Mapping the wood-wide web: mycorrhizal networks link multiple Douglas-fir cohorts

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Attempts to understand forest dynamics have focused primarily on competitive interactions among trees[1], resulting in poor predictions of forest regeneration, succession and productivity in changing environments[2]. Mycorrhizal networks are increasingly recognized as mediating interactions among trees through their effects on survival, growth, and competitive ability[3,4]. However, the effects of mycorrhizal networks on whole forest dynamics remain hindered by the elusiveness of their underlying spatial structure[5,6]. Here we show that two ectomycorrhizal fungi, *Rhizopogon vesiculosus* and *R. vinicolor,* connected all tree cohorts in an uneven-aged old-growth forest, with young Douglas-fir saplings established within the extensive mycorrhizal network of old Douglas-fir veterans. The two *Rhizopogon* species each formed 13-14 genets, with each genet connecting up to 19 trees in the 30 x 30 m stand. The largest hub (i.e. most highly connected tree) was linked to 61% of the younger trees in the stand, suggesting that mycorrhizal networks played a role in facilitating understory regeneration. For the first time, our results indicate that the mycorrhizal symbiosis is not just between a single plant and its fungal partners, but involves many trees of multiple ages in a forest. Because mycorrhizal networks are important in the self-regeneration of old-growth forests[7], ignoring or re-organizing them through poor forest management practices could undermine forest resilience in the changing climate.

Mycorrhizal networks (MNs), fungal mycelial connections between two or more plants, have sparked much controversy[8] and have been the focus of recent reviews[6,8-10]. Interest in MNs has focused on their formation and function in both controlled artificial systems[11] and in natural ecosystems[12,13], but new discoveries in the field have been hindered by equivocal evidence for the existence of MNs in nature. Most recent research on MNs, for example, has focused on empirical evidence for regeneration facilitation without examining the underlying network[6-13]. Recent breakthroughs in molecular techniques, including the use of microsatellite DNA markers[14-16], now enable

us to characterize MNs to the precision necessary for the field to move forward. The application of network theory[17,18] also provides a useful framework for describing the structure, function, and ecological significance of MNs[5,6]. Structure includes the genetic complexity, architecture, and spatial extent of the network, and describing it is a prerequisite to understanding how MNs function (e.g., in fungal colonization of plants, mycelial growth dynamics, nutrient uptake or exchange by plants) and how they affect forest dynamics (e.g., tree regeneration, competition, mortality)[6,9]. Description of network architecture is based on links and nodes, and we have chosen to represent mycorrhizal fungi as links and trees as nodes or hubs because it is most relevant for understanding MN influences on Douglas-fir forest dynamics. Network architecture is thus described using the number of links a node has to other nodes (i.e. node degree), the distribution of links among nodes (i.e. degree distribution), the accessibility of links to nodes (i.e. clustering coefficients), the number of link steps separating nodes (i.e. path lengths), and the contribution of nodes to network connectivity with respect to their topological position (centrality)[5,17,19]. In regular and random networks, links tend to distribute equally among nodes, whereas scale-free networks have some nodes (i.e. hubs) that are highly linked and more central to the network[17,19]. Overall, the structure of a MN is indicative of its robustness, such that a scale-free network is vulnerable to targeted attacks but can maintain its integrity with random perturbations[17,19], and can efficiently shuttle resources to an expanding mycelial frontier or to regenerating seedlings.

The old-growth interior Douglas-fir forests (*Pseudotsuga menziesii* var. *glauca*) where we studied MN structure undergo gap-phase regeneration[1,20], resulting in self-perpetuating, multi-cohort climax forests[21]. These forests today are struggling to regenerate after a decade of summer drought and increased disturbance severity[20,21], and their regenerative capacity and resilience to disturbance are predicted to decline further with climate change[22]. Understanding MN robustness, and how it affects forest

regeneration, may improve our predictions of forest dynamics and help us design management practices that maintain forest resilience.

In this study, we used multi-locus, microsatellite DNA markers to discriminate among individuals of interior Douglas-fir and genets of *Rhizopogon vesiculosus* and *R. vinicolor* and to characterize the structure of the MN. Using this approach, we considered a network link as the presence of a single *Rhizopogon* genet on roots of two different trees. A concurrent small-scaled study indicates that these fungal genets are continuous links rather than fractioned ramets (data not shown). Based on previous studies involving *Rhizopogon* spp.[16,23] and network theory[5], we hypothesized that 1) *R. vesiculosus* and *R. vinicolor* associate with many trees of different cohorts, 2) *R. vesiculosus* has larger genets and associates with more trees than *R. vinicolor,* and 3) older, larger trees are linked to more neighbours than are smaller trees. We also hypothesized that variable degrees of connectivity among trees (based on the size and distribution of trees) would result in a scale-free network, where some trees act as hubs and others have fewer, regularly distributed links[17,19,].

A total of 56 tree genotypes encountered as roots in *Rhizopogon* spp. mycorrhizas were successfully matched to reference tree boles based on microsatellite DNA analysis (Table 1, Fig. 1). They included 43 of the 65 trees inside the plot, with an additional 13 genotypes matching tree boles outside the plot. The trees were grouped into four cohorts ($\leq$15 years-old, n = 6 trees; 16-50 years-old, n = 38 trees; 51-85 years-old, n = 23 trees; and $\geq$86 years-old, n = 11 trees) that ranged widely in height (1.2-31.1 m), diameter (0.1-56.8 cm) and maximum observed root span (0.9-22.9 m). We found that up to 19 trees, including trees from all age classes, were linked together in a single *Rhizopogon* genet, supporting our first hypothesis. Moreover, *R. vesiculosus* genets were linked with a greater number of host trees (2-19 trees; mean = 10.2 ± 6.6) than *R. vinicolor* genets (3-10 trees; mean = 4.4 ± 2.2) ($n_1 = 9$, $n_2 = 9$; $P = 0.03$), supporting our

second hypothesis. Likewise, *R. vesiculosus* genets had a larger span (20.9 m maximum; mean = 13.9 ± 5.4 m) than *R. vinicolor* (12.1m maximum; mean = 5.4 ± 3.7 m) ($P < 0.01$) and covered a larger geometric area (*R. vesiculosus*: 3.6-135.3 m^2; mean = 35.9 ± 42.8 m^2; versus *R. vinicolor*: 0.3-10.0 m^2; mean = 3.4 ± 3.7 m^2; $P < 0.01$) (Fig. 1). Overall, these results support previous findings that *R. vesiculosus* genets are larger than those of *R. vinicolor*[16,23]. *Rhizopogon vesiculosus* genets also occurred at greater depths (1-34 cm; mean = 10.8 ± 1.1 cm) than *R. vinicolor* (1-18cm; mean = 8.7 ± 2.0 cm) ($n_1 = 14$, $n_2 = 13$; $P = 0.01$), showing evidence of vertical partitioning. The number of Douglas-fir trees linked by *R. vesiculosus* was over twice that reported for *Pinus densiflora* linked by *Tricholoma matsutake* in an even-aged stand in Japan[15] (or for *R. vinicolor* in this study). Our novel result that multiple tree cohorts rather than a single age class were included in the MN implies that the *Rhizopogon* fungi have a secure and diverse long-term energy source and play a fundamental role in forest structural development.

Our results also supported our third hypothesis: the degree to which a tree was linked with neighbouring trees was positively correlated with its cohort class ($\rho = 0.57$, $P < 0.01$), height ($\rho = 0.58$, $P < 0.01$), diameter ($\rho = 0.60$, $P < 0.01$), or maximum root length ($\rho = 0.78$, $P < 0.01$) (Figs. 1 and 2). Additionally, there was a strong positive association between a tree's node degree (i.e. number of other trees with which it was linked) and the number of *Rhizopogon spp*. genets colonizing it ($\rho = 0.91$, $P < 0.01$), mostly accounted for by *R. vesiculosus* alone ($\rho = 0.91$, $P < 0.01$). Ninety-nine percent of trees with roots encountered in the plot were linked to one or more neighbouring trees. The tree with the highest node degree, and thus the most central to the MN, was a mature tree (94 years-old) located 4.2 m outside the plot boundary (Fig. 1). This tree was directly linked to 47 other trees through its association with eight *R. vesiculosus* genets and three *R. vinicolor* genets in the plot. Given that this mature tree had a node

degree three times higher than average despite only a portion of its roots being sampled suggests it may be an even stronger hub at a larger spatial scale.

Our final hypothesis, that the MN was scale-free, was also supported. Though trees from all cohorts were highly interconnected, large veteran trees acted as hubs with a more central role in the MN (Table 1, Figs. 1 and 2). This resulted in a skewed distribution of node degrees characteristic of the scale-free model, with the connectivity of large trees well above average. The spatial diameter of the MN (43.2 m) was traversed through only two fungal links, and no more than three linkage steps separated any two trees in the network regardless of their spatial location or physical dimensions. Thus, the MN was also highly interconnected and easily traversed.

Our study expands concepts of symbioses, from interactions between two organisms, to a complex system involving multiple fungal and tree individuals affecting forest stand dynamics. We uncovered an extensive *Rhizopogon spp*. network that linked trees of all ages in an uneven-aged old-growth forest, where 62% of Douglas-fir trees from the two youngest cohorts were established within the extensive network of all old veteran trees. The MN was comprised of *R. vesiculosus* and *R. vinicolor*, each with unique horizontal and vertical spatial patterning in the soil, with implications for niche partitioning regarding fundamental processes such as nutrient uptake[24]. The MN is likely even more extensive and complex than what we describe here because we examined only two of up to 105 ectomycorrhizal fungal species previously described in interior Douglas-fir forests[25].

Our discovery, taken together with nearby experiments showing substantially greater establishment of Douglas-fir germinants when linked into the MN of larger trees, imply that MNs formed by the old trees were important in understory regeneration[7,26] and in functional continuity across the stand. Other studies provide

evidence that greater establishment of Douglas-fir seedlings is associated with carbon, nitrogen or water transfer from networked trees[7,12,27], and that this occurs at little cost to the larger trees[7,12]. Even without resource transfer, however, the extensive MN can mediate interactions among individual trees[28], challenging ecological theory that stand dynamics are driven primarily by inter-tree competition[1]. The increasing connectivity we found with tree size suggests a foundational role for large trees in fostering conspecific understory regeneration, which is a defining characteristic of climax old-growth forests[1].

Evidence from other studies using less precise methods suggests that MNs are a common phenomenon across temperate forests[5,9,10]. Our findings thus have important implications for conservation strategies and management of temperate forests in the changing climate. The historic management paradigm for Douglas-fir forests has been to harvest the largest trees for their high value (i.e. high-grading)[1], remove stumps and roots to reduce root disease, and leave the thickets of understory trees or plant seedlings to grow into future stands[20,21]. This management approach, when combined with the summer drought[22], episodic seed dispersal[21] and natural gap-phase disturbance regime characteristic of these forests[20], has led to regeneration problems across the range of interior Douglas-fir[21]. Tree mortality is expected to increase even further, particularly at the tree range margins, as summer drought and disturbance severity increase with climate change[22]. Our finding that large trees are hubs for MNs, together with evidence that such networks are important in facilitating regeneration[3,7], suggests a dramatically different management approach that aims to conserve large trees or groups of trees and their fungal associates. This paradigm shift in forest management is needed to prevent further degradation of interior Douglas-fir forests.

Methods

This study was conducted in a dry, cool interior Douglas-fir forest near Kamloops, Canada (51˚51'7''N latitude, 120˚31'46''W longitude). In a 30 x 30 m plot, *Rhizopogon* tuberculate mycorrhizas were collected in the four cardinal directions from 61 of the 65 trees, and between trees where cover was sparse (n = 401). Fresh needle or cambium tissue was collected from the 65 trees within the plot and from an additional 62 border trees to provide reference DNA for identifying tree roots. Both tree and fungal DNA were extracted from samples using the Qiagen DNeasy® plant extraction kit and protocols (QIAGEN Inc.), followed by PCR amplification and fragment analysis using a DNA sequencer (3130XL Genetic Analyzer, ABI). Microsatellite markers for interior Douglas-fir[29] and *R. vesiculosus* and *R. vinicolor*[16,23] were used to distinguish tree and fungal individuals. Two or more samples were considered to represent an individual if they had matching alleles at all microsatellite loci analyzed. Every tree had unique genotypes based on three loci amplified by the primer sets *PmOSU*_1C3, *PmOSU*_1F9, and *PmOSU*_2D4[29]. Genets of *Rhizopogon* were distinguished using the primer sets *Rv02*, *Rv15*, *Rv46*, *Rv1.34*, *Rve2.10*, *Rve2.77* and *Rve3.21* for both species, plus *Rve1.21* and *Rve2.44* for *R. vesiculosus*, and *Rv53* and *Rv2.14* for *R. vinicolor*[16,23]. The probability that two individuals could have identical multilocus genotypes by chance ranged from 5.2×10^{-8} to 9.3×10^{-4} for *R. vesiculosus* genets and from 2.7×10^{-10} to 4.7×10^{-4} for *R. vinicolor* genets[16,23], which is in the range of other fungal studies.

The number of trees colonized by individual genets was compared between *Rhizopogon* species using a Wilcoxon rank-sum test. Two-sample t-tests were used to compare genet size measures and mean depth of occurrence between *Rhizopogon* species ($\alpha = 0.05$). The maximum width and geometric area of each *Rhizopogon* genet were calculated using ArcMap (ArcGIS V9.1). All descriptive statistics are reported as a range with mean and standard deviation. Associations between tree cohort class, height, diameter, fungal genet frequency, and tree node degree were tested using Spearman's Rank Correlation tests. Mycorrhizal network structure was modelled using

Pajek[30], with Douglas-fir trees portrayed as nodes linked through one or more *Rhizopogon* genet.

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Table 1 Attributes of Douglas-fir trees and *Rhizopogon* genets used to characterize a mycorrhizal network in a 30 x 30 m plot in an uneven-aged Douglas-fir stand; mean values are reported ± one standard deviation.

***P. menziesii* var. *glauca* trees (nodes):**	
number of standing trees in plot	65
number of standing trees linked	43
no. of tree genotypes from EM roots in plot	56
mean no. of *R. vesiculosus* genets per tree	1.23 (±1.55)
mean no. of *R. vinicolor* genets per tree	0.46 (±0.75)
***Rhizopogon spp.* fungi (links):**	
number of genotyped *Rhizopogon* mycorrhizas	338
number of linking *R. vesiculosus* genets	9
mean no. of trees per *R. vesiculosus* genet	7.39 (±6.95)
number of linking *R. vinicolor* genets	9
mean no. of trees per *R. vinicolor* genet	3.58 (±2.43)
Network attributes:	
node degree range	0-47
mean node degree	13.7 (±12.9)
total number of links (excluding multiple links)	536
network density[1] (excluding multiple links)	0.18
network diameter[2] (measured in link steps)	3
mean path length[3] between linked tree pairs	1.69
network centralization[4]	0.44
network clustering coefficient[5]	0.59 (±0.41)

[1] the number of links between nodes in the network, relative to the maximum possible number of links

[2]the longest of the shortest paths between any two nodes in the network

[3]the minimum number of link steps separating two nodes, averaged across all node pairs in the network

[4]the variation among node degrees divided by the maximum variation possible in the network

[5]the mean clustering coefficient (density of links a node has relative to that of its neighbours) among all nodes in the network

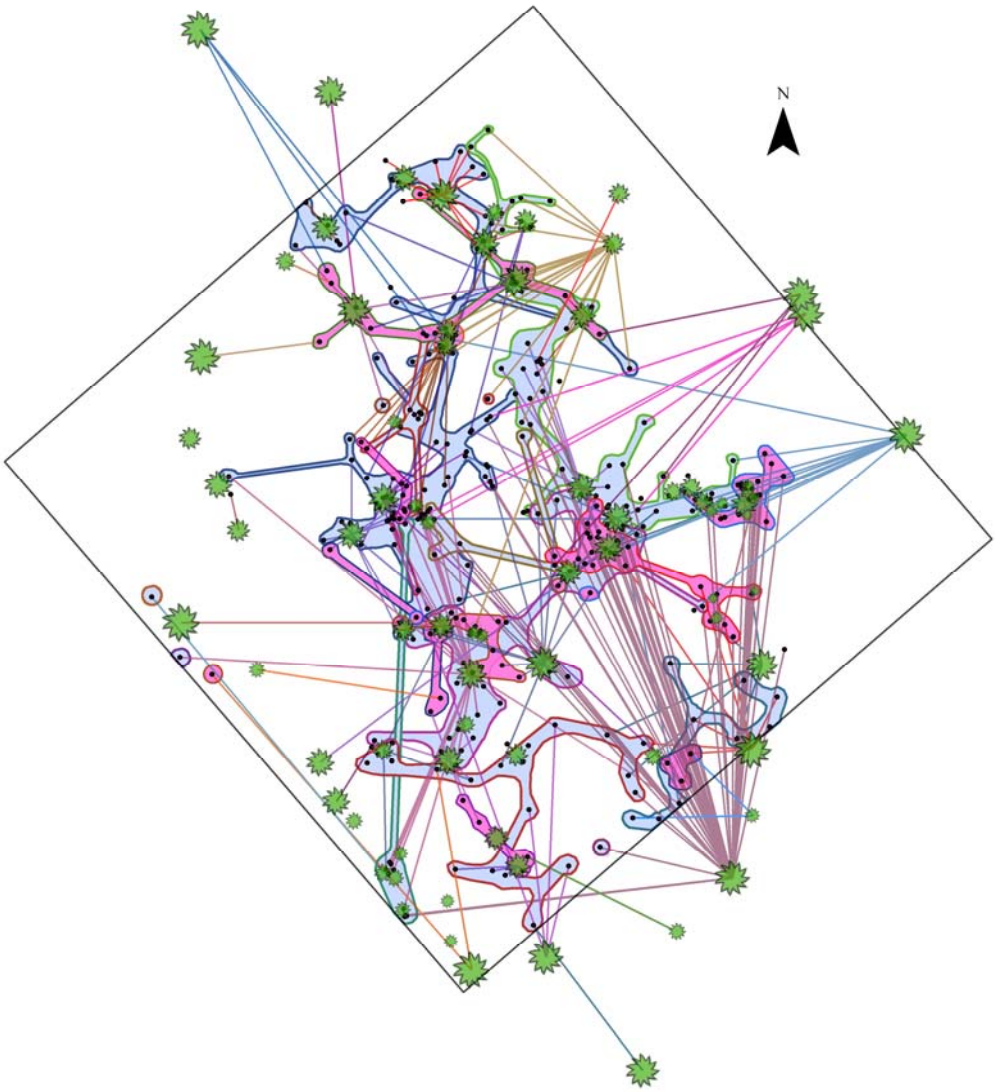

Figure 1. The top-down spatial topology of *Rhizopogon spp*. genet and Douglas-fir tree locations in a 30 x 30 m plot. The plot (square outline) lies on a southeastern slope and contains 65 trees of mixed ages (green shapes, sized relative to each tree's diameter). Small black dots mark *Rhizopogon* ectomycorrhiza samples (n = 401), with samples representative of each fungal

genet outlined in a different colour. *R. vesiculosus* genets (n = 14) are shaded with a blue background, and *R. vinicolor* genets (n = 13) with pink. Lines illustrate the linkages between tree roots encountered in *Rhizopogon* ectomycorrhizas and corresponding source trees aboveground (“root lengths”) and are coloured according to tree genotype. Some trees, mycorrhiza samples and genets are obscured by overlapping features.

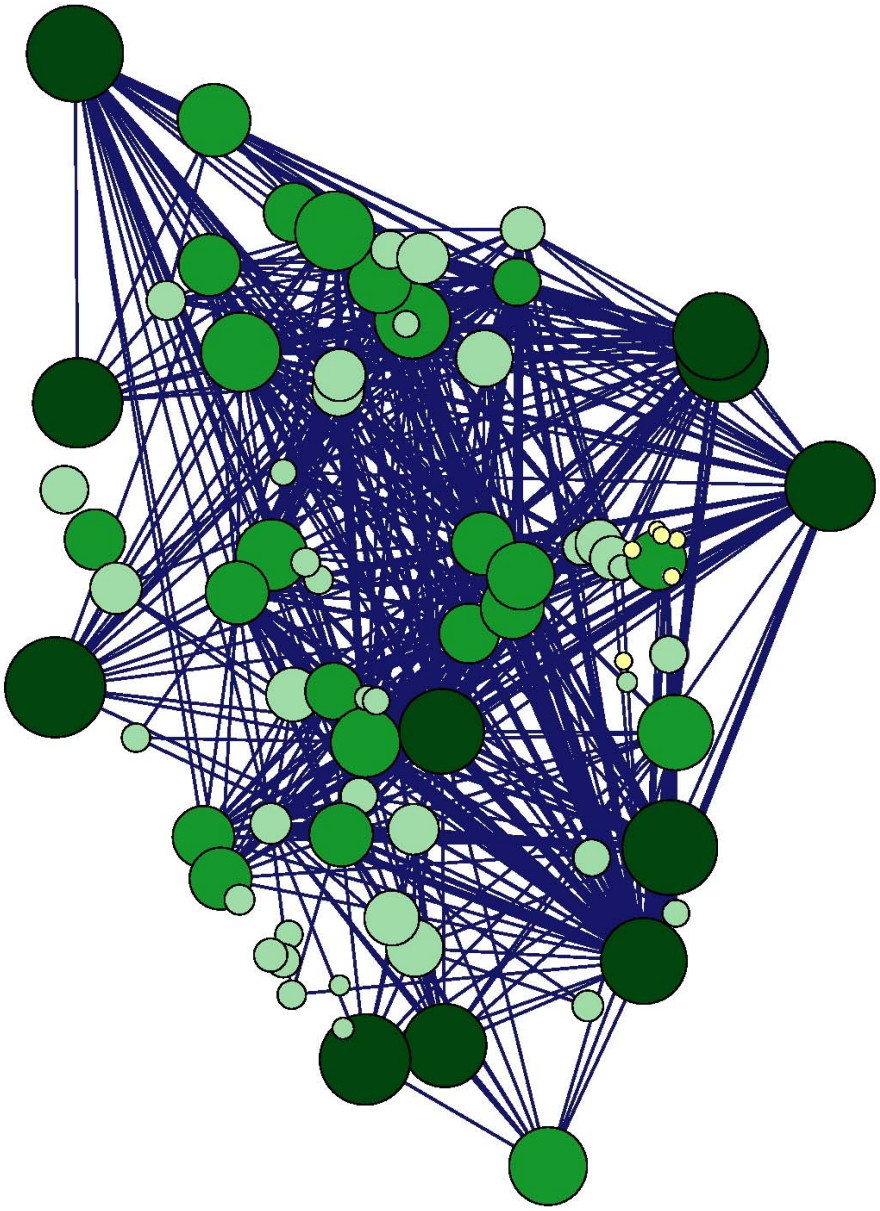

Figure 2. Spatially explicit network model showing linkages between interior Douglas-fir trees via shared colonization by *Rhizopogon vesiculosus* and *R. vinicolor* genets. Circles represent tree nodes, sized according to the tree's diameter, and coloured with four different shades of yellow or green that increase in darkness with increasing age class. Lines represent the Euclidean distances between trees that are linked. Line width increases with the number of links between tree pairs (i.e. repeated links through multiple fungal genets).