Architecture of the wood-wide web: *Rhizopogon* spp. genets link multiple Douglas-fir cohorts

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Summary

• The role of mycorrhizal networks in forest dynamics is poorly understood because of the elusiveness of their spatial structure. We mapped the belowground distribution of the fungi *Rhizopogon vesiculosus* and *Rhizopogon vinicolor* and interior Douglas-fir trees (*Pseudotsuga menziesii* var. *glauca*) to determine the architecture of a mycorrhizal network in a multi-aged old-growth forest.

• *Rhizopogon* spp. mycorrhizas were collected within a 30 × 30 m plot. Trees and fungal genets were identified using multi-locus microsatellite DNA analysis. Tree genotypes from mycorrhizas were matched to reference trees aboveground. Two trees were considered linked if they shared the same fungal genet(s).

• The two *Rhizopogon* species each formed 13–14 genets, each colonizing up to 19 trees in the plot. *Rhizopogon vesiculosus* genets were larger, occurred at greater depths, and linked more trees than genets of *R. vinicolor*. Multiple tree cohorts were linked, with young saplings established within the mycorrhizal network of Douglas-fir veterans. A strong positive relationship was found between tree size and connectivity, resulting in a scale-free network architecture with small-world properties.

• This mycorrhizal network architecture suggests an efficient and robust network, where large trees play a foundational role in facilitating conspecific regeneration and stabilizing the ecosystem.

Introduction

Mycorrhizal networks (MNs), or the mycorrhizal fungal mycelia that connect two or more plants, are increasingly recognized as mediators of interactions among trees through their effects on tree survival, growth and competitive ability (Simard & Durall, 2004; Selosse *et al.*, 2006; Whitfield, 2007). Mycorrhizal networks provide a source of mycorrhizal fungal inoculum for establishing seedlings (Finlay & Read, 1986; Nara, 2006), and a potential conduit for interplant transfer of water, carbon and nutrients (Simard *et al.*, 1997; He *et al.*, 2005; Smith & Read, 2008; Warren *et al.*, 2008). Three major challenges of MN research are determining the architecture, function and ecological significance of MNs; considerable debate exists on all levels (Whitfield, 2007). Historically, interest in MNs has focused on their formation and function in controlled artificial systems (Wu *et al.*, 2001) and in natural ecosystems (Simard *et al.*, 1997; Lerat *et al.*, 2002). However, little remains known regarding the architecture of MNs in the field. Architecture includes the physical components (e.g. nodes and links) of the network and their genetic complexity, the relationships among the components, and the spatial extent and topology of the components and their relationships. Describing these attributes is a prerequisite to understanding how MNs function (e.g. in fungal colonization of plants, mycelial growth dynamics, or nutrient uptake/exchange between plants) and how they affect plant populations, communities, and forest dynamics (e.g. in tree regeneration, competition and mortality) (Nara & Hogetsu, 2004; Simard & Durall, 2004; Selosse *et al.*, 2006).

Network theory provides a useful framework for describing the structure, function and ecology of MNs (Bray, 2003;

Southworth *et al.*, 2005; Selosse *et al.*, 2006). The architecture of networks is described using the node degree (i.e. number of links a node forms with other nodes), degree distribution (i.e. the distribution of links among nodes), clustering coefficients (i.e. the accessibility of links to nodes), path lengths (i.e. the number of link steps separating nodes) and the contribution of nodes to network connectivity with respect to their topological position or centrality (Table 1) (Albert *et al.*, 2000; Bray, 2003; Southworth *et al.*, 2005). With MNs, architecture depends largely on the taxonomic, spatial and temporal scales investigated (Southworth *et al.*, 2005; Selosse *et al.*, 2006). For example, whether one uses plants, fungi or both as nodes can lead to different structural interpretations (Southworth *et al.*, 2005). In this study, we describe MN architecture by representing trees as nodes and mycorrhizal fungi as links.

The architecture of biological networks often follows one of three models: regular, random or scale-free (Table 1). In both regular and random networks, links tend to be distributed equally among nodes, but the topology of regular networks is generally more cliquish and harder to traverse than that of random networks. In scale-free networks, some nodes (i.e. hubs) are highly linked and more central to the network, resulting in a skewed node degree distribution (Albert *et al.*, 2000; Bray, 2003). Scale-free networks are both cliquish and easily traversed, and tend to have a 'small world property', where most nodes can be accessed from every other node by a small number of hops or steps (i.e. a small path length). They also tend to be more robust to perturbations than regular or random networks. For example, the random deletion of a node would usually have little effect on the overall connectivity of the network, unless hubs were specifically targeted for removal (Albert *et al.*, 2000; Bray, 2003).

Ectomycorrhizal fungi are diverse in their form, function and ecology (Smith & Read, 2008), and the networking capabilities of individual species or genets are expected to vary accordingly. For example, the host specificity of a fungal species and the size, longevity, morphology and continuity of its individual genets are all factors that influence the architecture and function of the resulting MN. The physical traits of *Rhizopogon vesiculosus* and *Rhizopogon vinicolor*

Table 1 Glossary of terms relating to mycorrhizal networks assuming that trees are nodes or hubs linked via shared colonization by an individual fungal genet

Terminology	Description
Centrality	(i.e. betweenness centrality) The degree of a node, relative to the distribution of degrees in the network; describes the contribution of nodes to network connectivity with respect to their topological position in the network
Degree	(i.e. connectivity) The number of links a node has to other nodes
Degree distribution	The probability distribution of degrees among nodes in a network
Genet	An individual fungal genotype or clone
Hub	A node with a high degree of connectivity relative to the average degree distribution
Link	A path (connection) between two vertices (nodes); in this case an undirected path (edge) representing a fungal genet e_{ij} that colonizes the roots of two trees *i* and *j* or more ('hyperlink')
Mycorrhizal network (MN)	An underground network where the mycelia of mycorrhizal fungi link the roots of plants of the same or different species
Node	Points or vertices in a graph that are joined by a physical, functional, or physiological pathway
Node clustering coefficient	The density of links a node has relative to that of its neighbours; indicates the cliquishness among nodes in the network
Network centralization	The variation among node degrees divided by the maximum variation possible in the network
Network clustering coefficient	The mean clustering coefficient among all nodes in the network; indicates the accessibility of links to nodes
Network density	The number of linkages in a network, relative to the maximum possible number of linkages
Network diameter	(i.e. eccentricity) The longest of the shortest paths between any two nodes in the network
Mean path length	The minimum number of link steps separating two nodes, averaged across all node pairs in the network
Path length	(i.e. geodesic) The number of link steps separating two nodes
Random network	A network model where nodes link randomly to other nodes, resulting in a probability degree distribution following a Gaussian curve; random networks are generally less cliquish and more easily traversed than regular networks
Regular network	A network characterized by nodes with limited connectivity, high clustering coefficients and a probability degree distribution following a Gaussian curve; regular networks are generally cliquish and not easily traversed
Scale-free network	A network in which some nodes (i.e. hubs) have a high centrality and degree of connectivity relative to the average among all nodes in the network, resulting in a skewed node degree distribution; they are both cliquish and easily traversed
Small-world property	A network with a small topological diameter (eccentricity) relative to the total number of nodes in the network where nonneighbouring nodes can still reach each other through a small number of link steps

(Basidiomycota, *Villosuli* group *sensu* Kretzer *et al.*, 2003) genets, combined with their known benefits to hosts in accessing water and nutrients, make them ideal candidates for studies of mycorrhizal networking. They have been consistently reported as dominant members of ectomycorrhizal communities throughout Douglas-fir (*Pseudotsuga menziesii*) forest development (Chu-Chou & Grace, 1981; Jones *et al.*, 1997; Twieg *et al.*, 2007) and have been shown to increase seedling establishment, growth, and resistance to root pathogens and drought (Molina *et al.*, 1999). They produce durable tuberculate mycorrhizas and form rhizomorphs estimated to span from metres (e.g. *R. vinicolor*) to several decametres (e.g. *R. vesiculosus*) in length (Kretzer *et al.*, 2004, 2005). Previous studies have shown that rhizomorph-forming fungi, including species of *Rhizopogon*, are particularly adept at translocating nutrients and water through continuous mycelial networks (Brownlee *et al.*, 1983; Egerton-Warburton *et al.*, 2007; Warren *et al.*, 2008).

The old-growth interior Douglas-fir forests (*Pseudotsuga menziesii* var. *glauca*) where we studied MN architecture undergo gap-phase regeneration (Newsome *et al.*, 1991), resulting in self-perpetuating, multi-cohort, climax forests (Vyse *et al.*, 2006). The regenerative capacity of these forests has decreased over the last decade, however, as a result of increasing temperatures, summer moisture deficits, and disturbances (Hamman & Wang, 2006; Griesbauer, 2008; Klenner *et al.*, 2008). Understanding MN architecture may provide an insight into how MNs affect forest regeneration, thus improving our predictions of forest dynamics and resilience to disturbance.

In this study, we used multi-locus microsatellite DNA markers to discriminate among individuals of interior Douglas-fir and genets of two ectomycorrhizal fungal species, *R. vesiculosus* and *R. vinicolor*. We considered the presence of a single *Rhizopogon* genet on roots of two different trees as a network link, and then used this approach to characterize the architecture of an MN. Our aims were: to determine if *R. vesiculosus* and *R. vinicolor* colonize multiple trees or tree cohorts; to describe the size and spatial pattern of the *Rhizopogon* spp. genets; to compare the networking qualities of *R. vesiculosus* and *R. vinicolor*; and to determine the architecture of the MN. Based on previous work showing that *R. vesiculosus* forms larger genets than *R. vinicolor* (Kretzer *et al.*, 2004, 2005), we predicted that *R. vesiculosus* would link more trees than *R. vinicolor*. We also predicted that the greater connectivity of large trees compared with small trees would result in a scale-free network where large trees serve as hubs.

Materials and Methods

Site description

This study was conducted in a dry, cool interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca* (Beissn.) Franco) forest near Kamloops, Canada (51°51′7″N latitude, 120°31′46″W longitude). The monotypic, multi-storied, multi-cohort interior Douglas-fir forest was located on a mesic, permesotrophic (zonal) site in the Thompson Dry, Cool Interior Douglas-fir biogeoclimatic variant (IDFdk2) (Lloyd *et al.*, 1990) (Supporting Information Figs S1 and S2). The elevation of the study plot was 1035 m above sea level, with a 10–40% slope and a southeast aspect. Approximately 44 cm of precipitation falls annually in the IDFdk2, with the majority as snow during winter months (Lloyd *et al.*, 1990). Mean maximum and minimum temperatures are 21.0°C and −4.2°C (mean annual = 3.4°C), respectively, with an average growing season of 166 d (April–October) (Environment Canada; Canadian climate norms, 1971–2000). The 30 × 30 m plot in which we conducted the study contained 67 live trees (722 stems ha^{-1}) with a basal area of 22.3 m^2 ha^{-1}. Trees were grouped into four cohorts (age groups) based on correlations between age and stem diameter in a subsample of trees and the diameter distribution of the whole stand. Additional details pertaining to tree measurements and mapping, as well as soil properties and per cent cover and frequency of plants within the study site, are provided in Methods S1.

Field sampling

We sampled tuberculate *Rhizopogon*-like (Basidiomycota; *Rhizopogon vesiculosus* and *Rhizopogon vinicolor sensu* Kretzer *et al.*, 2003) mycorrhizas (when encountered; $n = 401$) from four sides of every tree in the plot, within the dripline or between trees where canopy cover was sparse. We used a dispersed, nonrandom sampling approach to maximize the probability of sampling roots from every tree in the plot. The location of each sample, derived from its distance and azimuth to two or more neighbouring trees, was plotted in ArcGIS (V9.1; ESRI, Redlands, CA, USA) as shown in (Fig. 1 and Fig. S3). The depth (cm) of each tubercule sample, measured from the surface of the forest floor, was recorded and used to compare the mean depths of occurrence of *R. vesiculosus* and *R. vinicolor* genets. Fresh needle or cambium tissue was collected from the 67 trees within the plot and from an additional 64 border trees to provide reference DNA for the identification of tree roots. Reference DNA was collected from border trees if their height was greater than their distance from the plot boundary, ensuring that all trees with roots potentially inside the plot were identified. After collection, mycorrhiza and tree tissue samples were placed in a refrigerated cooler for transport and stored at 4°C for no more than 2 wk before further processing. Tuberculate mycorrhizas were washed in deionized H_2O and their identity confirmed as *Rhizopogon* spp. based on morphological characters under a stereomicroscope at ×20 magnification. Tree cambial tissue was placed immediately on dry ice and extracted within 24 h of collection.

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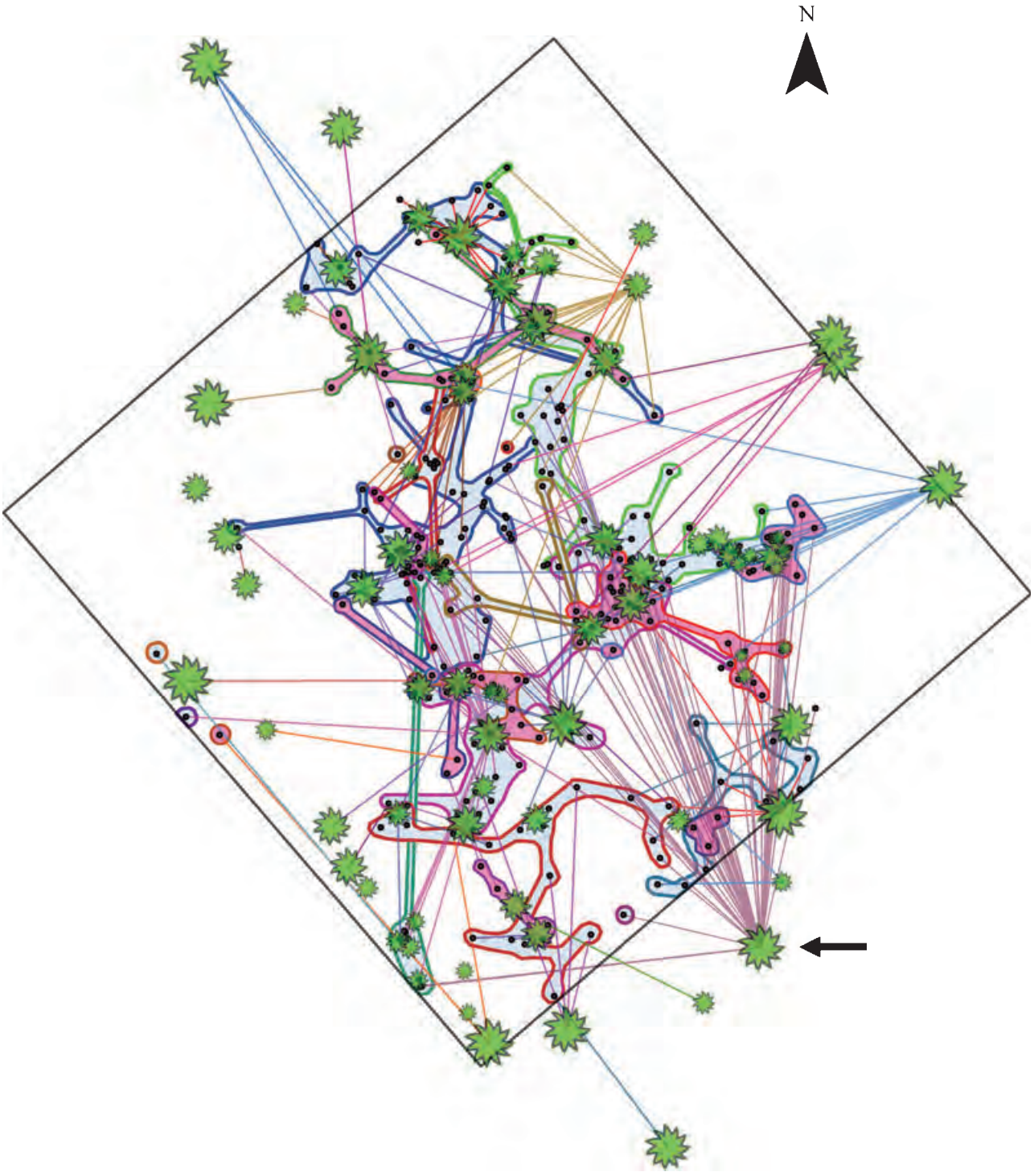

Fig. 1 The top-down spatial topology of *Rhizopogon* spp. genets and Douglas-fir trees in a 30 × 30 m plot. The plot (square outline) lies on a southeastern slope and contains 67 trees of various ages (green shapes; sized relative to each tree's diameter). Small black dots mark *Rhizopogon* ectomycorrhiza sample locations (n = 401), 338 of which were associated with a specific tree and fungal genet based on microsatellite DNA analysis. Samples representative of each fungal genet are outlined in different colours. *Rhizopogon vesiculosus* genets (n = 14) are shaded with a blue background, and *Rhizopogon 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. An arrow points to the most highly connected tree, which was linked to 47 other trees through eight *R. vesiculosus* genets and three *R. vinicolor* genets inside the plot. Some trees, mycorrhiza samples, and/or genets may be obscured by overlapping features.

Molecular methods and analysis

Both tree and fungal DNA was extracted from samples using the Qiagen DNeasy® plant extraction kit and protocols (Qiagen Inc., Mississauga, Canada), followed by PCR amplification and analysis of microsatellite DNA loci (for details see Methods S1 and Table S1). Douglas-fir trees were distinguished using microsatellite markers developed by Slavov *et al.* (2004) targeting the loci *PmOSU* (Oregon State University)_1C3, *PmOSU*_1F9 and *PmOSU*_2D4. Genets of *R. vesiculosus*/*R. vinicolor* were distinguished by the loci *Rv02*, *Rv15*, *Rv46*, *Rv1.34*, *Rve2.77* and *Rve3.21* for both species, plus *Rve2.10*, *Rve1.21* and *Rve2.44* for *R. vesiculosus*, and *Rv53* and *Rv2.14* for *R. vinicolor* (Kretzer *et al.*, 2004). Microsatellite loci were amplified together in PCR multiplex groups of two to four primer sets each, with forward primers labelled at the 5′ end with 6FAM (Integrated DNA Technologies Inc., Coralville, IA, USA), NED, VIC or PET (Applied Biosystems, Foster City, CA, USA) fluorescent dyes. A 'PIG-tail' tack-on sequence was applied to the 5′ end of reverse primers targeting Douglas-fir microsatellite DNA to reduce stutter banding as per Brownstein *et al.* (1996). PCR amplifications were conducted in 10-μl reaction volumes containing ultrapure H_2O, GeneAmp 10× PCR Buffer (Applied Biosystems), 10 mg ml^{-1} bovine serum albumin (BSA), 1 mM GeneAmp dNTP mixture (Applied Biosystems), 2.5 mM $MgCl_2$, 0.25–0.35 mM of each primer, 10 ng of DNA template and 5 U ml^{-1} AmpliTaq Gold polymerase (Applied Biosystems). Temperature cycles began with a 95°C hot-start for 10 min; followed by 30 cycles of 45 s at 93°C, 30 s at 55°C, and 30 s at 72°C; and a final hold for 7 min at 72°C. Microsatellite loci were analysed using a DNA sequencer (3130XL genetic analyser; Applied Biosystems) and then genotyped using Gene Mapper software (V4.0; Applied Biosystems). Two or more samples were considered to represent an individual tree or fungus if they had matching alleles at all microsatellite loci analysed. Every tree, including border trees that were not encountered as roots in the plot, had a unique genotype based on the targeted microsatellite loci. More than 30 tree genotypes were compared among needle, bud, root and cambium tissue sources to confirm consistency among tissue types. For *Rhizopogon*, additional tubercule samples were collected in the vicinity of the study site to determine allele frequencies and to test the power of genet delineations within the site (these samples represented an additional 37 *R. vesiculosus* and 33 *R. vinicolor* genets) (Table S1). The estimated probability that two unrelated individuals could have identical multilocus genotypes by chance (probability of identity) was 1.4×10^{-6} for Douglas-fir trees (n = 131), 2.7×10^{-5} for *R. vesiculosus* (n = 51) and 6.4×10^{-6} for *R. vinicolor* (n = 46). All population genetic statistics were calculated using GenAlEx software version 6.1 available at http://www.anu.edu.au/BoZo/GenAlEx/.

Statistics and network modelling

All statistical tests were performed using JMP software (V6; SAS Institute Inc., Cary, NC, USA) unless otherwise noted, with α = 0.05. Descriptive statistics are reported as a range with mean and SD. The number of trees colonized by individual genets was compared between *R. vesiculosus* and *R. vinicolor* using a Wilcoxon rank-sum test. The maximum width and geometric area of *Rhizopogon* genets were calculated using ArcGIS (V9.1) and compared between *Rhizopogon* species using two-sample *t*-tests. The mean depth of occurrence of genets was compared between *Rhizopogon* species using one-way ANOVA with subsampling in the R statistical computing environment available at http://www.r-project.org. Associations among tree cohort class, tree height, tree diameter, fungal genet frequency and tree node degree were tested using Spearman's rank correlation tests. The mean node degree and centrality of trees were compared between cohorts using ANOVA.

Mycorrhizal network architecture was modelled using Pajek (de Nooy *et al.*, 2005). Network measures were simplified by the exclusion of loops (a tree linked to itself through a single fungal genet) and multiple links between nodes (two or more trees linked through more than one genet) unless otherwise noted. The network model was classified as regular, random or scale-free based primarily on the degree distribution, centrality and clustering coefficients of nodes (Bray, 2003; Southworth *et al.*, 2005). The spatial orientation of tree nodes was integrated with graph-theoretical measures for network analysis and was used to produce a spatially explicit model of network topology.

Results

Characteristics of trees and fungi involved in the network

A total of 56 Douglas-fir genotypes encountered as roots forming mycorrhizas with *Rhizopogon* species were matched to reference tree boles based on microsatellite DNA analysis (Table 2). They included 45 of the 67 trees inside the 30 × 30 m plot, with an additional 11 genotypes matching tree boles outside the plot (Fig. 1). We found that up to 19 trees, and trees from all age classes, were linked together by a single *Rhizopogon* genet (Table S2). All 44 of the colonized trees from the youngest two cohorts shared one or more *Rhizopogon* genets with trees from older cohorts (Table S2). In addition, young trees from which *Rhizopogon* species were not sampled were primarily distributed within the area occupied by established fungal genets (Fig. 1). There were no associations between individual *Rhizopogon* spp. genets and any specific tree cohort class or genotype.

Of the 401 *Rhizopogon* spp. tubercules collected, 338 were matched to both a tree and fungal genet (Table S2).

Table 2 Size and networking characteristics of tree nodes by cohort class in a 30 × 30 m plot of interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca*) forest

	Cohort 1	Cohort 2	Cohort 3	Cohort 4
Estimated age (yr)	≤ 15	16–50	51–85	≥ 86
Number of tree boles in 30 × 30 m plot	6	36	21	4
Number of trees with roots colonized by *Rhizopogon* in the plot	1	25	19	11
Height (m)	1.2–1.9	1.2–22.8	9.6–26.4	20.6–31.1
Mean height	1.6 (± 0.3)	9.0 (± 5.3)	20.4 (± 4.4)	27.4 (± 2.7)
Diameter at breast height (cm)	0.0–1.5	0.7–18.6	12.9–35.6	39.3–56.8
Mean diameter at breast height	0.7 (± 0.68)	8.1 (± 5.2)	24.5 (± 6.2)	46.4 (± 5.3)
Maximum observed root length[a] (m)	0.9–0.9	0.3–15.6	1.4–17.6	5.1–22.9
Mean of maximum lengths	0.9 (NA)	4.5 (± 4.3)	7.6 (± 5.1)	15.1 (± 5.9)
Rhizopogon vesiculosus genets per tree	0–1	0–3	0–7	0–8
Mean *R. vesiculosus* genets per tree	0.2	0.6	1.9	2.5
Rhizopogon vinicolor genets per tree	0–1	0–2	0–3	0–3
Mean *R. vinicolor* genets per tree	0.2	0.4	0.7	1.0
Node degree range	0–4	0–30	0–38	4–47
Mean node degree (ANOVA: $P < 0.01$)	0.7 (± 1.6)	5.7 (± 7.8)	16.3 (± 13.0)	19.8 (± 13.8)
Mean node centrality (ANOVA: $P < 0.01$)	< 0.01	< 0.01	0.01	0.02

Values are means ± 1 SD.

[a]Root lengths were estimated based on the occurrence of *Rhizopogon* spp. mycorrhizas; cohort 1 root data are based on one sample only. NA, not applicable.

We encountered a total of 14 *R. vesiculosus* genets and 13 *R. vinicolor* genets (Fig. 1). Nine genets from each species were found on roots from more than one tree and were thus considered a link. *Rhizopogon vesiculosus* genets were linked with a greater number of host trees (2–19 trees; mean = 10.2 ± 6.6; $n = 9$) than *R. vinicolor* genets (3–10 trees; mean = 4.4 ± 2.2; $n = 9$) ($P = 0.03$). Also, *R. vesiculosus* genets had a larger span (0–20.9 m; mean = 13.9 ± 5.4 m) than *R. vinicolor* genets (0–12.1 m; mean = 5.4 ± 3.7 m) ($P < 0.01$) and covered a larger geometric area (*R. vesiculosus*: 0–135.3 m^2, mean = 35.9 ± 42.8 m^2; vs *R. vinicolor*: 0–10.0 m^2, mean = 3.4 ± 3.7 m^2; $P < 0.01$) (Fig. 1). We found that *R. vesiculosus* genets also occurred at greater mean depths (1–34 cm; overall mean = 10.8 ± 1.1 cm; $n = 14$) than *R. vinicolor* (1–18 cm; overall mean = 8.7 ± 2.0 cm; $n = 13$) ($F = 6.52$, df = 1, $P = 0.01$).

Mycorrhizal network architecture

Fifty-five of the 56 tree genotypes identified from *Rhizopogon* spp. tubercules were linked to one or more trees in the plot (Table S2). The maximum distance between any two trees in the network (43.2 m) was traversed through only two fungal links, and the maximum path length between any two trees in the network was three fungal links, regardless of the trees' spatial locations or size (representing a small-world property; Table 1 and Table S3; Fig. 2). The degree to which a tree was linked with other trees (i.e. node degree) was positively correlated with its cohort class ($\rho = 0.57$, $P < 0.01$), height ($\rho = 0.58$, $P < 0.01$), diameter ($\rho = 0.60$, $P < 0.01$) and maximum root length ($\rho = 0.78$, $P < 0.01$). Additionally, there was a strong positive association between the node degree of a tree and the number of *Rhizopogon* spp. genets colonizing it ($\rho = 0.91$, $P < 0.01$). Although trees from all cohorts were linked, large mature trees acted as hubs with a higher degree of connectivity (ANOVA comparing node degree among cohorts: $F = 9.94$, df = 3, $P < 0.01$) and a more central position in the MN (ANOVA comparing node centrality among cohorts: $F = 6.70$, df = 3, $P < 0.01$) (Table 2). This resulted in a skewed distribution of node degrees, characteristic of a scale-free model, with the connectivity of some large trees well above average (Fig. 3). The tree with the highest node degree ($k = 47$) and centrality (0.07) in the MN was a mature tree (94 yr old) located 4.2 m outside the plot boundary (marked with an arrow in Figs 1 and 2; see also tree no. 23 in Table S2). This tree was colonized by eight *R. vesiculosus* genets and three *R. vinicolor* genets in the plot.

Discussion

Mycorrhizal networks link multiple tree cohorts

We uncovered an extensive 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 network of veteran trees. The MN was comprised of *R. vesiculosus* and *R. vinicolor*, each with unique horizontal and vertical spatial patterning in the soil. Our finding that multiple tree cohorts rather than a single age class were included in the MN implies that *Rhizopogon* fungi have a diverse energy source that is secure over space and time. While linked trees collectively supply carbon to

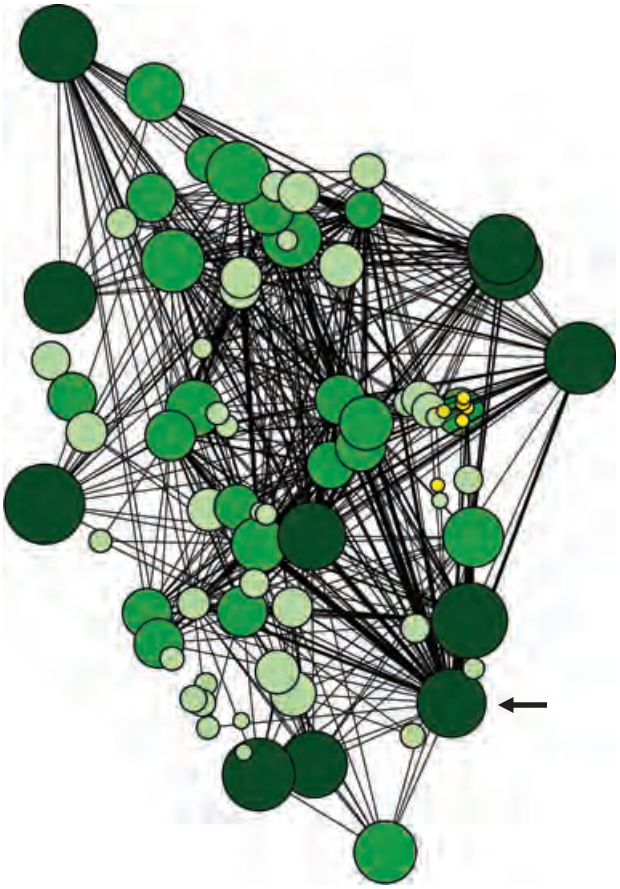

Fig. 2 Spatially explicit network model showing linkages between interior Douglas-fir trees via shared colonization by *Rhizopogon vesiculosus* and *Rhizopogon vinicolor* genets. Circles represent tree nodes, sized according to the tree's diameter, and coloured with four different shades of yellow or 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). An arrow points to the most highly connected tree, which was linked to 47 other trees through eight *R. vesiculosus* genets and three *R. vinicolor* genets inside the plot. Some tree nodes and their links may be obscured by overlapping features.

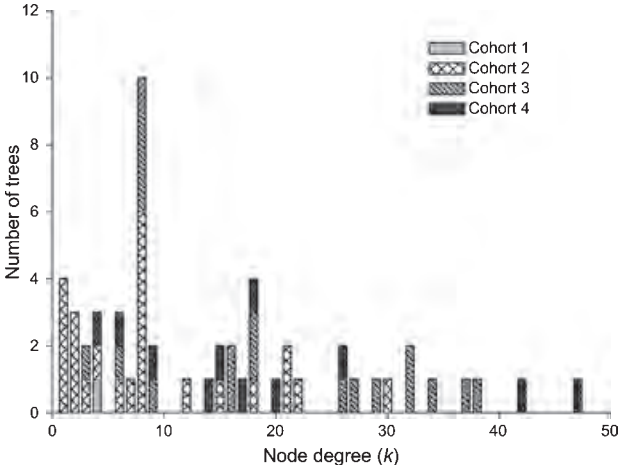

Fig. 3 The degree distribution of tree nodes linked through a mycorrhizal network, showing the number of trees linked to *x* number of other trees with roots colonized by *Rhizopogon* spp. in a 30 × 30 m plot of uneven-aged Douglas-fir forest. Two or more trees were considered linked if they were colonized by the same fungal genet. Node degrees were higher on average among older trees (cohorts 3–4) than younger trees (cohorts 1–2), leading to a skewed degree distribution characteristic of a scale-free network model.

the fungus, young trees in turn gain access to an established fungal inoculum source (Finlay & Read, 1986; Nara, 2006). Our study demonstrates that the mycorrhizal symbiosis is not just between two or more organisms, but is a complex assemblage of fungal and plant individuals that spans multiple generations (Selosse *et al.*, 2006).

Fungal genet parameters

The maximum span of genets in our study was 20.1 m for *R. vesiculosus* and 12.1 m for *R. vinicolor,* supporting the finding of Kretzer *et al.* (2004, 2005) that *R. vesiculosus* has larger genets (≥ 13.4 m) than *R. vinicolor* (2.0 m maximum) in 40- to 80-yr-old second-growth Douglas-fir forests in Oregon. The span of *R. vesiculosus* genets in our study was also in the range reported for other rhizomorph-forming species such as *Suillus bovinus* (17.5 m; Dahlberg & Stenlid, 1994) and *Suillus variegatus* (27 m; Dahlberg, 1997) in a > 100-yr-old *Pinus sylvestris* forest. Studies of ectomycorrhizal fungi have reported genet sizes ranging from ≤ 1 m span (Gherbi *et al.*, 1999) to ≥ 40 m (Bonello *et al.*, 1998), and often attribute these size differences to the life history traits of the fungi. For example, species forming small genets are thought to be short-lived, early-successional species dispersed primarily through sexual reproduction (e.g. spores), while large genet sizes are considered to represent late-stage, perennial species spreading primarily through vegetative growth (Dahlberg & Stenlid, 1994; Bergemann & Miller, 2002). However, most of these studies were based on the occurrence of sporocarps, which may misrepresent the size and distribution of fungal mycelia belowground (Gardes & Bruns, 1996; Zhou *et al.*, 2001). Moreover, associations among species presence, genet size and stand successional stage are not always consistent (Guidot *et al.*, 2001; Redecker *et al.*, 2001; Dunham *et al.*, 2003; Twieg *et al.*, 2007). Thus, more work is needed to determine whether the size differences we observed between *R. vesiculosus* and *R. vinicolor* are attributable to differences in genet longevity, growth rate, or mode of dispersal.

The number of host trees linked by a single genet was greater for *R. vesiculosus* (mean = 10.2) than *R. vinicolor* (mean = 4.4), which is consistent with their size differences. The potential for linkages between Douglas-fir trees and *R. vinicolor* genets in our study was similar to that reported by Lian *et al.* (2006) for *Tricholoma matsutake* in an 85-yr-old

Pinus densiflora forest (4.5 pine trees per genet); however, the average number of host trees linked through associations with *R. vesiculosus* genets was more than twice that of *T. matsutake* or *R. vinicolor*. Moreover, *Rhizopogon* genets in our study linked recent natural regeneration with old trees, with important implications for old-growth forest dynamics. By contrast, *T. matsutake* linked similar-aged trees in an even-aged forest. In addition, we found that *R. vesiculosus* tubercules occurred at greater mean depths than those of *R. vinicolor*, showing evidence of vertical niche differentiation, with implications for fundamental processes such as nutrient uptake (Dickie *et al.*, 2002; Neville *et al.*, 2002; Tedersoo *et al.*, 2003). For example, a greater depth range may provide *R. vesiculosus* with greater access to water and nutrients deeper in the soil during dry periods (Baier *et al.*, 2006; Genney *et al.*, 2006), thereby promoting the water stress tolerance of its hosts.

We represented *Rhizopogon* genets as continuous links across space, although it is possible that some genets may be fractioned as a result of clonal propagation or disruption over time. The continuity of *Rhizopogon* genets was supported by a concurrent study using fine-scaled sampling of 20-cm^3 soil blocks in contiguous transects, where we found the mycelia of both species to colonize and form continuous connections between multiple trees within a 2-m span (K. J. Beiler *et al.*, unpublished). Previous studies have shown that strand-forming fungi similar to *Rhizopogon* provide continuous transport pathways across ecologically significant distances (Brownlee *et al.*, 1983; Finlay & Read, 1986) and that severed linkages in the hyphal network are able to reconnect via anastamosis (Bebber *et al.*, 2007; Fricker *et al.*, 2008; Rotheray *et al.*, 2008). There was also a high degree of linkage redundancy between trees in our study. For example, most trees were colonized more than once by individual *Rhizopogon* genets, and multiple links between trees (the same trees linked through two or more genets) represented 22% of all linkages in the network (Tables S2 and S3).

Network parameters

We found that the node degree of a tree was positively correlated with its size and cohort class. This is not surprising given that the probability of a tree encountering fungal genets increases with the extent of its root system, but to our knowledge this has never been empirically demonstrated. Accordingly, the association between tree size/cohort class and connectivity resulted in a scale-free network as we had predicted. This highlights the importance of large mature trees in the architecture of the MN, where they accounted for most of the connectivity and centrality among nodes in the network. In addition, the network was easily traversed both spatially and topologically (i.e. path lengths were small relative to the number of nodes), giving the network a small-world property. These results are in contrast to those of Southworth *et al.* (2005), who described a random network in *Quercus garryana* forests of California. The difference in results may be a consequence of different approaches in defining a link. We defined a link as a single fungal genet found on two different trees, whereas Southworth *et al.* defined a link as the presence of a morphotype (near species-level identification based on the morphology of a mycorrhiza) on a tree, such that the node degree of a tree was equal to its morphotype richness. Stand structure also differed between these studies: our interior Douglas-fir forest was dense and had a skewed diameter distribution, with a few large, old hub trees and many young trees, whereas Southworth *et al.*'s savanna forest had fewer, larger, widely spaced trees that were interspersed with *Ceanothus* shrubland.

In our study, the tree with the highest node degree was directly linked to 47 other trees through its association with eight *R. vesiculosus* genets and three *R. vinicolor* genets in the plot. This corresponded to 84% of potential linkages between this tree and all other trees encountered in the plot, and was three times higher than the mean node degree among trees. The influence of this tree as a network component, despite only a portion of its roots being sampled (the bole of the tree was located 4.2 m outside the plot), suggests that it would be an even stronger hub at a larger spatial scale. Overall, MNs are probably even more extensive and complex than what we describe here because we examined only two of up to 65 ectomycorrhizal fungal species previously described in interior Douglas-fir forests of British Columbia (Twieg *et al.*, 2007).

Implications for network functioning and forest management

The scale-free architecture and small-world property of the MN suggest that resources could be efficiently shuttled to expanding mycelial fronts, including those associated with regenerating seedlings (Bray, 2003). For example, carbon and water resources have been shown to move from multiple plants into a common mycelium (Leake *et al.*, 2001; Wu *et al.*, 2001; Querejeta *et al.*, 2003). When a young seedling connects into this common mycelium, as shown in this study, it has direct access to these resources, potentially facilitating its establishment. This premise is supported by field studies showing greater establishment of germinants when linked into the MN of larger trees (Nara, 2006; McGuire, 2007), and by those showing belowground resource transfer among plants facilitated by mycorrhizal fungi (Simard *et al.*, 1997; Lerat *et al.*, 2002; Teste *et al.*, 2009). Even if resource transfer does not occur between trees, seedlings would benefit from colonization by *Rhizopogon* genets already being supplied with carbon by other trees in the network. In turn, *Rhizopogon* genets would benefit

from colonizing a range of hosts because their large carbon demands are unlikely to be met by young understorey trees alone. This is implied by the results of Högberg *et al.* (1999), where ectomycorrhizal fungi specific to pine hosts acquired 87–100% of their carbon from overstorey trees and relatively little from understorey trees.

The architecture of our network also suggests that it is a relatively robust system, where it would be resilient against random perturbations (Albert *et al.*, 2000; Bray, 2003) but susceptible to targeted removals of hub trees (e.g. by high-grade harvesting, insects or disease) (see Figs S4, S5). For example, if hub trees were removed in our study (equal to 20% of networking trees), the capacity for resource transfer and MN facilitation of forest regeneration would be greatly reduced compared with 20% of networking trees removed at random (Figs S4c,d, S5c,d). Because large trees sustained a greater number of fungal genets than small trees, the removal of hub trees may also have a large effect on the genetic diversity of *R. vesiculosus* and *R. vinicolor* populations. In summary, we found that most trees in a multi-cohort old-growth forest were linked in a scale-free MN, where large trees served as hubs, with implications for understorey regeneration and functional continuity in the stand. To ensure that old-growth Douglas-fir forests remain resilient and self-regenerative following disturbance, our findings support a management approach that conserves large trees or groups of trees and their mycorrhizal fungal associates.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Methods S1. Soil and vegetation cover, tree measurements, DNA extraction and DNA amplification and microsatellite analysis.

Fig. S1 Photograph showing the mixed-aged, multi-storey stand structure characteristic of *Pseudotsuga menziesii* var. *glauca* trees in the Dry, Cool Interior Douglas-fir biogeoclimatic zone.

Fig. S2 The relative height and spatial distribution of Douglas-fir trees in the 30 × 30 m study plot depicted using the Stand Visualization System.

Fig. S3 The location of Douglas-fir trees and *Rhizopogon* spp. mycorrhiza samples in the 30 × 30 m plot and the spatial distribution of (a) Douglas-fir tree roots, (b) *Rhizopogon vesiculosus* genets, and (c) *Rhizopogon vinicolor* genets.

Fig. S4 Mycorrhizal network topology showing the loss of connectivity between Douglas-fir trees linked through *Rhizopogon* spp. genets with the removal of (a) the most highly linked hub tree (k = 47), (b) the most linking fungal genet (colonized 19 trees), (c) 20% of networking trees selected randomly, or (d) trees from the oldest cohort (also 20% of networking trees).

Fig. S5 Histograms showing the degree distributions of Douglas-fir tree nodes linked through *Rhizopogon* spp. genets following the hypothetical removal of (a) the most highly linked hub tree (k = 47), (b) the most linking fungal genet (linked 19 trees), (c) 20% of networking trees selected randomly, or (d) trees from the oldest cohort (also 20% of networking trees).

Table S1 Properties of microsatellite DNA loci used to discriminate among individual Douglas-fir trees and *Rhizopogon* spp. fungal genets

Table S2 Incidence matrix showing the number of times each *Rhizopogon vesiculosus* and/or *Rhizopogon vinicolor* genet was encountered on the roots of each Douglas-fir tree

Table S3 Attributes of Douglas-fir trees linked through *Rhizopogon* spp. genets in the plot and resulting network characteristics

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