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Topology of tree–mycorrhizal fungus interaction networks in xeric and mesic Douglas-fir forests

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Summary

1. From the phytocentric perspective, a mycorrhizal network (MN) is formed when the roots of two or more plants are colonized by the same fungal genet. MNs can be modelled as interaction networks with plants as nodes and fungal genets as links. The potential effects of MNs on facilitation or competition between plants are increasingly recognized, but their network topologies remain largely unknown. This information is needed to understand the ecological significance of MN functional traits.

2. The objectives of this study were to describe the interaction network topologies of MNs formed between two ectomycorrhizal fungal species, *Rhizopogon vesiculosus* and *R. vinicolor*, and interior Douglas-fir trees at the forest stand scale, identify factors leading to this structure and to contrast MN structures between forest plots with xeric versus mesic soil moisture regimes.

3. Tuberculate mycorrhizas were sampled in six 10 × 10 m plots with either xeric or mesic soil moisture regimes. Microsatellite DNA markers were used to identify tree and fungal genotypes isolated from mycorrhizas and for comparison with reference tree boles above-ground.

4. In all six plots, trees and fungal genets were highly interconnected. Size asymmetries between different tree cohorts led to non-random MN topologies, while differences in size and connectivity between *Rhizopogon* species-specific subnetwork components contributed towards MN nestedness. Large mature trees acted as network hubs with a significantly higher node degree compared to smaller trees. MNs representing trees linked by *R. vinicolor* genets were mostly nested within larger, more highly connected *R. vesiculosus*-linked MNs.

5. Attributes of network nodes showed that hub trees were more important to MN topology on xeric than mesic sites, but the emergent structures of MNs were similar in the two soil moisture regimes.

6. *Synthesis*. This study suggests MNs formed between interior Douglas-fir trees and *R. vesiculosus* and *R. vinicolor* genets are resilient to the random loss of participants, and to soil water stress, but may be susceptible to the loss of large trees or fungal genets. Our results regarding the topology of MNs contribute to the understanding of forest stand dynamics and the resilience of forests to stress or disturbance.

Key-words: complex adaptive system, fungal genet, microsatellites, mutualistic network, mycorrhiza, mycorrhizal network, plant–soil (below-ground) interactions, *Pseudotsuga menziesii* (Douglas-fir), self-organization, soil water stress

Introduction

Network analysis is increasingly used to describe and analyse the dynamics of interaction networks in ecology, where biotic or abiotic entities can be viewed as nodes linked through pairwise associations (Bascompte 2009; Anand *et al.* 2010). It provides a tool for holistically describing the myriad of positive and negative interactions between organisms, or between organisms and their environment, which generate the system's patterns and processes (Levin 1992, 2005; Lau *et al.* 2010). Most work to date has focused on community trophic dynamics using predator–prey, plant–pathogen or food-web models (Ings *et al.* 2009; Parrott 2010). In contrast, little attention has been paid to interaction networks at the population level,

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where nodes represent individuals and links represent intraspecific interactions or shared attributes (Jordano 1987; Ohtsuki *et al.* 2006; Beiler *et al.* 2010). This focal scale provides an indication of how phenotypic and phenological heterogeneities within and among populations influence network structure and dynamics at coarser scales (Hewitt *et al.* 2007; Bascompte 2009; Ings *et al.* 2009; Peters *et al.* 2009).

Which nodes interact with each other, the direction, frequency or intensity of those interactions, and the accessibility of nodes to others at a given time form the basic topology of an interaction network. In addition to providing a basis for describing and comparing complex systems, a network's topology provides an indication of its resilience to the loss of specific nodes or groups of nodes. This information can be used to assess the overall robustness of the system represented, as well as to identify participants that are critical for maintaining the integrity of the network (Holling 1973; Albert, Hawoong & Barabási 2000; Dunne, Williams & Martinez 2002; Montoya, Pimm & Sole 2006). For example, the number of links a node has to other nodes (node degree) and the degree to which nodes that are linked to a particular node are themselves linked (node clustering coefficient), provide metrics for identifying key participants (e.g. individuals), components (e.g. species, functional guilds) or processes that contribute to the network's structure and modularity (Bascompte 2009; Parrott 2010). Attributes such as the density of links among nodes (network density), the frequency distribution of links among nodes (node degree distribution) and the cliquishness of links among nodes (network clustering coefficient) can indicate the robustness of a network and the extent and efficiency at which materials or information are able to traverse it (Watts & Strogatz 1998; Barabási & Albert 1999; Barabási 2009). A glossary of terms related to network analysis in the context of MNs is provided in Table S1 in the Supporting Information.

Pseudotsuga menziesii var. *glauca* (Beissn.) Franco (interior Douglas-fir) predominates in the dry, cool climate regions of western North America, where it forms single-species, multicohort stands through gap-phase regeneration following natural mortality or disturbance (Oliver & Larson 1996; Huggard *et al.* 2005; LeMay, Pommerening & Marshall 2009). These forests are considered complex self-organizing ecosystems where resource limitations determine the current structural state (Simard 2009). For example, precipitation levels below the lower threshold cause transition from forests dominated by interior Douglas-fir to grasslands at lower elevations, and exceeding the upper threshold causes transition to mixed-species forests at higher elevations (Vyse, Smith & Bondar 1990; Meidinger & Pojar 1991; Huggard *et al.* 2005). Thus, pure stands of interior Douglas-fir tend to develop in semi-arid climates, where water availability is limiting. Paradoxically, seasonal water deficits limit the regeneration and growth of interior Douglas-fir forests, and unusual drought events make them more susceptible to stand-replacing wildfires, outbreaks of insects and disease or diebacks (Lopushinsky 1990; Klenner *et al.* 2008; Littell, Peterson & Tjoelker 2008). Projections of future climatic conditions in this region suggest an increased frequency and severity of such abiotic and biotic disturbances (Nitschke & Innes 2008; Walker & Sydneysmith 2008; Rodenhuis *et al.* 2009; Griesbauer, Green & O'Neill 2011).

A mycorrhiza is a symbiotic association between a fungus and a plant root. Ectomycorrhizal fungi (EMF), a specific type of mycorrhizal fungi that envelops root tips but does not penetrate root cortical cells, provides trees with increased access to water and nutrients and protection from root pathogens in exchange for carbon from the tree (Bruns, Bidartondo & Taylor 2002; Allen *et al.* 2003). A mycorrhizal network (MN) refers to interconnections between plant roots and mycorrhizal fungal hyphae below-ground (Molina & Trappe 1982; Brownlee *et al.* 1983). This is distinct from a mycelial network which describes the architecture specific to fungal mycelial systems and does not include plant roots (Heaton *et al.* 2012). Mycorrhizal networks can be modelled from the phytocentric perspective, with plants as nodes and fungi as links in spatially explicit, implicit or aspatial multiplex networks (Simard *et al.* 2012). For this study, MNs are modelled as interaction networks involving multiple plant hosts that are colonized by at least one shared mycorrhizal fungal individual (genet).

Studies have demonstrated that linkage into a MN with residual trees or shrubs can improve the survival and productivity of newly establishing seedlings (Nara 2006; McGuire 2007; Bai *et al.* 2009). In interior Douglas-fir forests, MNs associated with residual trees in cutover areas appear to facilitate new regeneration by providing mycorrhizal inoculum and carbon, water or nutrient subsidies from the mature trees (Teste, Simard & Durall 2009; Teste *et al.* 2010). With or without the direct transfer of materials between plants, seedlings gain access to water and nutrients through EMF mycelia that are predominantly subsidized by mature trees (Horton, Molina & Hood 2005). The existence of an MN may also intensify competition between trees (Kytoviita, Vestberg & Tuomi 2003). By altering competition between trees and/or by altering the surrounding environment, MNs can influence the spatial and genetic structure of forest communities (Dickie, Koide & Steiner 2002; Booth 2004; Nara & Hogetsu 2004; Nara 2006). However, little is known regarding the spatial, temporal or social architectures of MNs (Lian *et al.* 2006; Beiler *et al.* 2010, 2012).

An important question to be answered with regard to MNs is how their structure may vary across local environmental gradients (e.g. water and nutrient availability or pH) or in response to disturbances or climate change at coarse spatiotemporal scales. Particularly relevant for interior Douglas-fir forests is the potential influence of soil moisture regime on mycorrhizal network structure. Soil moisture regimes refer to the average amount of soil water annually available for evapotranspiration by vascular plants over multiple years (Pojar, Klinka & Meidinger 1987). Recent field experiments have found that mycorrhizal network facilitation between mature and regenerating interior Douglas-fir trees increases under water deficit conditions (Bingham & Simard 2012). These findings support the stress-gradient hypothesis, which infers

that net effects of community interactions shift in importance from competition to facilitation with increasing environmental stress (Bertness & Callaway 1994; Callaway 1995; Brooker *et al.* 2008). Nonetheless, the outcomes of ectomycorrhizal symbioses are known to vary in response to soil water stress at the species level (Theodorou 1978; Parladé *et al.* 2001; Ortega *et al.* 2004; Kennedy & Peay 2007).

Rhizopogon vesiculosus and *R. vinicolor sensu* Kretzer *et al.* (2003) (Basidiomycota) are 'sister' EMF species that share narrow host specificity for Douglas-fir trees (Molina & Trappe 1994). They are among the most frequent and abundant EMF encountered on Douglas-fir roots throughout all forest developmental stages (Twieg, Durall & Simard 2007). *R. vesiculosus* and *R. vinicolor* are readily distinguishable from other co-occurring EMF species by their unique tuberculate mycorrhizas, a complex of densely packed fine-root tips encased by a fungal hyphal sheath. They form highly differentiated rhizomorphs capable of transporting water and dissolved nutrients over ecologically relevant distances (Brownlee *et al.* 1983; Egerton-Warburton, Querejeta & Allen 2007) and have been found to colonize the roots of multiple trees, including young and old cohorts, within a spatially continuous mycelium (Beiler *et al.* 2012).

The objectives of this study were threefold: (i) to describe the emergent structural properties of MNs at the forest stand scale, (ii) contrast the structure of subnetwork components linked by *R. vesiculosus* genets with those linked by *R. vinicolor* genets and (iii) to contrast the structure of MNs between forest plots with xeric versus mesic soil moisture regimes. We predicted that tree size asymmetries would lead to non-random network topologies where large trees influence network connectivity more than small trees. This was based on the mixed-aged structure of the forest stands where the study was conducted, together with the finding that older interior Douglas-fir tree cohorts sustain more fungal genets than younger cohorts (Beiler *et al.* 2010). We predicted that *R. vesiculosus*-linked MN components would have greater connectivity than *R. vinicolor*-linked components, because *R. vesiculosus* mycelial systems have been found to be more pervasive and associate with more trees across contiguous units of space relative to *R. vinicolor* (Beiler *et al.* 2012). Lastly, we expected greater MN connectivity in xeric stands relative to mesic stands in keeping with the stress-gradient hypothesis (e.g. Bertness & Callaway 1994; Callaway 1995; Bingham & Simard 2012).

Materials and methods

PLOTS AND SAMPLING

Six independent 10 × 10 m plots (each separated by ≥150 m) were selected within a multistoried, multicohort old-growth interior Douglas-fir forest (*P. menziesii* var. glauca (Beissn.) Franco). Geographic information and stand characteristics are described for each plot in Appendix S1. To compare MN attributes between xeric and mesic soil moisture regimes, three plots were selected in upper slope positions and three plots in lower slope positions with plant communities indicative of the respective soil moisture regimes as per Lloyd *et al.* (1990). These ecosystem attributes are represented by contrasting site series classifications in the Thompson Dry, Cool Interior Douglas-fir biogeoclimatic variant of southern-interior BC, Canada (Pojar, Klinka & Meidinger 1987). Mesoslope position and the relative abundance of *Calamagrostis rubescens* Buckl. and *Pleurozium schreberi* Brid. served as indicators of soil moisture regime, with xeric plots (plots 1–3) having a greater proportion of *C. rubescens* in the understorey, and mesic plots (plots 4–6) having a greater proportion of *P. schreberi* (Appendix S1). The mean number of tree boles (stems m^{-2}), tree basal area (m^2 ha^{-1}) and the frequency distribution of tree cohort classes (five classes) were contrasted between plots with xeric versus mesic soil moisture regimes to obtain baseline information regarding their potential influences on network measures (Appendix S1). Soils were Orthic Dystric Brunisols with a sandy loam texture and Hemimor humus form based on soil pits located adjacent to plots (Soil Classification Working Group 1998).

In each of the six 10 × 10 m plots, 200 person hours were spent collecting *Rhizopogon* spp. mycorrhizas between May and June, 2008. In order to meet network modelling objectives and avoid undersampling the root systems of small trees relative to large trees, *Rhizopogon* spp. mycorrhizas were sampled purposively to obtain samples from a range of tree roots representative of the mixed-aged stand structure (Hewitt *et al.* 2007). For this, a visual search of the forest floor was conducted at four sampling points surrounding every tree in each plot, with sampling points oriented in the four cardinal directions and at the inside edge of the crown dripline surrounding each tree. This was accompanied by dispersed sampling of plot interspaces with no canopy cover. When encountered, two or more *Rhizopogon* spp. tuberculate mycorrhizas were collected from each sampling location, placed in 2 ml polypropylene tubes, and frozen at −20°C for molecular analysis. To provide a reference DNA library of tree genotypes for matching with tree roots isolated from *Rhizopogon* spp. mycorrhiza samples in the 10 × 10 m plots, needle and/or cambium tissue was collected from 646 trees, including all standing trees within the 10 × 10 m plots and surrounding trees with a height greater than their distance to the plot boundary (height was used to estimate potential rooting extent).

MOLECULAR ANALYSIS

A subsample of approximately 0.1-g tree needle or cambium tissue and fine-root tissue from within *Rhizopogon* spp. tuberculate mycorrhizas was processed in the laboratory. The molecular analysis workflow included DNA extraction, amplification of microsatellite loci in multiplex PCRs and fragment analysis using a capillary sequencer (3130XL genetic analyzer, Applied Biosystems, Foster City, CA, USA) with GeneMapper software (V4.0, Applied Biosystems) as detailed in Beiler *et al.* (2010) and Appendix S1. Interior Douglas-fir tree DNA samples isolated from needles, cambium tissue, and from roots within *Rhizopogon* tubercles were genotyped at microsatellite loci *PmOSU*_1C3, *PmOSU*_1F9 and *PmOSU*_2D4 using primers developed by Slavov *et al.* (2004). *Rhizopogon* DNA was identified to species using the microsatellite loci *Rve2.10* and *Rve2.14*. *R. vesiculosus* was then further genotyped at loci *Rv02*, *Rv15*, *Rv46*, *Rve1.21*, *Rv1.34*, *Rve2.44*, *Rve2.77* and *Rve3.21*; and *R. vinicolor* at loci *Rv02*, *Rv15*, *Rv46*, *Rv53*, *Rv1.34*, *Rve2.77* and *Rve3.21* using primers developed by Kretzer *et al.* (2003). Two or more samples were considered to represent the same individual when they had identical multilocus genotypes based on amplified microsatellite loci.

Tests for deviations from Hardy–Weinberg expectations, expected heterozygosity and FIS values were implemented using GENEPOP version 4.1.3 (Rousset 2008). The probability of identity for multilocus genotypes of *R. vesiculosus*, *R. vinicolor* and interior Douglas-fir were obtained using GENALEX software version 6.41 (Peakall & Smouse 2006).

STATISTICS AND NETWORK MODELLING

Statistical tests were performed using SAS version 9.2 (Statistical Analysis Software, SAS Institute, Cary, NC, USA) except where otherwise noted. A Bonferroni adjustment (alpha/the number of pairwise comparisons) was used to control familywise error rates when determining the significance of tests involving multiple comparisons. Generalized linear mixed models (described later) were obtained using SAS Proc GLMMIX, based on pseudo-likelihood estimation with parameter convergence criterion set at 1E-7 and a singularity tolerance of 1E-12. Prior to making any plot-level MN comparisons, the assumptions that sample sizes were reasonably balanced between soil moisture regimes and between MN components (subnetworks) linked through *R. vesiculosus* versus *R. vinicolor* genets were tested (Appendix S1).

Network analysis was conducted using PAJEK version 2.04 (Batagelj & Mvar, Ljubljana, Slovenia). MNs were modelled as interaction networks from the phytocentric perspective, with tree boles as spatially explicit nodes that were assumed to be linked when colonized by the same fungal genet. Specifically, we describe potential MNs because the spatial continuity of genet links between tree roots is assumed rather than demonstrated. Network models are based solely on *Rhizopogon* spp. mycorrhiza samples collected inside the 10 × 10 m plots. However, some tree boles outside the plot boundaries had roots growing into the plot where they were colonized by *Rhizopogon* spp. and are thus included as nodes in the network models. Replicate tripartite network motifs between tree pairs linked through the same fungal genet (i.e. multiple associations across space) are represented by a single link in the models (genets represent hyperlinks). Multiple links representing tree pairs sharing more than one *Rhizopogon* spp. genet are shown by increasing line weights in MN illustrations, but are reduced to a single link for network analysis. MN model topologies were classified based on the density of links, degree distribution and mean clustering coefficient among nodes in each network (see Table S1) (Bray 2003; de Nooy, Mrvar & Batagelj 2005). Network classifications were verified by contrasting empirical networks against undirected random Erdos-Renyi configurations (ER networks) in Pajek, using average node degree as input. As such, the ER network models illustrate the expected topology of a network having the same number of nodes and mean node degree as the empirical networks, when pairs of nodes are linked at random. Spearman's rank correlation coefficient (*rho*) was used to indicate the strengths of pairwise associations between tree cohort class and tree height, dbh, node degree and node clustering coefficients, and between tree node degree and node clustering coefficients using SPSS (IBM Corporation, Armonk, NY, USA). Tree dbh data were square root transformed to achieve linearity. Node clustering coefficient data could not be successfully transformed to achieve linearity. However, in all cases, relationships were monotonically increasing or decreasing. Therefore, the Spearman's rank correlation coefficients indicate the strength of relationships.

Mycorrhizal network models were partitioned into separate components linked through either *R. vesiculosus* or *R. vinicolor*; these are referred to as *Rhizopogon* sp. subnetwork components. Network densities, mean node degrees and network clustering coefficients were contrasted between *Rhizopogon* sp. subnetwork components pairwise by plot using Wilcoxon signed-rank tests in SPSS. The numbers of trees colonized by fungal genets were contrasted between *R. vesiculosus* and *R. vinicolor* in a Wilcoxon rank sum test in SPSS. The nestedness of *R. vesiculosus* and *R. vinicolor* subnetwork components within the MNs was estimated based on the NODF (nestedness metric based on overlap and decreasing fill, per Almeida-Neto *et al.* 2008) of columns in the respective tree–*Rhizopogon* sp. incidence matrices using the software ANINHADO (Guimarães & Guimarães 2006). The NODF of the six empirical MNs was contrasted with the average NODF of random replicates generated from null models using a Wilcoxon signed-rank test (see Appendix S1).

Mycorrhizal network topological properties (as listed above) were contrasted between xeric and mesic soil moisture regimes in separate Wilcoxon rank sum tests using SPSS. Generalized linear mixed models were obtained to assess the effects of *Rhizopogon* species and soil moisture regime on the networking attributes of tree subsamples, including the response variables: tree node degree, normalized node degree and node clustering coefficient. A negative binomial distribution with log link function was used to fit 'tree node degree', and binomial distributions with logit link functions were used to fit the proportional response variables. Each model included tree dbh as a fixed-effect covariate to account for tree size differences, and species, soil moisture regime, interactions between fixed effects and the variable 'plot' as a random effect. Variables that were not significant at alpha = 0.05 were removed stepwise from the model based on −2 log pseudo-likelihood fit statistics, beginning with the 3-way interaction, then 2-way interactions starting with the highest p-value if more than one were not significant and finally class variables, if it and all 2-way interactions involving that variable were dropped. The process stopped when remaining variables were significant.

Results

STAND CHARACTERISTICS AND SAMPLING OF MYCORRHIZAS

Eighty-one tree boles occurred within the six 10 × 10 m plots and were grouped into 5 cohort classes based on the modality of tree height and diameter distributions. A total of 478 *R. vesiculosus* and 280 *R. vinicolor* mycorrhizas collected within the six 10 × 10 m plots were successfully genotyped based on the fungal and tree microsatellite DNA loci targeted. These corresponded to 28 *R. vesiculosus* genets, 27 *R. vinicolor* genets, and 166 tree genotypes that included all tree cohorts. With one exception, all non-singleton fungal genets (i.e. genets represented by more than one mycorrhiza sample) were associated with multiple trees. Thus, of the 166 tree genotypes isolated from *Rhizopogon* sp. mycorrhizas, 165 were potentially linked to other trees through a *Rhizopogon* sp. genet. This included 101 tree genotypes associated with *Rhizopogon* spp. mycorrhizas inside 10 × 10 m plot boundaries that originated from tree boles located outside the plots. Among the 81 tree boles located within plot boundaries (excluding tree genotypes matched to boles outside plot boundaries), 65 were found in association with *Rhizopogon spp.* mycorrhizas. This corresponds to 78–89% of tree boles in each plot except plot 2 where *Rhizopogon* spp. mycorrhizas

were found on 50% of trees (Table 1). Maps illustrating the spatial distribution of tree boles and *Rhizopogon* spp. mycorrhiza samples, including the identity of fungal genets and tree roots isolated from mycorrhizas, are shown in Fig. S1. The probability of encountering two unrelated individuals (*Rhizopogon* spp. genets or trees) with identical multilocus genotypes by chance was very low ($< 10^{-6}$), regardless of whether plots were analysed separately or as one population.

NETWORK TOPOLOGY OF INTERIOR DOUGLAS-FIR-*RHIZOPOGON* SPP. MNS

Figure 1 illustrates the interaction network topology of interior Douglas-fir trees linked through shared associations with *Rhizopogon* spp. genets, including trees with boles or roots occurring in each plot and the distribution of fungal links among them. Mycorrhizal network attributes are summarized by plot in Table 1. Networking trees were highly interconnected, but the frequency distribution of links between trees was skewed towards larger trees. Specifically, the shape and spread of tree node degree distributions resembled that of multimodal tree diameter distributions, with most networking trees having an average node degree plus a few large trees with substantially higher numbers of links to other trees. MN topological properties (e.g. node degree distributions and node clustering coefficients) were markedly divergent from random ER network models based on the same number of nodes and average node degree as empirical MNs (see Fig. S2). There was an overall inverse relationship between node degrees and node clustering coefficients, which is characteristic of scale-free network models ($r = -0.528$, $P < 0.001$; Fig. 2). Moreover, the boles of most large trees were located outside plot boundaries (i.e. cohorts 4 & 5, Fig. 1, Appendix S1), and their node degrees were disproportionately reduced by the plot edge effect relative to younger cohorts, suggesting node degree distributions would have a stronger positive skew at larger spatial extents.

Among all tree genotypes in the 10×10 m plots ($n = 182$), significant relationships were found between tree cohort class and $(\text{dbh})^{1/2}$ ($r = 0.945$, $P < 0.001$), height ($r = 0.845$, $P < 0.001$) and node degree ($r = 0.252$, $P = 0.001$) based on Spearman's *rho*. The relationship between the cohort class and node clustering coefficient of trees was weak ($r = -0.018$, $P > 0.008$). Spearman's rank correlations between tree cohort class and $(\text{dbh})^{1/2}$, height, node degree and node clustering coefficient, and between tree node degree and clustering coefficient, are shown independently for each plot in Table 1.

RHIZOPOGON VESICULOSUS AND *R. VINICOLOR* SUBNETWORK COMPONENTS

The number of trees colonized by *R. vesiculosus* and *R. vinicolor* genets differed significantly based on a Wilcoxon rank sum test ($W = 27$, $P = 0.054$, $n_{1,2} = 6$), with *R. vesiculosus* genets tending to colonize more trees than *R. vinicolor* genets

Table 1. Attributes of mycorrhizal network formed between mixed-aged interior Douglas-fir trees linked through *Rhizopogon vesiculosus* and *R. vinicolor* fungal genets

	Xeric soil moisture regime			Mesic soil moisture regime		
	Plot 1	Plot 2	Plot 3	Plot 4	Plot 5	Plot 6
No. of nodes (trees linked in MN)	26	13	31	27	27	41
No. of 'links' between tree pairs	258	66	283	254	177	434
No. *R. vesiculosus*/*R. vinicolor* mycorrhizas sampled in 10×10 m	69/7	29/39	79/48	59/53	112/12	130/121
No. *R. vesiculosus*/*R. vinicolor* genets	4/2	2/4	4/5	4/2	7/4	7/10
No. *R. vesiculosus* genets per tree	0–2	0–2	0–2	0–4	0–2	0–4
Mean ± SD	0.92 ± 0.64	0.50 ± 0.67	0.81 ± 0.66	1.15 ± 0.80	1.22 ± 1.30	1.33 ± 1.19
No. *R. vinicolor* genets per tree	0–1	0–3	0–3	0–1	0–2	0–5
Mean ± SD	0.15 ± 0.38	0.67 ± 0.98	0.75 ± 0.93	0.85 ± 0.69	0.56 ± 0.53	1.39 ± 1.29
Proportion of trees in 10×10 m colonized by *Rhizopogon spp.*	0.846	0.500	0.875	0.846	0.778	0.889
Node degree range	0–24	0–12	0–29	0–26	0–24	0–37
Mean node degree ± SD	18.00 ± 8.49	6.95 ± 4.94	17.15 ± 8.92	17.52 ± 7.34	12.21 ± 7.03	20.19 ± 10.10
Normalized node degree range	0–0.86	0–0.67	0–0.91	0–0.93	0–0.86	0–0.88
Network (i.e. link) density ± SD	0.64 ± 0.30	0.39 ± 0.27	0.54 ± 0.28	0.62 ± 0.26	0.44 ± 0.25	0.48 ± 0.24
Node clustering coefficient (cc') range	0–1	0–1	0–1	0–1	0–1	0–1
Mean cc' ± SD	0.84 ± 0.37	0.63 ± 0.43	0.84 ± 0.28	0.85 ± 0.25	0.83 ± 0.27	0.80 ± 0.26
Spearman's rho						
Tree cohort vs. (dbh)1/2	0.959*	0.979*	0.983*	0.867*	0.940*	0.909*
Tree cohort vs. height	0.931*	0.918*	0.895*	0.673*	0.913*	0.861*
Tree cohort vs. node degree	0.498*	0.710*	0.327	0.410	0.523*	0.025
Tree cohort vs. betweenness centrality	0.279	0.331	0.389	0.374	0.494*	0.064
Tree cohort vs. node cc'	0.225	0.700*	−0.285	0.025	−0.322	−0.094

*Correlation is significant at $P \leq 0.008$ (Bonferroni adjusted α) level.

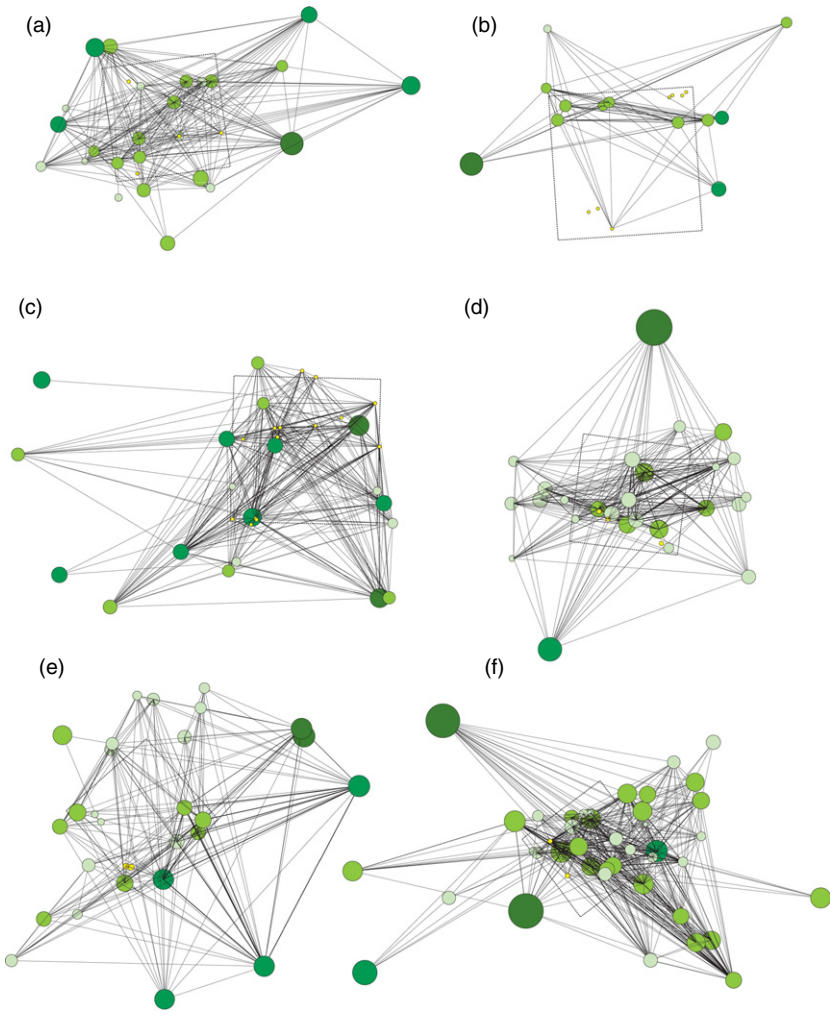

Fig. 1. Network models depicting the socio-spatial topology of tree–mycorrhizal fungus interaction networks in 100 m^2 plots with xeric (a–c) or mesic (d–f) soil moisture regimes (squares show plot boundaries, models shown at different scales). Nodes (circles) are *Psuedotsuga menziesii* trees, sized relative to tree diameter and darkening in colour with increasing age class, and links represent the number of *Rhizopogon* spp. genotypes shared between trees (shown by line heaviness).

(Table 2). This corresponded with topological differences between *R. vesiculosus* and *R. vinicolor* subnetwork components (Fig. 3). With *R. vesiculosus* and *R. vinicolor* MN components compared pairwise by plot, the mean number of links that tree nodes had to other nodes, the density of links among nodes and the clustering of those links among node cliques significantly differed (respectively, mean node degree: W = 21, $P = 0.028$, $n_{1,2} = 6$, network density: W = 19, $P = 0.046$, $n_{1,2} = 6$, network clustering coefficient: W = 19, $P = 0.046$, $n_{1,2} = 6$, Wilcoxon signed-rank tests). In all of the above pairwise comparisons, the median values associated with *R. vesiculosus* MN components were numerically greater than those of *R. vinicolor* MN components. Lastly, *R. vinicolor* subnetwork components were moderately to highly nested within *R. vesiculosus* network components based on the overlap and decreasing fill of corresponding tree *Rhizopogon* spp. incidence matrices from each plot (NODF range 57.14–80.00, Table 2). Across all six plots, the nestedness of *Rhizopogon* sp. subnetwork components in empirical MNs differed significantly from the average columnwise NODFs (i.e. *Rhizopogon* spp. occurrences on trees) of random replicates generated from ER null models (W = 0, $P < 0.01$, $n_{1,2} = 6$, Wilcoxon-signed rank tests).

NETWORK PROPERTIES IN XERIC VERSUS MESIC SOIL MOISTURE REGIMES

No significant differences were found between soil moisture regimes regarding the topological properties of MNs, including network density, mean node degree or network clustering coefficients (all with $P > 0.05$, $n = 3$ plots per soil moisture regime, in separate Wilcoxon rank sum tests; Table 1). Conversely, interactions between soil moisture regime and other fixed effects variables in generalized linear mixed models were commonly significant, indicating that soil moisture affects networking attributes of tree nodes (node degree, normalized node degree or node clustering coefficient; Table S2). Each of the response variables showed a positive relationship with tree dbh, and their values were higher among trees linked through *R. vesiculosus* compared to *R. vinicolor* (Fig. 4). The slope for 'tree node degree' on tree dbh was greater for xeric compared to mesic soils. In the model with 'normalized node degree' as the response variable, a significant interaction between *Rhizopogon* species and soil moisture regime was found. Normalized node degrees were higher in xeric plots compared to mesic plots among *R. vesiculosus* links, but the opposite trend was found for *R. vinicolor* links

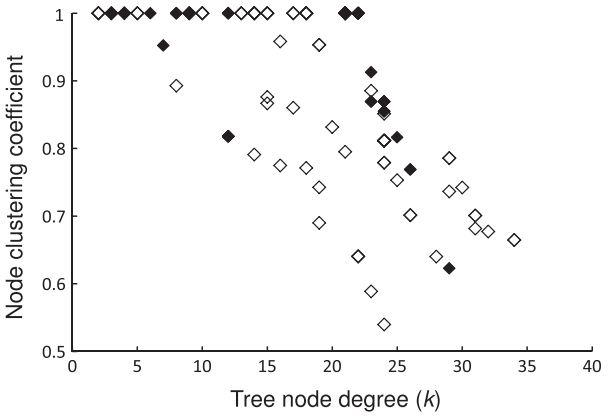

Fig. 2. Scatterplot showing the monotonically negative relationship between the number of links a tree had to other trees (node degree), and the degree to which trees it was linked to were themselves linked (node clustering coefficient; Spearman's *rho* = −0.469, $P < 0.01$). Links represent mycorrhizal fungal genotypes shared between two trees based on tuberculate mycorrhizas sampled in 100 m^2 plots with xeric (trees as filled shapes) or mesic (trees as open shapes) soil moisture regimes.

(soil moisture regime changed the y-intercepts of *Rhizopogon* species). The rates at which normalized node degrees increased with tree dbh were the same among each combination of *Rhizopogon* species and soil moisture regime. In the model with 'node clustering coefficient' as the response variable, the positive trend with tree dbh was influenced by *Rhizopogon* species and by soil moisture regime, owing to significant 2-way interactions between *Rhizopogon* species × dbh and soil moisture regime × dbh. In general, the relationship between tree node clustering coefficients and dbh was stronger in xeric compared to mesic soils, but the strength of these effects was not independent.

Discussion

NETWORK TOPOLOGY OF MNS AND *RHIZOPOGON* SP. SUBNETWORK COMPONENTS

In all six plots, trees were highly interconnected through their associations with *R. vesiculosus* and *R. vinicolor* genets. As predicted, tree size asymmetries led to non-random MN topologies, where large mature trees contributed more to network connectivity than smaller trees. The high density of links among nodes, together with the skewed distribution of those links towards large mature trees, resulted in MNs with complex topologies. Highly connected networks consisting of asymmetric or otherwise heterogeneous interactions among entities are generally associated with self-organized complex adaptive systems (see Table S1) (Parrott 2010). The connectivity of the MNs suggests materials could be shuttled efficiently between numerous trees, including mature veterans and newly established trees, when those trees are colonized by the same EMF genets (Bray 2003; Leake *et al.* 2004).

Subnetwork components comprised of trees associating with *R. vesiculosus* genets were larger and more connected than those based on *R. vinicolor* genets. This confirms our prediction, based on previous studies focusing on a single 30 × 30 m plot (Beiler *et al.* 2010) and replicated 2 × 0.2 × 0.2 m soil slices (Beiler *et al.* 2012), in which *R. vesiculosus* genets were found to associate with more trees

Table 2. The frequency distribution of interior Douglas-fir tree cohorts linked through either *Rhizopogon vesiculosus* or *R. vinicolor* genets in mycorrhizal networks, including all trees with roots associating with *Rhizopogon* spp. mycorrhizas inside 10 × 10 m plots with xeric or mesic soil moisture regimes (SMR)

	Xeric SMR			Mesic SMR		
	Plot 1	Plot 2	Plot 3	Plot 4	Plot 5	Plot 6
Number of trees in each cohort linked through *R. vesiculosus* (*VESI*) genets						
Cohort 1	2	0	9	1	0	2
Cohort 2	6	0	3	13	11	12
Cohort 3	11	8	5	6	8	15
Cohort 4	4	1	7	1	4	2
Cohort 5	1	1	2	1	2	2
Total	24	10	26	22	25	33
% of trees in 100 m^2 linked by *VESI*	76.92	41.67	68.75	76.92	55.56	72.22
% of networking trees linked by *VESI*	92.31	76.92	83.87	81.48	92.59	80.49
Number of trees in each cohort linked through *R. vinicolor* (*VINI*) genets						
Cohort 1	0	1	7	0	2	1
Cohort 2	2	1	1	12	1	9
Cohort 3	1	6	2	5	3	14
Cohort 4	1	2	5	0	2	1
Cohort 5	1	1	2	0	0	1
Total	5	11	17	17	8	26
% of trees in 100 m^2 linked by *VINI*	7.69	41.67	50.00	69.23	44.44	66.67
% of networking trees linked by *VINI*	19.23	84.62	54.84	62.96	29.63	63.41
Nestedness (i.e. NODF*) of *VESI*– and *VINI*–tree associations	57.14	80.00	70.59	70.59	77.78	70.37

*NODF, nestedness metric based on overlap and decreasing fill, ranging from 0 (random, modular, etc.) to 100 (perfect nestedness).

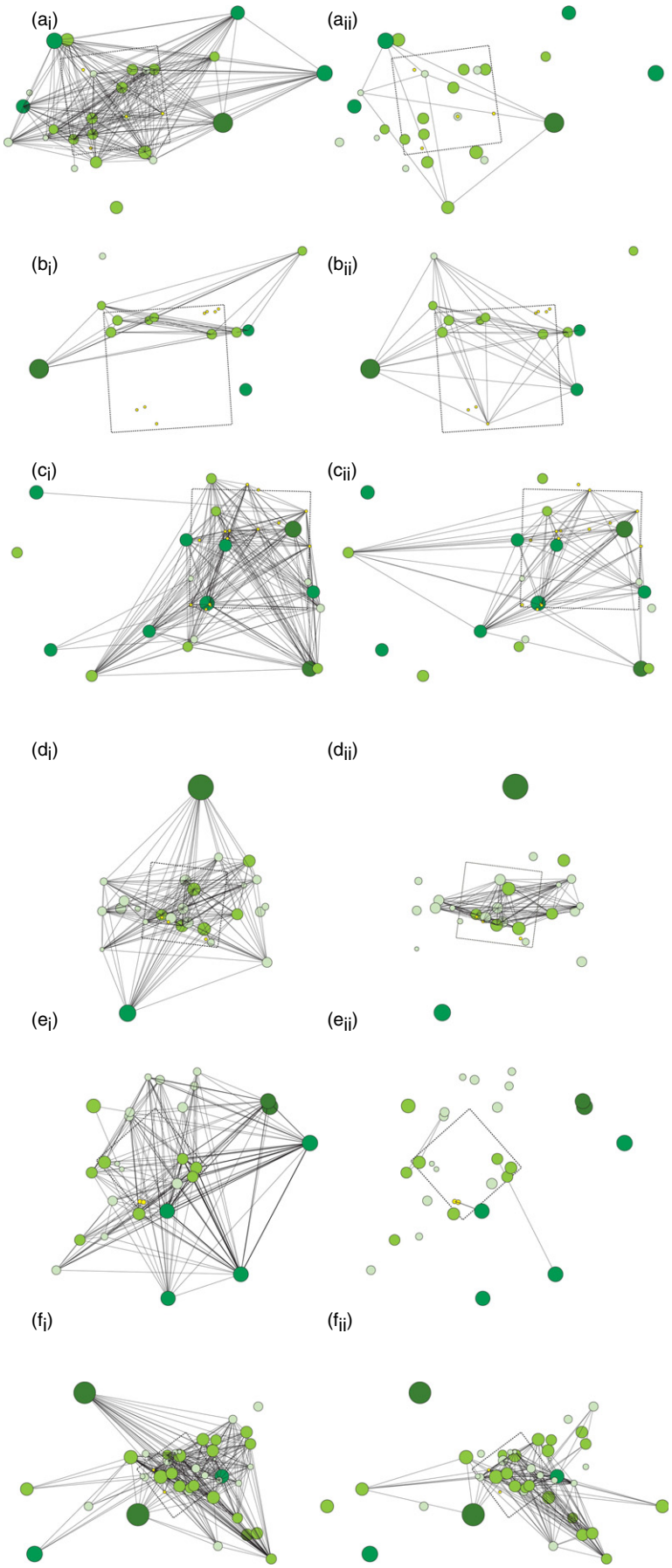

Fig. 3. *Rhizopogon vesiculosus* (i) and *R. vinicolor* (ii) subnetwork components of tree–mycorrhizal fungus interaction networks in 100 m^2 plots with xeric (a–c) or mesic (d–f) soil moisture regimes (squares show plot boundaries, models shown at different scales). Nodes (circles) are *Psuedotsuga menziesii* trees, sized relative to trunk diameter and darkening in colour with increasing age class, and links represent the number of *Rhizopogon* spp. genotypes shared between trees (shown by line heaviness). The mean node degree and proportion of trees that were linked (i.e. network density) differed significantly between *R. vesiculosus* and *R. vinicolor* linked network components, with *R. vesiculosus* forming more links between more trees than *R. vinicolor* (see main text).

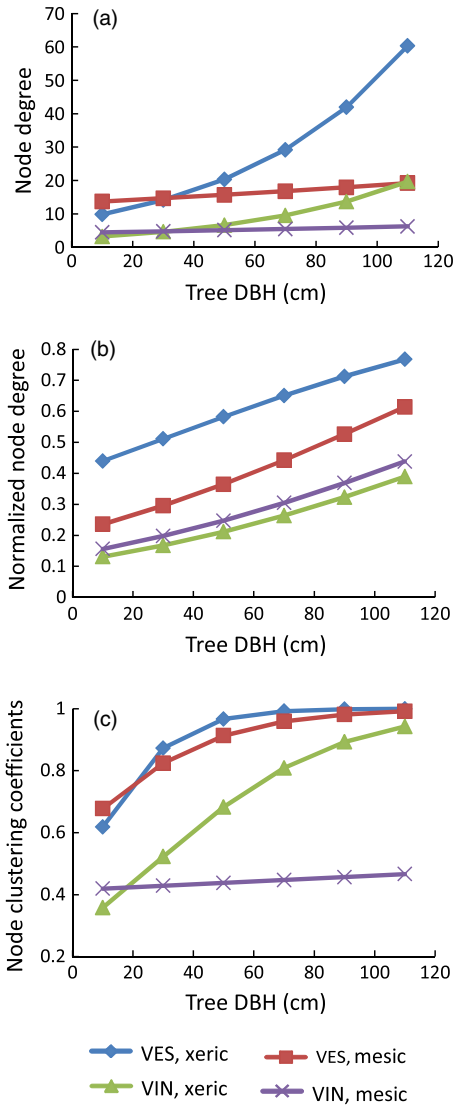

Fig. 4. Generalized linear mixed model results showing the predicted values for response variables (a) tree node degree, (b) normalized node degree and (c) node clustering coefficient in relation to increasing tree DBH in cm, for trees linked in mycorrhizal networks through *Rhizopogon vesiculosus* ('VES') or *R. vinicolor* ('VIN') in plots with xeric or mesic soil moisture regimes (see main text).

per unit space than *R. vinicolor* genets. Together, the MNs represent meta-networks in which *R. vinicolor*-linked network components are mostly nested within larger *R. vesiculosus*-linked MN components. This multiplicity contributes to the cohesiveness and stability of the MNs by increasing the overall density of links between trees, and by spreading the cliquishness of links between tree nodes across multiple spatial and temporal scales (Levin 2005; Montoya, Pimm & Sole 2006). The nestedness of interactions between plant and fungal species in the mycorrhizal symbiosis is an indicator of self-organization, which occurs when a global pattern (i.e. emergent property) results from interactions between various components of the system (Gunderson & Holling 2002). Thus, MNs provide a means through which the structure of EMF communities can influence the diversity, stability and evolutionary dynamics of forest communities and vice versa. The evolutionary significance of nestedness in mutualistic networks has been, for example, demonstrated for plant–pollinator networks (Bascompte & Jordano 2007). Interestingly, genets of *R. vesiculosus* and *R. vinicolor* were encountered in similar frequencies in each plot, which is consistent with previous studies showing co-occurrence of these species at both fine (0.2 × 0.2 m, Beiler *et al.* 2012) and coarse (30 × 30 m, Beiler *et al.* 2010) spatial scales.

The highly interconnected, nested topology of these MNs suggest they are robust systems that would be resilient to random perturbations targeting participants indiscriminately, because most trees in the network would remain connected following a moderate loss of participants (Albert, Hawoong & Barabási 2000; Barabási 2009). On the contrary, the MNs could be susceptible to the loss of critical participants such as large hub trees or hyperlinking fungal genets (see Fig. S4 in Beiler *et al.* 2010). For example, the exclusion of *R. vinicolor* genets would have little effect on MN topology, but the loss of *R. vesiculosus* genets would dramatically reduce connectivity, and in Plot 4, effectively break the MN into isolated components. A loss of key nodes or links could have cascading effects throughout the system that ultimately lead to a reduction in MN stability (Dunne, Williams & Martinez 2002; Ellison *et al.* 2005; Bascompte 2009). For example, if the largest trees were removed from a stand due to selective harvesting or insect attacks, the remaining trees may not adequately meet the carbon demands of EMF species forming large perennial genets such as *R. vesiculosus* (Dosskey, Linderman & Boersma 1990; Kretzer *et al.* 2003; Beiler *et al.* 2010). Remaining trees would still have a diverse suite of EMF species to associate with (e.g., Twieg, Durall & Simard 2007), but the regenerative capacity of the stand could be suppressed if access to mycorrhizal networks becomes limited (Teste, Simard & Durall 2009).

Despite the structural complexity of these MNs, they represent only a small part of a more expansive system in terms of space, time and the complexity of MNs as a whole. The spatial patterns of tree roots and *Rhizopogon* spp. genets are dynamic and form a continuous matrix spanning multiple scales of space and time, making them inherently difficult to measure with adequate sampling resolution. The longevity of individual *Rhizopogon* spp. mycorrhizal root tips remains unknown and genets may become fragmented over time, or alternately, reach such extents that the links they represent may no longer be functionally relevant to trees. Nonetheless, the maximum node degree of trees and the overall spatial extent and complexity of the MNs are likely underestimated in this study. More than half of the tree roots sampled in 10 × 10 m plots were matched to tree boles outside plot boundaries, meaning only a portion of their root systems were sampled. A tree located over 20 m distance from the centre of Plot 6 (number 116 in order of distance to centre) had roots sharing *Rhizopogon* spp. genets with 30 other trees inside the plot. Considering the observed root lengths of trees in this study, their distance and orientation from plots, tree bole densities surrounding the plots and the density of links

among trees inside the plots, these trees could be directly linked to 250 or more trees through genets of a single EMF species such as *R. vesiculosus*. Yet, only two species among a genetically diverse and functionally multifarious community of EMF that associate with *P. menziesii* var. *glauca* trees were sampled (Twieg, Durall & Simard 2007).

DETERMINANTS OF NETWORK STRUCTURE

With increasing tree sizes, the probability of a tree encountering genets and accumulating links to other trees increased. Large, mature trees acted as hubs in the network and tended to have higher node degrees compared to younger trees. While associations between tree cohort and networking parameters were not strong, they were enough to reject the null hypothesis that no linear relationship exists, which would be expected if the network had a regular or random topology. Nonetheless, young trees could still become linked to many other trees if colonized by a hyper-linking *Rhizopogon* sp. genet. This was evident in plot six, where a tree had direct links to 37 other trees (88.1% of trees in the network), despite belonging to the youngest cohort.

The inverse relationship between node degree and node clustering coefficients suggests that large hub trees provide network paths bridging the gaps between otherwise distinct cliques of densely interconnected smaller trees. Through their influence on MN connectivity, large hub trees could play a foundational role in the self-regeneration of complex stand structures in these forests (Simard 2009). The presence of large trees can influence the ambient temperature and moisture of local environments, modify local edaphic conditions (soil pH, nutrient status, etc.) and sustain rich assemblages of EMF species that provide a diverse inoculum source to regenerating seedlings (Flores & Jurado 2003; Querejeta, Egerton-Warburton & Allen 2007; Teste & Simard 2008). When seedlings become linked into a MN with veteran trees, they gain access to hydraulically lifted water and patchily distributed nutrients that would otherwise be limiting resources (Leake *et al.* 2004; Egerton-Warburton, Querejeta & Allen 2007; Warren *et al.* 2008).

NETWORK STRUCTURE IN XERIC AND MESIC SOIL MOISTURE REGIMES

Recently, Bingham & Simard (2012) reported the increased importance of MN regeneration facilitation in drier soils compared to moist soils. Based on this finding, we predicted greater MN connectivity in xeric stands relative to mesic stands in keeping with the stress-gradient hypothesis. In support of this hypothesis, we found that soil moisture regime affected the node degree, normalized node degree and node clustering coefficients of trees linked through *Rhizopogon* spp. genets. There was a stronger positive trend between the dbh and node degree of trees in xeric plots compared to mesic plots, suggesting the role of large trees as network hubs is more pronounced under water stress (Fig. 4a). Similarly, the proportion of links between networking trees accounted for by hub trees (i.e. normalized node degrees) was greater in xeric plots relative to mesic plots for trees linked by *R. vesiculosus*, but not *R. vinicolor* (Fig. 4b). Lastly, the relationship between the dbh and node clustering coefficient of trees suggests that a greater proportion of trees linked to hub trees in xeric plots was themselves linked, compared to associates of hub trees in mesic plots (Fig. 4c). This suggests that cliques of nodes that include large trees were more densely linked, and thus more cohesive, in xeric plots relative to mesic plots. Nonetheless, MN topologies were complex and highly interconnected in every plot sampled, with no significant differences found between xeric and mesic soil moisture regimes. This suggests the structural thresholds of these MNs extend beyond the soil moisture constraints represented in this study, and points to *R. vesiculosus* and *R. vinicolor* being well adapted to frequent soil water deficiencies. This supposition is consistent with the evolutionary history of *Rhizopogon* spp. and hypotheses regarding the ecological advantages of hypogeous fruiting habit in xeric environments (Thiers 1984; Lilleskov *et al.* 2009). Further evidence was provided by Parke, Linderman & Black (1983) in microcosms, where *R. vinicolor* was found to increase the water use efficiency of individual *P. menziesii* var. *menziesii* trees and to speed their recovery following periods of water stress.

In water-limited interior Douglas-fir forests, the potential for hydraulically lifted water from deep-rooted mature trees to be redistributed through MNs to shallow-rooted seedlings could be of paramount importance to the self-maintenance of mixed-cohort stand structures (Querejeta, Egerton-Warburton & Allen 2003; Egerton-Warburton, Querejeta & Allen 2007; Meinzer, Warren & Brooks 2007; Schoonmaker *et al.* 2007; Warren *et al.* 2008). It would be insightful to examine the structure of *Rhizopogon* spp. MNs in wetter and drier soil moisture conditions across the broader geographic range of *P. menziesii* var *glauca* (e.g. where *P. menziesii* var *glauca* occurs as a component in wet cedar–hemlock forests and in dry mixed-conifer forests) and to contrast MN structure between interior and coastal variants of *P. menziesii*. Further studies are also needed to elucidate the ecological significance of MN topologies, which currently remains conjecture.

SUMMARY

This study demonstrates the utility of network analysis for simplifying the description of complex ecological systems, and for comparing the emergent properties of these systems between independent locations. Likewise, it demonstrates the use of network analysis to identify key components of these systems (i.e. individuals or species) and for predicting the potential consequences of losing these components. To our knowledge, this is the first study to contrast the emergent structural properties of multitrophic interaction networks between different points along an environmental gradient, while accounting for population-level size and spatial pattern heterogeneities among participants.

In all six study plots, interaction networks representing interior Douglas-fir trees linked through shared associations

with *Rhizopogon* spp. genets had complex, nested topologies. The observed topologies were self-organizing via a positive relationship between the physical size of trees and their probability of association with fungal symbionts, combined with the number of trees those symbionts colonized. This suggests the structure of MNs between interior Douglas-fir trees and their EMF associates is cohesive and robust against the random loss of participants, but may be susceptible to the targeted loss of large trees or hyper-linking fungal genets. Soil moisture regime affected the networking attributes of tree nodes, but MN topologies were similar in mesic and xeric forest stands, suggesting *Rhizopogon* spp. links between trees are resilient to soil water stress.

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Data accessibility

Spatial reference data for trees and EMF samples, and population genetic analysis and microsatellite data are available in the Dryad Digital Repository (Beiler, Durall & Simard 2015).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Maps of tree and fungal genotypes associated with tuberculate mycorrhiza samples in the six 10 × 10 m study plots.

Figure S2. MNs illustrated aspatially to contrast the topologies of empirical MNs with those of corresponding randomly generated ER networks.

Table S1. Glossary of network analysis terms pertaining to mycorrhizal networks.

Table S2. Results of generalized linear models fit to predict the number of links a tree has to other trees (node degree), the proportion of potential links to other trees this represents (normalized node degree) and the density of links among trees linked to that tree (node clustering coefficient).

Appendix S1. Auxiliary methods and results pertaining to plot characteristics, *Rhizopogon* spp. mycorrhiza sampling, and molecular and statistical analysis.