal network of the harvested forest would have reduced connectedness as compared to an unharvested neighbouring forest. We based this prediction on the network disturbance model included in Beiler et al. 2010, supplemental information.

2. Dominance of a *Rhizopogon* sister species corresponds to forest age structure

2.1 Synopsis

Rhizopogon spp. are among the most dominant members of ectomycorrhizal fungal (EMF) communities in temperate coniferous forests (Zak 1971, Molina et al. 1999, Grubisha et al. 2002). Members of the genus form long-distance exploration type mycelial systems, which are characterized by differentiated rhizomorphs and diffuse, undifferentiated hyphae (Agerer 2001). Rhizomorphs aid in the long-distance transport of water and nutrients (Brownlee et al. 1983, Egerton-Warburton et al. 2007) and may contribute to survival and drought tolerance of conifer seedlings (Parke et al. 1983, Dosskey et al. 1991). In a study mapping mycorrhizal associations between Douglas-fir (DF) (*Pseudotsuga menziesii* var. *glauca* (Beissn.) Franco) roots and *Rhizopogon* spp., a single fungal genotype in a 30×30-m plot was found to associate with 19 DF, and 67 % of the DF in the plot shared at least one common fungal genotype (Beiler et al. 2010). Such belowground networks, formed between trees and ectomycorrhizal fungi, may enhance water and nutrient transfer at a landscape level (Simard 2009, Teste et al. 2010).

The previously mentioned study by Beiler et al. 2010 focused on a pair of cryptic species, *Rhizopogon vesiculosus* A.H. Smith and *Rhizopogon vinicolor* A.H. Smith (Basidiomycota, Villosuli group sensu Kretzer et al. 2003b), which form unique tuberculate mycorrhizas and associate exclusively with DF roots. Based on morphological characters of their fruiting bodies, they were originally identified as separate species (Smith 1964). Nevertheless, in studies sampling mycorrhizas, the two species were not distinguished until the ITS regions for the type specimens were sequenced (Kretzer et al. 2003a). Because of the difficulty in morphologically differentiating between *R. vesiculosus* and *R. vinicolor* (Louma et al. 2011), they continue to be grouped in descriptive ectomycorrhizal community studies (Twieg et al. 2007). However, recent studies, focusing on the spatial patterns of individual *R. vesiculosus* and *R. vinicolor* genotypes, have uncovered differences in the autecological traits of these species. For example, *R. vesiculosus* genotypes have been found to span greater distances than those of *R. vinicolor* in both coastal and interior DF forests

(Kretzer et al. 2005; Dunham et al. 2013; Beiler et al. 2015). Population genetic analysis of an Oregon population revealed that *R. vesiculosus* genotypes are closely related to their neighbors, whereas *R. vinicolor* appears to disperse away from related individuals (Dunham et al. 2013). At fine spatial scales (20-cm cubes over a 1×2-m area), the two species have been observed to overlap in horizontal space and partition vertically in the soil profile (Beiler et al. 2012); *R. vesiculosus* genotypes were always lower in the soil horizon than *R. vinicolor* when both species were present at the same sampling point. Research comparing *R. vesiculosus* and *R. vinicolor* has been conducted in ecologically and geographically separated forests. The coastal DF (*Pseudotsuga menziesii* var. *menziesii*) forest at Mary's Peak, Oregon, experiences a relatively high annual precipitation, has a dense tree and shrub understory, and consists primarily of 40- to 80-year-old trees, which established following a stand-replacing disturbance (Kretzer et al. 2005). In contrast, the interior DF (var. *glauca*) forest, studied by Beiler et al. (2010, 2012, 2015), is subjected to strong seasonal moisture deficits and gap-phase regeneration following natural mortality or disturbance, which results in a patchy, mixed-cohort stand structure (LeMay et al. 2009). Despite these different ecological conditions and different DF subspecies, there was a consistent pattern; both species of *Rhizopogon* were frequently encountered, and *R. vesiculosus* genotypes reached greater spatial extents and formed mycorrhizas in greater abundance than *R. vinicolor*.

2.2 Materials and methods

2.2.1 Site description

This study took place in two separate forests north of Kamloops, British Columbia (50° 51' 7" N, 120° 31' 46" W and 50° 55' 19.86" N, 120° 17' 37.09" W). The forests were located 17 km apart and represent differing biogeoclimatic subzone variants (the Interior Douglas-fir Thompson dry, cool IDF-dk1, and Cascade dry, cool IDF-dk2, respectively; Lloyd et al. 1990). The two sites, characterized in the IDFdk1 and IDF-dk2 (1981–2009 Climate BC http://climatewna.com/climatena_map/ClimateBC_Map.aspx), had a mean annual precipitation of 400 and 500 mm, a mean annual temperature of 4.3 and 5.7 °C, and a mean elevation of 900 and 1150 m, respectively. In each of the two forests, six plots were selected in stands of mono-dominant Douglas-fir with a similar

aspect and slope. Douglas-fir ranged in age from 1–223 years old with trunk diameters from <1 to 119 cm DBH. Plots were ranked from 1–12 according to the level of Douglas-fir regeneration (percent of DF<50 years old), with rank 1 having the lowest percent of DF<50 years old and rank 12 having the highest. In this document, we will refer to this ranking of DF age structure as the DF regeneration gradient. We used 50 years as a partition point because it best delineated trees in dominant and subordinate canopy layer positions in the forests. In the multi-storied, mixed-age IDFdk forests where our study took place, tree growth and mortality are largely influenced by size and proximity to other trees (LeMay et al. 2009). In the first of the two forests sampled, six 10×10-m square plots were used (Beiler et al. 2015). These plots contained the entire range of DF ages from seedlings to individuals over 100 years old. In the second forest, six 10-m diameter circular plots were used. Three of these plots were located in selectively logged gaps that had substantial recruitment of Douglas-fir seedlings/saplings, but were surrounded by mature trees. The other three plots were located where there was a wide range of tree sizes, but there were no DF<15 years old and no signs of mature tree removal. Within their respective forests, plots were positioned between 150 and 1000 m apart.

2.2.2 Field sample collection

Rhizopogon tubercles were excavated from the forest floor and from the mineral soil horizons to a depth of 30cm. Initial sampling targeted microhabitats where tubercles have been found to proliferate (e.g., the underside of rocks and within coarse woody debris). Following targeted sampling, we sampled within every square meter of the plot to a total sampling effort of 2h/m². The 10×10-m plots were sampled in May–June 2008 and the 10-m circular plots were sampled in May–July 2011. When multiple tubercles were found on the same root and observed to be connected by fungal rhizomorphs, they were treated as one sample. If the physical connection could not be visually confirmed, multiple tubercles in the same location were stored and labeled separately. The entire tubercle was collected, labeled, stored in a cooler for transport, and transferred to a freezer (–20°C) awaiting DNA extraction. At the completion of the sampling period, all DF and tubercle locations were mapped using a laser and digital compass as in Beiler et al. (2010). Tree core samples were collected at 1.3-m height

from a subsample of 62 DF in square plots, and from all DF in circular plots, as well as, from all DF within 25 m of the plot edge. Rings were counted under a dissecting microscope to determine tree age \pm 5 years for categorizing into cohorts (0–15, 16–50, 51–85, <86 years). All seedlings and saplings (<20 years old) within and surrounding the plots were recorded, and the number of whorls of branches was used as an indicator of their age.

2.2.3 DNA extraction, amplification and fragment length analysis

Plant and fungal DNA was extracted from single root tips excised from within *Rhizopogon* spp. tubercles using a Qiagen DNeasy Plant kit, a MoBio Power Plant Pro kit or a Machery Nagel NucleoSpin® Plant II Maxi kit using the manufacturer's protocol. Fungi were genotyped at seven microsatellite loci using primer sets developed by Kretzer et al. (2000; 2003a) and amplified in two multiplex PCR reactions (Rv15, Rv46, Rve2.10, and Rv53, Rve1.34, Rve2.77, Rve3.21), which were described by Beiler et al. (2010). The reactions were evaluated using a 1 % agarose gel; successful amplifications were analyzed with a capillary sequencer (3130XL genetic analyzer, Applied Biosystems, Foster City, USA) and identified to genotype using GeneMapper software (V4.0, Applied Biosystems). The GenalEx software add-in for Excel (Peakall and Smouse 2012) was used to find 100 % multi-locus matching samples within each site and to identify sample pairs that differed from each other at a single locus. Samples that were unmatched or differed at a single locus were manually screened and were compared to check for missed calls. Genotypes were delineated to *Rhizopogon* species using an AluI digest of the ITS region following the criteria developed by Kretzer et al. (2003b). The *R. vesiculosus* genotype samples were then amplified with two additional species-specific primers (Rve1.21 and Rve2.44) to ensure sufficient resolution (Kretzer et al. 2003a; Beiler et al. 2010). GenalEx software was used to calculate the multilocus probability of identity value for each plot.

2.2.4 Data analysis

Simple linear regression was used to examine the relationships between the DF regeneration gradient and the response variables: number of *R. vesiculosus* or *R. vinicolor* tubercles, genotypes, and the relative abundance of tubercles and

genotypes for each species. The assumption of normality of errors was not met in the regression models with the number of *R. vinicolor* tubercles and number of *R. vesiculosus* genotypes as response variables, so Spearman's rank correlation coefficient was used to examine the relationships between these variables and the DF regeneration gradient. Data analysis and visualization was conducted in R, with $\alpha=0.05$ (R version 3.1.0, R Core team 2012) and the R package ggplot2 (Wickham 2009).

2.3 Results

In total, 82 genetically distinct genotypes of *R. vinicolor* and 66 genotypes of *R. vesiculosus* were identified from the 1178 tubercle samples. The probability of different individuals having identical genotypes by chance in each plot was low, ranging from $5.8\text{E}-6$ to $1\text{E}-5$ for *R. vesiculosus* and $3.20\text{E}-04$ to $3.00\text{E}-02$ for *R. vinicolor*. Plots varied in the number of fungal tubercles collected (34–251), fungal genotypes (6–25), and the number of DF root genotypes encountered (24–141) (Fig. 1). The number of *R. vesiculosus* tubercles correlated positively with an increasing proportion of DF in a regenerating phase ($y=1.32x-3.65$, $r^2=0.32$, $p=0.055$). A Spearman's rank correlation test showed no significant correlation between *R. vinicolor* tubercle number and the DF regeneration gradient ($\rho=0.042$, ρ critical= 0.587 ($\alpha=0.05$), $p=0.897$). The number of *R. vesiculosus* genotypes was variable among plots and was not significantly correlated with the DF regeneration gradient (Spearman's rank correlation, $\rho=0.415$, ρ critical= 0.587 ($\alpha=0.05$), $p=0.180$). In contrast, there was a significant negative correlation between the number of *R. vinicolor* genotypes and an increasing proportion of DF in a regenerating phase ($y=-0.13x+11.8$, $r^2=0.43$, $p=0.020$). In summary, when the plots were ranked by the percent of DF of <50 years old, a pattern in tubercle number appeared for *R. vesiculosus*, whereas a pattern in genotype number appeared for *R. vinicolor* (Fig. 1a, b).

When we accounted for the potential influence of one species on the other and expressed the number of *R. vesiculosus* tubercles and genotypes as a relative abundance of the total tubercles and genotypes of both species, significant trends for both tubercles and genotypes became clear. The percent of genotypes that were *R. vesiculosus* were positively correlated with an increasing proportion of DF in a

regenerating phase ($y=12.3+0.89x$, $r^2=0.499$, $p=0.010$; Fig. 2a), whereas those with *R. vinicolor* were negatively correlated. Similarly, the percent of tubercles that were *R. vesiculosus* were positively correlated with an increasing proportion of DF in a regenerating phase ($y=3.57+1.08x$, $r^2=0.433$, $p=0.020$; Fig. 2b), whereas those with *R. vinicolor* were negatively correlated.

2.4 Discussion

Both *R. vesiculosus* and *R. vinicolor* are known to associate with roots from DF of all ages (Beiler et al. 2010), but to our knowledge, there have not been any studies that have investigated relationships between the abundance of *Rhizopogon* spp. and forest age structure. Our initial prediction was that neither the number nor the relative abundance of *R. vesiculosus* and *R. vinicolor* tubercles and genotypes would be altered by differences in stand age structure among study plots. On the contrary, the DF regeneration gradient, or ecosystem factors related to DF regeneration, influenced the population dynamics of *R. vesiculosus* and *R. vinicolor*. Previous studies in a DF forest, comprised of 40 to 80 year-old trees, reported higher frequencies of *R. vesiculosus* tubercles per unit area compared with *R. vinicolor* and a greater number of *R. vinicolor* genotypes per unit area compared with *R. vesiculosus* (Kretzer et al. 2005; Dunham et al. 2013). In our study, the number of *R. vesiculosus* tubercles varied among plots, and *R. vinicolor* accounted for the majority of tubercles encountered in five out of 12 plots. Simple linear regression indicated that an increasing abundance of *R. vesiculosus* tubercles strongly corresponded with an increasing proportion of DF in the stand that were <50 years old. We also found a significant negative correlation between the number of *R. vinicolor* genotypes and an increasing proportion of DF <50 years old in the stand. Thus, the number of *R. vesiculosus* tubercles increased, whereas the number of *R. vinicolor* genotypes decreased along the DF regeneration gradient.

Vertical partitioning has been documented for these species, where *R. vesiculosus* appeared to be excluded from the upper soil strata whenever *R. vinicolor* was present (Beiler et al. 2012). Furthermore, Mujic et al. (2016) inoculated DF seedlings with both species and found that *R. vesiculosus* dominated the root tips, except when multiple *R. vinicolor* genotypes survived. In those cases, *R. vinicolor* was

more dominant. The idea that the coexistence of these species could be influenced by competitive interactions or niche differentiation prompted us to examine *R. vesiculosus* and *R. vinicolor* tubercles and genotypes as a relative abundance of the two species. In these regression models, we found opposing relationships for the two species, where the relative abundance of *R. vesiculosus* tubercles and genotypes was positively correlated with the DF regeneration gradient and *R. vinicolor* was negatively correlated. This suggests that the realized niches of *R. vesiculosus* and *R. vinicolor* may differentiate not only in space but also over time or stages in forest development. Peay et al. (2011) have presented root density and hyphal exploration types as predictors for zonation in ectomycorrhizal communities. Both species in our study are considered long distance exploration type (Agerer 2001), but *R. vesiculosus* genets are known to extend decameters in the soil (≤ 20.9 -m span) and likely rely on long-distance vegetative growth to colonize root tips, whereas genets of *R. vinicolor* are smaller (≤ 12.1 -m span) and are hypothesized to allocate more resources to spore production (Kretzer et al. 2005, Beiler et al. 2010). According to Richardson et al. (2003), live root biomass (kg/m^2) is significantly lower beneath regenerating clumps of interior DF than root density beneath mature DF with no tree understory. Peay et al. (2011) suggest that young trees with low root density are typically colonized by fungi with abundant spore inoculation and short distance hyphal exploration (in our study, this would be *R. vinicolor*) whereas mature trees with higher root density would be colonized by long distance exploration species (in our study, the longer distance species, *R. vesiculosus*). In contrast, we encountered more genets of *R. vesiculosus* in stands with a greater proportion of regenerating trees (low root biomass) and more *R. vinicolor* in mature stands with high root biomass. Therefore, the broad ecological concept suggested by Peay et al. (2011) does not clarify the zonation we observed between closely related species.

Although we found a relationship between tubercles and the DF regeneration gradient, we do not know if a greater abundance of *R. vesiculosus* directly supports survivorship of young DF, or vice versa. Evidence supporting a direct relationship includes the facilitation of seedling recruitment in DF forests by ectomycorrhizal fungi, where “*R. vinicolor*” (*R. vesiculosus*/*R. vinicolor* complex) was the second most abundant species colonizing seedlings (Teste et al. 2010). In inoculation trials, “*R.*

vinicolor” has been shown to promote seedling growth and survival (Castellano and Trappe 1985) and to increase the net photosynthesis rates and stomatal conductance of inoculated seedlings (Dosskey et al. 1991). However, these studies did not differentiate between *R. vesiculosus* and *R. vinicolor*, so it is unknown which species was being observed or used in inoculation trials. It is possible that the two species differ in their effects on DF seedling recruitment, growth, or survival, and further research in this area is warranted. Alternately, it is possible that environmental conditions that support the development of young DF are the same conditions that give *R. vesiculosus* a competitive advantage over *R. vinicolor*, and vice versa. The environmental conditions we observed at the sites, which support young DF, were moderately disturbed by elk or by selective logging equipment and had exposed mineral soil and canopy gaps to allow for penetration of light and moisture. Several *Rhizopogon* species have been shown to respond positively to experimental manipulations mimicking disturbances such as fire (Izzo et al. 2006, Peay et al. 2009). “*R. vinicolor*” tubercles have also been found in great abundances following forest clear-cutting, thinning, or burning (Molina et al. 1999). Again, no differentiation was made between *R. vesiculosus* and *R. vinicolor* in these studies, so further research is needed to determine if these species respond differently to disturbance or habitat conditions that support DF regeneration.

In summary, we found that the number of *R. vesiculosus* tubercles increased, whereas the number of *R. vinicolor* genotypes decreased along the gradient of increasing DF regeneration. We do not know what is driving these relationships. Further research investigating the respective life-spans of *R. vesiculosus* and *R. vinicolor* individuals, their response to disturbance, and their interactions with each other and other soil biota in controlled experiments is needed to better understand the dynamics of these closely related species and their co-occurrence through every developmental stage of DF forests. Our findings highlight the idea that considerable autecological trait variation may exist between closely related ectomycorrhizal fungi, which should be considered when assigning functional roles based on exploration type. Failing to distinguish between cryptic species likely confounds attempts to measure and determine functional attributes of ectomycorrhizas.

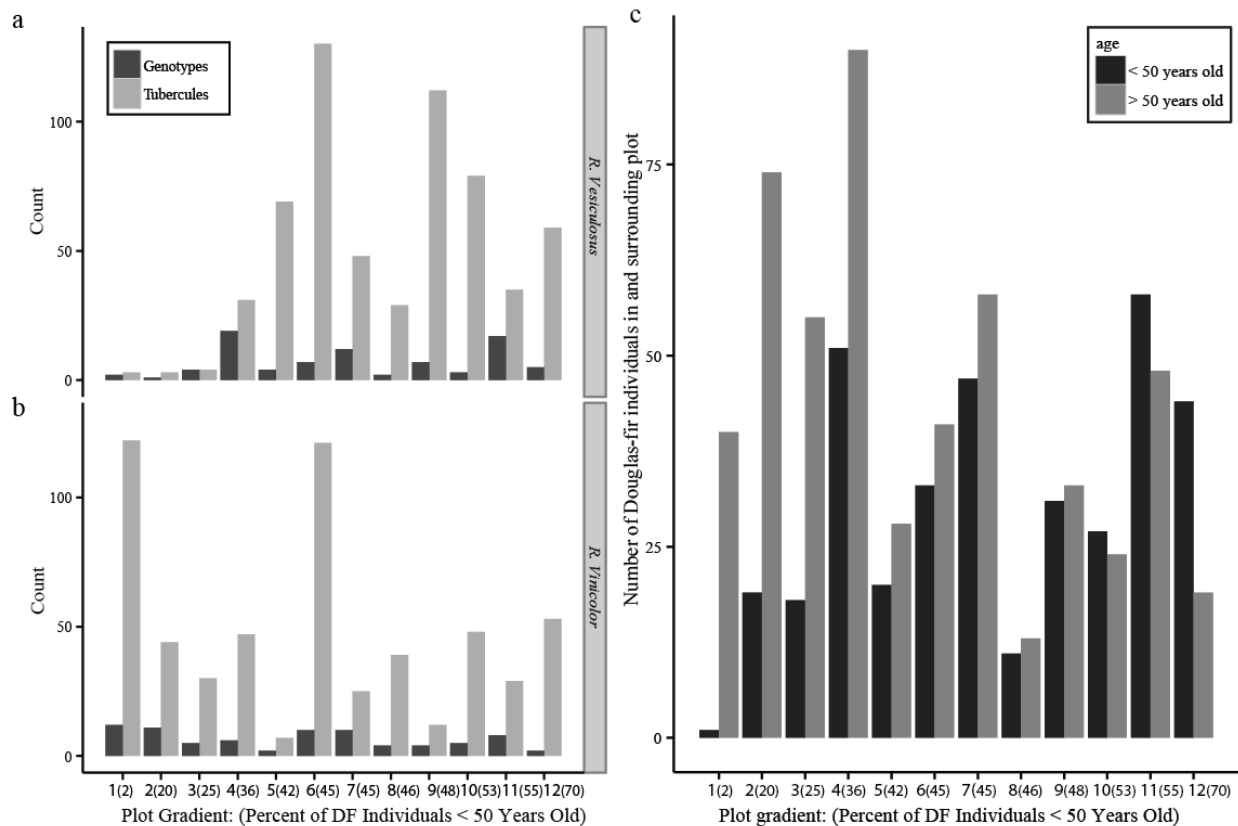


Fig. 2.1 Counts of *Rhizopogon vesiculosus*, *R. vinicolor*, and Douglas-fir in each plot, arranged on the x-axis as plot number according to the percentage of DF<50 years old (% in parentheses). a The number of distinct genotypes and tubercles of *R. vesiculosus* in each plot. b The number of distinct genotypes and tubercles of *R. vinicolor* in each plot. c The number of <50 year old and >50 year old DF trees that potentially have roots in each plot.

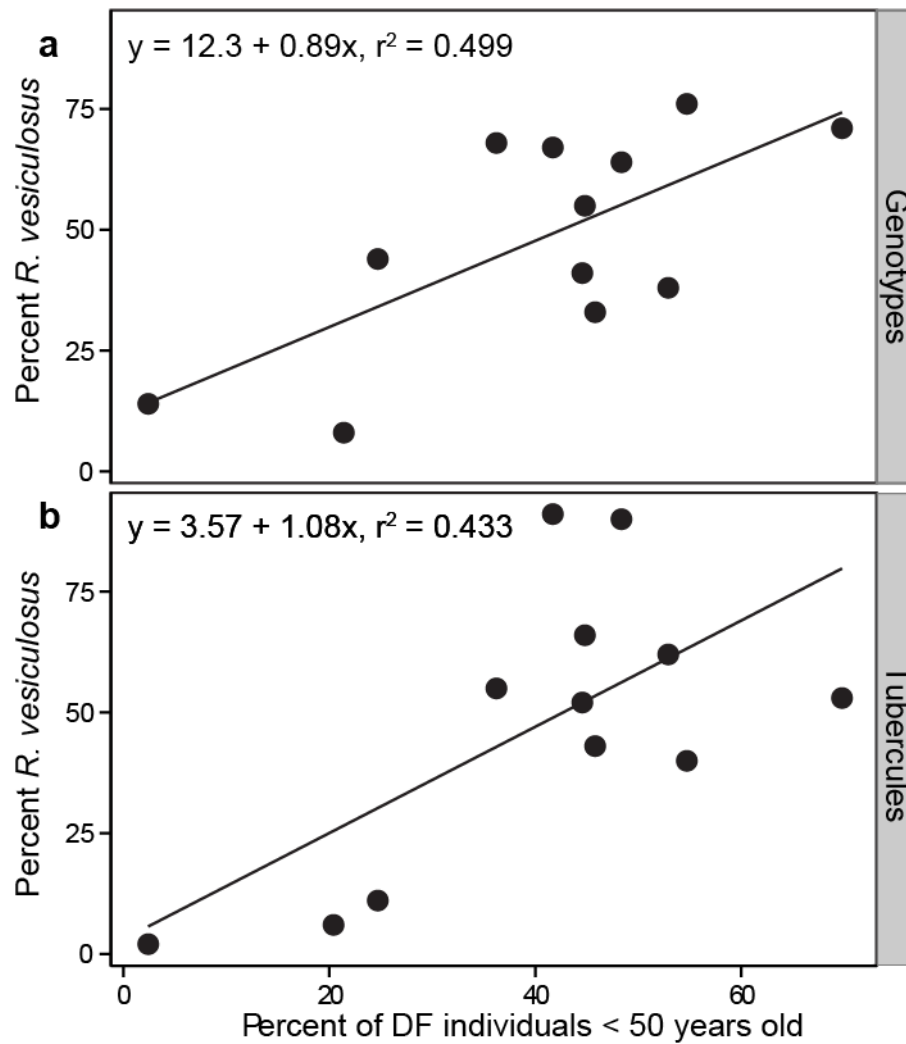


Fig. 2.2 *Rhizopogon vesiculosus* dominance in relation to forest age distribution. a Regression between the percentage of all tuberculate ECM genotypes that are *R. vesiculosus* and the percentage of DF < 50 years old ($p=0.010$). b Regression between the percentage of all tubercles that are *R. vesiculosus* and the percentage of DF < 50 years old ($p=0.020$).

3. Resilience of *Rhizopogon*-Douglas-Fir mycorrhizal networks 25-years after selective logging.

3.1 Synopsis

Mycorrhizae are abundant and influential in most terrestrial belowground ecosystems. In trying to understand their function and strategy, it has been demonstrated that many mycorrhizal fungi associate with the roots of more than one plant species or plant individual, which results in the formation of a belowground network of plants and fungi termed the Common Mycorrhizal Network (CMN). Numerous studies have demonstrated that being connected to the CMN influences plant survival (Teste & Simard 2008, Song et al. 2010, 2015). Ectomycorrhizal fungi can be part of the CMN, but the physiology of some species allows us to study the mycorrhizal network (MN) formed between individual species and even individual organisms. *Rhizopogon* fungi have specialized rhizomorph mycelium that allow these fungi to transport water and nutrients long distances (Brownlee et al. 1983, Egerton-Warburton et al. 2007) and to form networks by colonizing multiple trees (Beiler et al. 2010, Beiler et al. 2011). The large extending mycelial network is likely why Douglas-fir and pine trees with *Rhizopogon* mycorrhizae have increased seedling survival and drought tolerance (Parke et al. 1983, Dosskey et al. 1991). Two closely related sister species within the *Rhizopogon* genus, *R. vinicolor* and *R. vesiculosus* form distinctive ectomycorrhizae called tubercles, which are fungal tissue wrapped around a proliferation of tree roots that are colonized by the fungus. These distinctive connections of the tree root with the fungus make these fungal species and DF a model system for studying belowground networks at the scale of species and individual interactions (Beiler 2010).

In the past two decades, advances in computational systems allow many disciplines to describe complex systems using network theory. Network analysis is a set of tools to describe systems with interacting components and quantify the importance of the components and the interactions. These theories have been used to describe the internet, social networks, cellular pathways, and ecosystem interactions between different species plants and the insects which pollinate them or mycorrhizal fungi and

their plant hosts (Bray 2003; Southworth et al. 2005). In analyzing the networks, one can identify the most influential components, the most vulnerable components of the system and the most efficient pathways through the system.

Network theory can be applied to ectomycorrhizal networks by assigning the trees as the node, which are linked by fungal individuals (genets) that colonize roots of more than one tree. Alternatively, one could assign the fungal individuals (genets) as the nodes, which are linked to other fungal individuals by common association with the roots of the same tree (Southworth et al. 2005). In the case of the mycorrhizal network of *R. vinicolor*, *R. vesiculosus* and Douglas-fir (DF) (*Pseudotsuga menziesii* var. *glauca*), trees can be viewed as nodes and the fungus individual as links (Beiler et al. 2010). Networks can be compared to each other by comparing the density of links, the number of subgroups, the number of unconnected individuals, and the ratio of highly to barely connected nodes. These comparisons reveal which networks are vulnerable to disturbances, and if networks formed by the same species have a different form under different conditions (which may result from different forest management practices). Previous research (Beiler et al, 2010, Supplemental Information) suggested that the largest trees in a forest are network hubs, where many different fungal genotypes are connected by forming mycorrhizae on the roots of the same tree. Using a model, Beiler demonstrated that when < 20% of trees were randomly removed that MN connectivity remained intact, but when hub trees were selectively removed, the MN connectivity was reduced. In our field study, the main objective was to determine the resilience of MNs after 25 years following removal of hubs. We predicted that the targeted removal of mature trees would have a significant lasting impact on the network; the mycorrhizal network of the harvested forest would have reduced connectedness as compared to an unharvested neighbouring forest.

3.2 Materials and Methods

3.2.1 Site Description

The study was conducted in the Interior Douglas-fir Cascade Dry cool (IDFdk2) Biogeoclimatic subzone variant, North of Kamloops, British Columbia. The mean annual precipitation is about 400mm and the mean annual temperature 5.7 °C (1981-2009

Climate BC). The plots are dominated by DF trees and a richly developed herbaceous understory layer with *Calamagrostis rubescens* (pine grass). As is characteristic of this forest type, the trees are a range of age and diameter classes (1-223 years, <1cm to 119cm DBH).

Six 10m diameter circular plots were established (as in chapter 2), 3 of which were located in logged gaps (selective logging occurred 25 years ago). The foresters removed 12-18% of mature trees to create gaps in the forest for variable tree density habitat for mule deer. These logged plots had significant recruitment of young DF seedlings and saplings which had regenerated in the last 25 years (Table 1). The other 3 plots were selected within an adjacent forest that was not logged and had no trees that were <15 years old (Table 1). Plots were between 150 and 1000m apart around 50°55'19.86"N, 120°17'37.09"W, at 900-1000m elevation (Illustration 3.1)

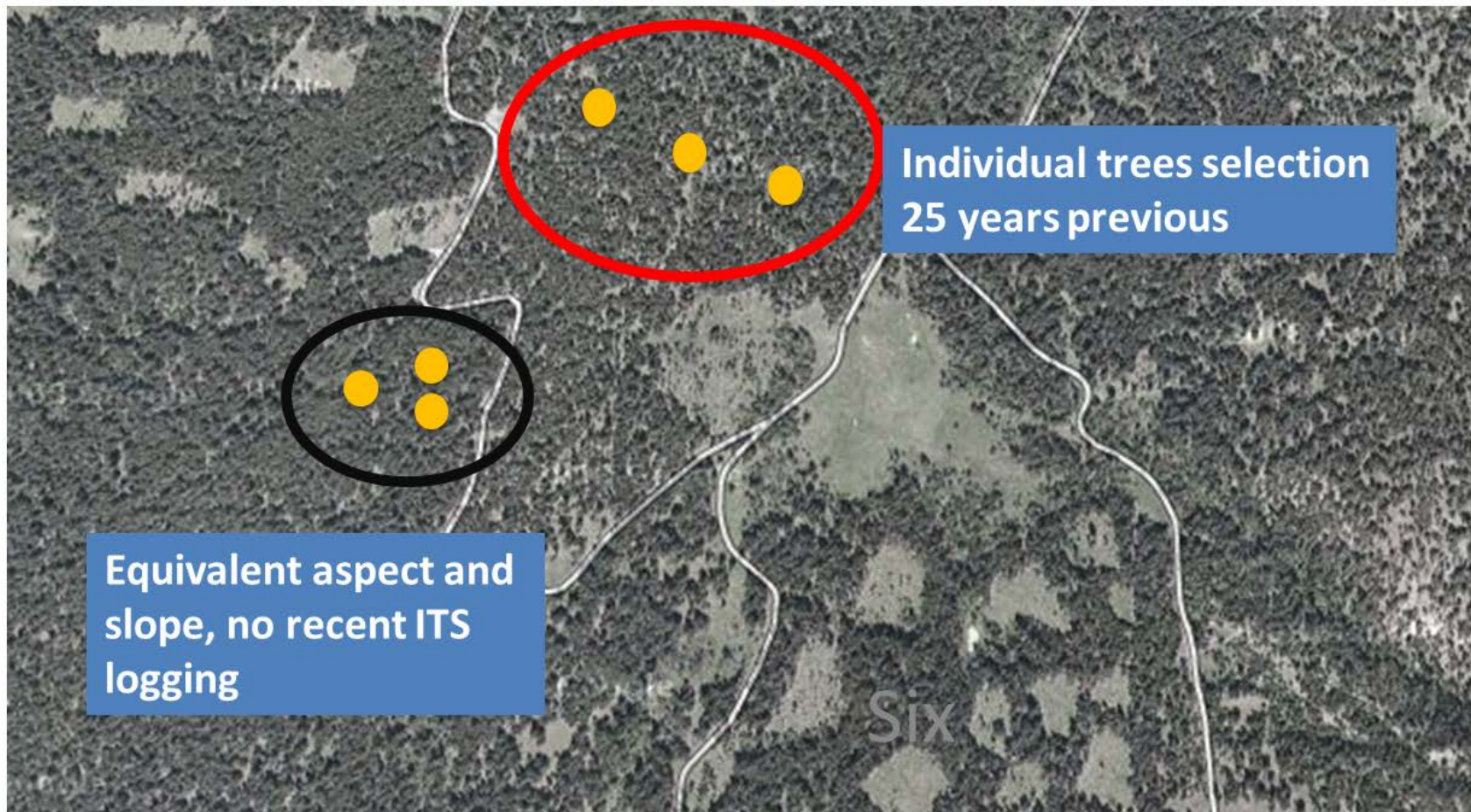


Illustration 3.1 Sample Sites: Aerial photograph with overlaid circles marking the location of 6 plots. The road visible in the bottom right quadrant of the image is Black Pines Road, North of Kamloops BC (off Jamieson Creek) The Plots were between 150 and 1000m apart around 50°55'19.86"N, 120°17'37.09"W, at 900-1000m elevation.

3.2.2 Field Sample Collection

Rhizopogon tubercles were collected from the soil surface to a depth of 30 cm wherever encountered within the plot. Initial sampling targeted micro-habitats where tubercles have been found to proliferate in the past, such as the underside of rocks and within coarse woody debris. In the remainder of plot, 10% of the forest floor was excavated to 30 cm depth to evenly sample in the areas which were not initial sampling sites. The plots were sampled in May through July 2011 for 150 hours/plot. When multiple tubercles were found on the same root and observed to be connected by fungal rhizomorphs, they were treated as one sample. If the physical connection could not be visually confirmed, multiple tubercles in the same location were stored and labeled separately.

The location of each tubercle collected was marked with a flag and the depth, microsite characteristics and position in the plot was recorded. The entire tubercle was collected in a 2mL micro-centrifuge tube, labelled, stored in a cooler, and transferred to a freezer (-20°C) awaiting DNA extraction. Tree needle tissue or cambium tissue was collected to create a tree DNA library to match the sampled roots from tubercles to their above ground tree. Needle tissue was collected after bud burst but before the needles elongated from all trees with accessible needles. Needle tissue was stored in a 1.5 mL micro-centrifuge tube until DNA extraction. We sampled cambium from all trees whose needles were inaccessible. To collect cambium we removed bark and collecting a 9.5 mm punch of tree cambium, which was immediately frozen on dry-ice and stored at -80°C until DNA extraction.

At the completion of the sampling period, all tubercle and tree locations were measured for mapping using an Impulse laser and digital compass (Laser technology Inc., Centennial Co, USA, by Forestec Forestry Consulting, Kamloops BC). The coordinates were adjusted for slope and tree diameter. Tree core samples were collected from all trees in the plot and within 25m of the plot edge. Rings were counted under a dissecting microscope to determine tree age ± 5 years for categorizing into cohorts (0-15, 16-50, 51-85, <86 years). All seedlings and saplings (<15yrs) within and

surrounding the plots were recorded and the number of whorls of branches were used as an indicator of tree age.

3.2.3 DNA extraction, Amplification and Fragment Length Analysis

At the time of DNA extraction, tubercle samples were thawed and viewed under a dissecting microscope to collect a single root tip free from soil and peridium hyphae. DNA was extracted from root tip samples with the Qiagen DNeasy Plant kit, the MoBio Power Plant Pro kit or the Machery Nagel NucleoSpin® Plant II Maxi kit and their suggested protocols. The DNA from the needle tip samples (approx. 5 g of tissue) was extracted using the MoBio Power Plant Pro Kit. The tree cambium tissue samples were frozen in liquid nitrogen and crushed in a MoBIO 96 plate shaker (Cat. 11996, MoBio MO BIO Laboratories, Inc. 2746 Loker Avenue West Carlsbad, CA 92010), then DNA was extracted using the Machery Nagel Nucleospin II 96 kit.

The DNA from tubercles was amplified using 7 primer sets developed by (Kretzer et al. 2000, Kretzer, et al. 2003a) for *Rhizopogon vinicolor* in two multiplex PCR reactions previously developed (Rv15, Rv46, Rve2.10, and Rv53, Rve1.34, Rve2.77, Rve3.21) (Beiler et al. 2010). The success of the reaction was evaluated using a 1% agarose gel and successful reactions were submitted to the Fragment Analysis and DNA Sequencing Service (FADSS) at the University of British Columbia Okanagan for fragment analysis with a capillary sequencer, (3130XL genetic analyzer, Applied Biosystems, Foster City, USA) and GeneMapper software (V4.0, Applied Biosystems). Based upon the results of these seven primers, the samples that had the same fragment lengths at all primers were grouped into fungal genets. Two representative samples of each genet were processed further to determine *Rhizopogon* species using an ALU1 digest of the ITS region following the criteria developed by Kretzer (Kretzer et al. 2003b). The *R. vesiculosus* genet samples were then further amplified with an additional two *R. vesiculosus*-specific primer sets (Rve1.21 and Rve2.44) to ensure sufficient resolution of those genets (Kretzer et al. 2003a, Beiler et al. 2010). The tubercle DNA was also amplified using three highly polymorphic primers developed by Slavov et al. (2004) to identify the DF root portion of the tubercle. These same primers were used to amplify both needle and cambium DNA.

3.2.4 Data Analysis

The fragment lengths of all the samples were determined using GENEMAPPER v4.0 (Applied Biosystems, Carlsbad, CA, USA). The GenalEx software add-in for Excel (Peakall & Smouse 2012) was used to find 100% multilocus matching samples within each site and to identify sample pairs which differed from each other at a single locus. Samples which were unmatched or differed at a single locus were manually screened and compared. When samples were found to have genotypes deviating by a single heterozygous allele, the fragment analysis output was manually re-examined. In cases where the difference was likely due to allelic drop-out (because the magnitude of the second signal was low) the samples were grouped and were referred to as a single genotype.

3.2.5 Network Analysis

All tree genotypes, having roots within the plot, were counted as nodes for network analysis. A common fungal genotype shared between two different tree genotypes was considered a link. The networks of trees in each plot were analyzed using Pajek version 3.0 (Batagelj and Mrvar 1998, Nooy et al. 2005). The 3 plots that were un-harvested had very few *R. vesiculosus* tubercles (see chapter 1). Thus network analysis was based only on networks formed by *R. vinicolor*, because they were well represented in both treatments. Because the number of *R. vinicolor* tubercles and genets were not evenly distributed between the sites, we used a null model comparison. For each plot, 1000 Erdos-Renyi random networks were generated with the same number of nodes and links as the sampled network density. Each of the random networks was analyzed, and were compared with the mean of the 1000 random networks to calculate the standard effect size. The mean standard effect size was used to test if the MN network parameters of logged forest were different to those of unlogged forests. The robustness of each plot network was evaluated using Pajek to find subcomponents and the articulation points of those sub components.

3.3 Results

In total, 420 tubercule samples were successfully identified as one of 47 genetically distinct genets of *R. vinicolor* or 38 genets of *R. vesiculosus*. The tubercules were also matched to 163 genetically distinct tree roots. The probability of different individuals having the same multi-locus fungal genotype by chance was low (1.8×10^{-6}) and for tree primers it was higher (1.8×10^{-3}).

Beginning with the sample data (Table 3.1), the total number of tubercules sampled did not differ significantly between non harvested and harvested plots. The number of *R. vinicolor* tubercules and genets was not significantly different between the treatment and control plots. However, there were significantly more *R. vesiculosus* tubercules and genets in the harvested plots (Table 3.1, see chapter 2). Also, the harvested plots had significantly more trees (<15 years old and <85 years-old), and one could assume there would be significantly more DF roots in the harvested plots than in the non-harvested plots.

When we consider a comparison of the sampled network analysis values (Table 3.2), the density of links was significantly different between the harvested plots and the non-harvest plots. The non-harvested plots had more links per node resulting in a more densely connected *R. vinicolor* - DF MN. The density and average node degree is foundational to all other values in the quantification of networks, so it was not surprising that the plot MNs look very different (Figure 3.1).

When the effect of density was accounted for using the null model analysis and comparing the standard effect size, our analysis to compare the architecture and connectivity of these networks revealed that the harvest plot sampled networks were not significantly different from the un-harvested plots. There was no measurable effect of harvesting on the network connectivity, centrality or diameter, clustering or distance (Table 3.2).

In the networks plots that are spatially arranged by the location of the root samples in the plots (Fig 3.1, blue lines, *R. vinicolor*), there is a pattern of vulnerability that becomes visible in the network. The network in the harvested sites appears to be

more vulnerable to fragmentation, as site 4 is already fragmented and sites 5 and 6 are each easily fragmented into two networks by the targeted removal of a single node. The subnetworks formed following this fragmentation are not easily fragmented. The non-harvested forest MN networks in plots 1 and 3 cannot be fragmented by the targeted removal of any nodes. Plot 2 can be fragmented in two by the targeted removal of two nodes. This vulnerability is related to the number of components which was significantly higher in the harvested networks than the unharvested networks sampled networks (Table 3.1). The number of components is related to network density (proportion of potential links which are real link). When the difference in density is taken into effect with the standard effect size null model analysis (Table 3.2) the number of components is not different..

When the plot networks are arranged with the nodes in a random circle (Figure 3.2), the difference in the number of roots in each plot becomes clear, and the resulting difference in network density. Plot 1 is particularly densely connected, but all three harvested plots are less densely connected than Plots 1-3.

We also performed analysis of the complete networks with both species of fungi, and found that there was variation within treatments, and no network parameters were significantly different between the un-harvested and the harvested plots (Table 3.3).

3.4 Discussion

The initial assessment of the sample data reveals that despite equal sampling, the number of tubercles and genets and the size of those genets were very different between plots of the same treatment (Table 3.1). Beiler (2011) also found that between plots there was variability in the network and there could be many factors that influence tubercle distribution beyond the test factor of selective logging activity. The only clear difference between non-harvested and harvested forest plots was that there was very little *R. vesiculosus* sampled in the non-harvested plots (as discussed in Chapter 2).

Despite this major difference, the network connectivity, centrality or density of links was not significantly different between the two treatments (Table 3.3). The number

of *R. vinicolor* tubercles and genets did not differ significantly between the treatments, so our analysis of the MN continued with the *R. vinicolor*- DF networks.

When we considered only *R. vinicolor* with DF networks, we found that the density of links in the unharvested forest was significantly higher than the density of links in the harvested forest. Many tree roots present in the non-harvested plots were not sampled as part of the DF–*R. vinicolor* network (Figure 3.2), so the number of links/node was lower in the harvested plots. This may seem to indicate that the networks in the forest where harvesting occurred have been diminished. However, because we chose to look at the forests 25 years after harvest, the forest had significant DF regeneration following the harvesting of mature trees. The young trees with smaller roots systems may not have yet encountered the *Rhizopogon vinicolor* mycelium and formed a *Rhizopogon* tubercle. *Rhizopogon vinicolor*-type fungi are dominant colonizers of DF seedlings, but they are not found 100% of the time (Twieg et al. 2007), and in this study we did not completely excavate the young seedlings and find every root in the plots. Therefore, we suggest that the difference in network density is likely related to the shift in forest age. The other confounding factor in drawing conclusions about the different reduction in link density is that this evaluation of density does not include the density of links in the DF- *R. vesiculosus* MN. We know that the two species do not tend to colonize the same space in the soil (Beiler et al. 2012, Mujic et al. 2016).

The density of links affects all other network measures at some level, so in order to evaluate whether other network architecture measures show patterns apart from density, we compared each network to 999 random networks with the same number of nodes and links. We tested if the harvested and non-harvested sampled networks as treatment groups were distinctive from the corresponding random networks. The standard effect size was not significant, so we did not discover distinctive network architecture of the non-harvested or harvested plots. The architecture of the logged forests vs unlogged forests were not different from what would happen by chance with that number of links and nodes.

Finally, when considering network resilience of *R. vinicolor*-DF networks, we did notice a trend that the harvested plot networks are more easily fragmented by the

targeted removal of a single tree. The component networks after initial fragmentation, however, are resistant to further fragmentation. The treatments were significantly different in the number of components but when density is accounted for (with standard effect size compared to null models) this difference was no longer significant. The difference in vulnerability to fragmentation may be a result of an incomplete representation of the *Rhizopogon* networks in the harvested plots. *R. vesiculosus* was not found in the un-harvested plots, so analysis could not compare networks of two fungal species with networks containing only one species. However, the species have been shown to partition in soil (Beiler et al. 2012, Mujic et al. 2016), so the *R. vinicolor* network of the harvested sites is likely reduced in some areas of the plots due to competition for roots and soil from *R. vesiculosus* genets. The presence of *R. vesiculosus* genets could exclude *R. vinicolor* from exploration and colonization, which would create fragmented components in the *R. vinicolor* network. When we examine the networks with both *Rhizopogon* species (Figure 3.1, Table 3.3), there is no longer a trend towards vulnerability in the harvested forest MN.

In summary, our results indicate that when a forest ceases to have understory regeneration of DF, the *Rhizopogon* belowground network will lose *R. vesiculosus*. Nevertheless, both the selectively harvested forest and unharvested forests have *Rhizopogon*-DF networks that are well-connected, and robust against loss of individual nodes. We found that the logged plots indeed had different MNs than unharvested plots, but we are unable to accept our hypothesis that these networks have reduced connectedness when considering both sister species of *Rhizopogon*.

Though there is not a significant treatment influence on the network connectivity, networks without *R. vesiculosus* may have reduced value to the trees involved, and less capacity to support regenerating seedlings. In previous studies *R. vesiculosus* has been found to be deeper and farther extending into the soil which may allow it to be more effective at water redistribution. Large trees access deep water sources through the established tap root, and MN have been demonstrated to move water between trees to mitigate water stress (Bingham and Simard, 2012). Further research is needed to investigate differences in water redistribution between *R. vesiculosus* and *R. vinicolor*.

This research also emphasizes the value of including multiple species to study network connectivity, but further research is needed to determine the functional contributions of all the species in a CMN, and how far those benefits are distributed through the network.

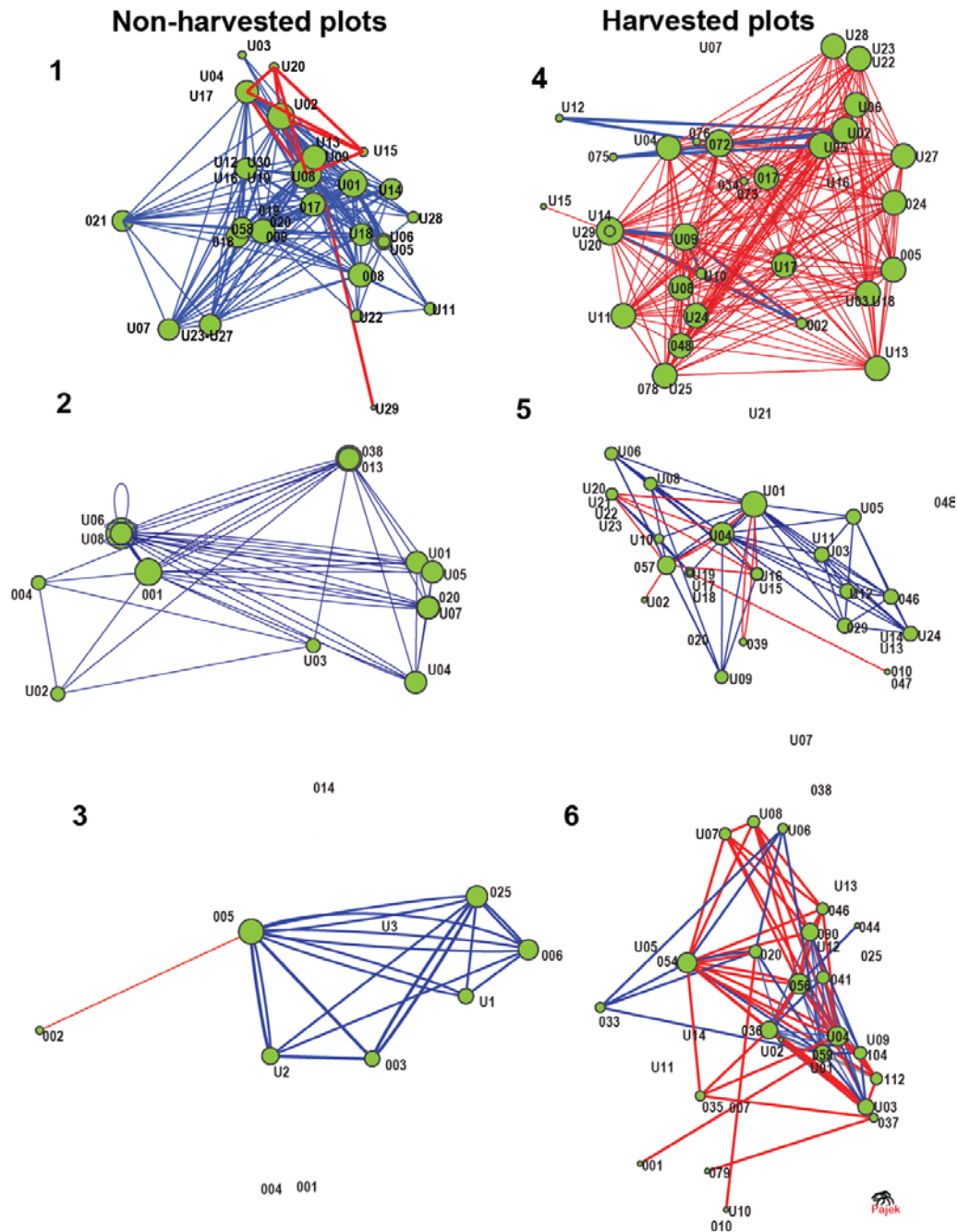


Fig. 3.1: Interaction Networks of Douglas-fir roots *R. vinicolor* (blue) and *R. vesiculosus* (red). The circular green nodes are the tree genotypes, and they are positioned according to the root location in the plot (or the average of the root positions when a single tree genotype was encountered in more than one location). The connecting lines were drawn when a fungal genotypes was identified in association with the roots of both tree genotypes.

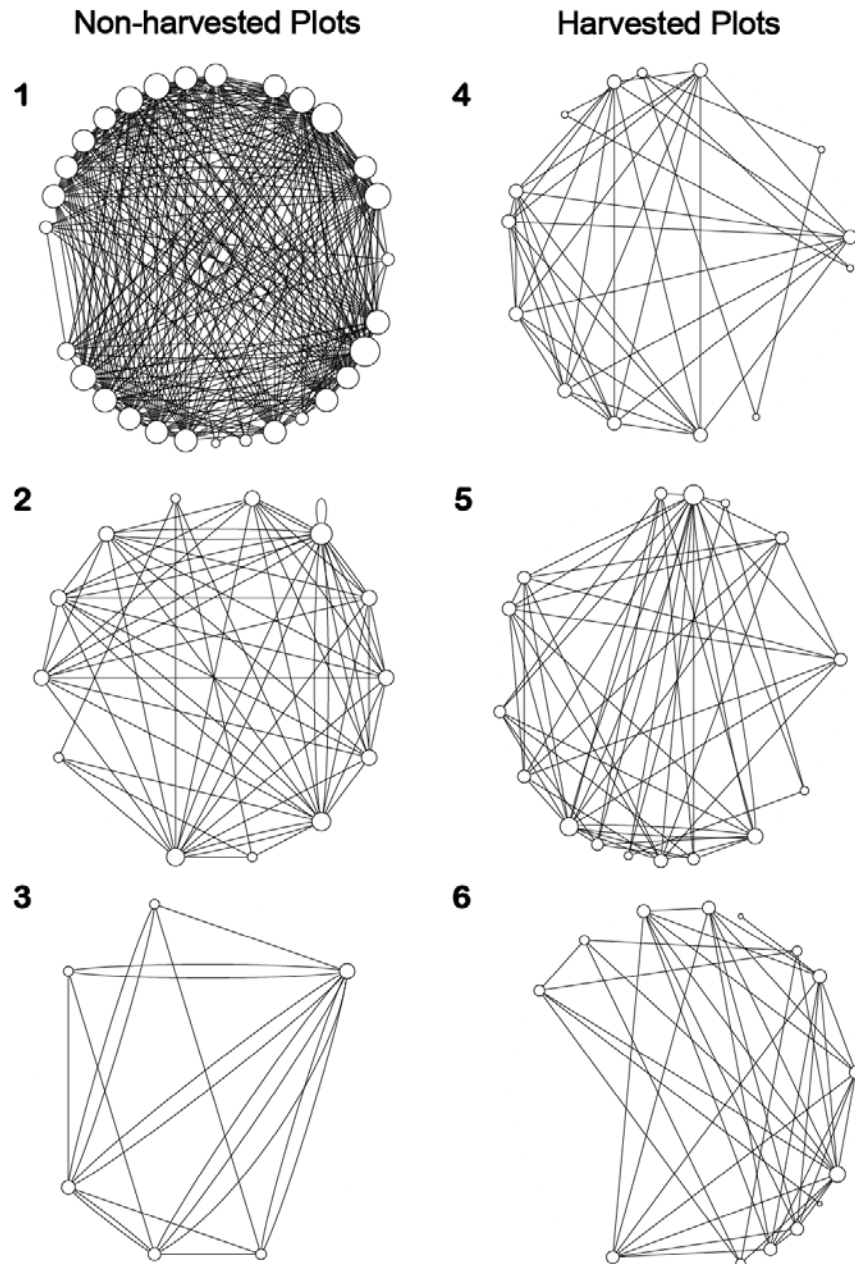


Fig. 3.2 Douglas-fir and *R. vinicolor* interaction network circular projection. Around the perimeter of the circle are the tree root genotypes encountered in the plot, positioned randomly (nodes). The diameter of the node corresponds to the degree centrality of each of tree genotype; the largest nodes are the most central to the network. Gaps in the perimeter represent trees with known roots in the plot which were not sampled as part of the network – they have no centrality so no node circle is drawn. The connecting lines represent an interaction connection and are drawn between tree genotypes when the same multilocus fungal genotype was found in association with the roots of both trees.

Table 3.1: *Rhizopogon* tubercule and DF sample data summary for each of each of six plots.

Parameter	N-Harvest Plot 1(X)	N-Harvest Plot 2 (L)	N-Harvest Plot 3 (A)	Harvest Plot 1 (J)	Harvest Plot (C)	Harvest Plot 3 (Z)	Test(2 tail alpha =0.5)	P value
Total tubercule samples	34	47	125	73	64	77	t-test	0.5126
Total genotypes	9	12	13	22	15	22	MannWhit	0.0495
<i>R. vinicolor</i> tubercules	30	44	122	25	29	26	t-test	0.2484
<i>R. vesiculosus</i> tubercules	4	3	3	48	35	51	MannWhit	0.0495
<i>R. vinicolor</i> genotypes	5	11	11	10	8	10	Mann Whit	0.5127
<i>R. vesiculosus</i> genotypes	4	1	2	12	7	12	Mann Whit	0.0495
DF root genotypes	36	14	10	37	33	33	Mann Whit	0.27523
No. of trees age <16	0	0	0	45	39	49	N/A	-
No. of trees age 16-50	19	1	18	2	19	2	Mann Whit	0.5
No. of trees age 51-85	52	17	47	27	25	38	Mann Whit	0.5127
No. of trees age >85	22	23	8	31	23	52	Mann Whit	0.0809
total trees within plot area	93	41	73	105	106	141	Mann Whit	0.0495

Table 3.2 *R. Vinicolor*-Douglas-Fir Network Analysis Data Summary. The sample values are the mean values of the network parameters, measured in three plots. The Standard Effect Size values are the parameter values compared with 999 randomly created networks with the same link number of vertices and average node degree as the measured networks.

Factor	Sample values			Standard effect Size		
	mean NH	mean H	K-W p-value	mean NH	mean H	K-W p-value
Vertices	20.00	34.33	0.28			
Edges	131.33	39.33	0.51	1.65	0.06	0.05
Multiple lines	12.00	0.33	0.08			
Density loops	0.41	0.14	0.05	0.01	12.11	0.51
Density no loops	0.44	0.07	0.05	0.01	0.01	0.51
Average node degree (links/node)	9.82	2.35	0.05			
Average degree (no multiple.lines)	8.74	2.33	0.13	0.05	0.79	0.83
#Components	4.00	22.33	0.05	8.48	9.44	0.83
Size of largest component	17.00	11.67	0.66	-8.19	-8.41	0.83
%Largest component	29.25	13.35	0.28	-8.09	137.51	0.51
Highest degree	17.33	10.33	0.51	6.54	4.47	0.83
Degree centrality (no m. lines)	0.44	0.26	0.05	3.40	4.74	0.83
Diameter	2.00	2.33	0.51	-1.01	-2.89	0.13
Average distance	1.36	1.43	0.51	-3.99	-3.46	0.83
Clustering Watts-Strogatz (no m. lines)	0.90	0.94	0.51	9.72	14.75	0.51
Network clustering coefficient	0.83	0.84	0.83	9.21	15.51	0.51
Betweenness centrality (no m. lines)	0.19	0.06	0.13	7.41	-1.16	0.28

Table 3.3 *R. Vinicolor* and *R. vesiculosus* Douglas-Fir Network Analysis Data Summary. The sample values are the mean values of the network parameters, measured in three plots.

Factor	Sample values						Mean Values		
	NH Plot 1(X)	NH Plot 2(L)	NH Plot 3(A)	Harvest Plot 4 (J)	Harvest Plot 5(C)	Harvest Plot 6(Z)	mean NH	mean H	K-W p-value
Vertices	36	14	10	37	33	33	20	34.333	0.275
Edges	322	52	22	299	78	84	132	153.667	0.513
Multiple lines	27	1	8	3	4	6	12	4.333	0.513
Density loops	0.497	0.531	0.28	0.432	0.136	0.143	0.436	0.237	0.127
Density no loops	0.511	0.571	0.311	0.444	0.140	0.148	0.465	0.244	0.127
Average degree	17.889	7.429	4.4	16.162	4.727	5.090	9.906	8.660	0.827
Avg degree (no m. lines)	16.389	7.286	2.8	16	4.485	4.727	8.825	8.404	1
#Components	5	2	3	5	11	8	3.333	8	0.081
Size of largest component	32	13	8	33	22	26	17.667	27	0.275
% Largest component	0.889	0.929	0.80	0.89	0.667	0.79	0.873	0.782	0.275
Highest degree	44	12	7.00		20.00	14	21	17	0.655
Degree centrality (no m. lines)	0.412	0.423	0.583	0.323	0.516	0.308	0.473	0.383	0.275
Diameter	3	2	2	3	3	5	2.333	3.667	0.127
Average distance	1.417	1.346	1.5	1.48	1.72845	2.157	1.421	1.788	0.127
Clustering Watts-Strogatz(no m. lines)	0.930	0.969	0.777	0.970	0.896	0.748	0.892	0.871	0.827
Network clustering coefficient	0.891	0.932	0.574	0.950	0.646	0.613	0.799	0.736	0.827
Betweenness centrality (no m. lines)	0.082	0.346	0.307	0.152	0.217	0.118	0.245	0.162	0.513

4. Conclusion and Further Research

The purpose of this research was to build upon the previous research of Beiler et al. 2010 and on the hypothesis that the removal of hub trees through selective logging would have a significant and negative impact on the connectivity of *Rhizopogon*-DF networks. We did not study the immediate impact of tree removal on network connectivity, but instead selected plots where logging had occurred 25 years ago in order to assess the networks at a time when the forest had recovered from the initial soil disturbance. The sampling and analysis revealed two discoveries that add to the body of knowledge about *R. vesiculosus* and *R. vinicolor*-IDF networks. These discoveries were presented in chapters 2 and 3 of this thesis.

In the research described in the first chapter, we discovered that *R. vesiculosus* was significantly less abundant in a forest with no regenerating seedlings, and when we expanded the analysis to include Beiler's sample data, we found a significant negative correlation between the age of the forest and the relative abundance of *R. vesiculosus*. Other studies, which differentiated between these two species had not been conducted in forests with little regeneration, and had found both *Rhizopogon* fungi to be well represented in all plots (Dunham et al. 2013, Beiler et al. 2015, Kretzer et al. 2005, Kretzer et al. 2005). A limitation of our study is that the plots of no regeneration and regeneration were grouped, instead of being interspersed. There may have been other factors which influenced the lack of *R. vesiculosus* related to the area of forest where the three older forests were located. Further research is needed to qualify this finding in other forests. The ranges of *R. vesiculosus* and *R. vinicolor* within the range of DF have not been established, and it may be that both species are not present in all ecotypes where DF is established. This finding also suggests new research could consider the functional differences between these two closely related species of fungi, and if that is at all related to tree age. The genomes of these two species have recently been sequenced so it is now possible to examine these questions at the genetic level (Grigoriev IV et al. (2014)).

The research presented in the second chapter revealed that the network structure of the complete networks sampled in 10m circular plots is very diverse in the

number of fungal genotypes, tree genotypes, the number of links, the density of links and the number of components. This diversity was across treatments, and not specifically related to the selective logging treatment. What was significantly different was that the logged forests had two species forming the network and the unharvested forest network was connected by a single species. When the *R. vinicolor* DF network was compared across treatments, the density of links was significantly higher in the unharvested forest. When I accounted for the effect of the density difference with the null model analysis, there were no significant differences between the harvested and unharvested network metrics. The *R. vinicolor*-DF networks of the harvested plots also appear to be more easily fragmented with the targeted removal of just one or two nodes, but when *R. vesiculosus* is included in the networks, the harvested plot networks are no longer easily fragmented. This indicates that forest capacity to be well connected by the *Rhizopogon* –DF networks was not significantly reduced or compromised by the selective removal of mature trees. This study is dependent upon length based measures of DNA fragments to differentiate species and individuals. The RFLP method was not tested against morphological species typing in this study. If the ALU 1 RFLP cutting reaction fails, samples would be considered *R. vesiculosus*. We found an absence of *R. vesiculosus* in a subset of the data so do not believe this was a problem in our research, but independent morphological classification of the samples would strengthen our findings. The microsatellite methods in fungi are limited because we do not know that the same genotype is actually physically connected in the forest. The limits of this methods bias towards grouping fungus into the genotypes which may bias the research towards larger genets with more connections. With advances it may be possible to add expressed sequence tags to individual fungi in order to determine their actual connectivity across the landscape. Further research is required on the longevity of fungal individuals in disturbed and undisturbed forests, as it would clarify to what extent networks recover or are newly established after selective logging. Further research is also required on forest areas that have been selectively logged but do not have DF regeneration in order to determine whether regeneration success is related to a well-connected MN.

This research also contributes to the growing body of knowledge for sustainable forest management. The post-harvest forests where we sampled were healthy examples of natural regeneration and had well connected networks with the *R. vesiculosus* and *R. vinicolor* fungi. Previous research indicates that the *R. vesiculosus* being larger (Dunham et al. 2013)) and deeper (Beiler et al. 2012), which would give it more value in network redistribution of deep soil water through hydraulic lift. Therefore, to encourage *R. vesiculosus* fungi, gaps selection logging to thin trees while leaving large deep rooted trees may reduce water stress on individuals and create light and moisture access to support regenerating seedling. Leaving these forests of large trees close together may limit their ability to survive intense competition for water sources as climate change advances, and leave us without trees to replace giants. The contribution ECM forest networks to seedling survival must be incorporated as values into the models being developed to inform silviculture decision making.

5. References

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