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Fungal Ecology

Community structure of ericoid mycorrhizas and root-associated fungi of *Vaccinium membranaceum* across an elevation gradient in the Canadian Rocky Mountains

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ABSTRACT

The ecological plasticity of *Vaccinium membranaceum* (Ericaceae) to grow from valley bottoms to alpine habitats allowed us to test if fungal community structure varies along an elevation gradient in east-central British Columbia. Using the shrub as an independent variable, and plant and soil features along the gradient to model a changing climate, communities of root-associated fungi were investigated. The colonized roots from 40 individuals were examined using culture-dependent sequencing and ARISA (automated ribosomal intergenic spacer analysis). Our results demonstrated that high elevation fungal communities, characterised by *Rhizoscyphus ericae*, differ from lower elevation communities, where *Phialocephala fortinii* was the most frequently isolated fungus. Co-occurrence analysis indicated that, overall, fungi tended to occur together more often than would be expected by chance. At the scale of the individual host plant, facilitation may play a more important role than competition in shaping fungal communities in these ecosystems.

Introduction

Vaccinium membranaceum (Ericaceae), commonly known as black huckleberry, is predominantly found in western North America, but has a broad distribution ranging from Alaska to Arizona and as far east as Ontario and Michigan (Vander Kloet 1988; Small & Catling 2005; Barclay-Estrup 2007). It is an important wild food collected in the Pacific Northwest (Kerns et al. 2004; Trusler & Johnson 2008). In British Columbia, it is widely distributed, growing from valley bottoms to high elevation sites in dry to moist coniferous forests (Mackinnon et al. 1992). Unlike other members of the genus, which includes blueberries, this shrub is not easily cultivated (Barney et al. 2007). *V. membranaceum* forms ericoid mycorrhizas,

symbiotic associations in which soil fungi colonize the host plant epidermal cells of very narrow lateral roots ('hair roots') (Peterson *et al.* 2004; Smith & Read 2008). Anatomically, fungi form characteristic hyphal coils visible under standard light microscopy (Peterson *et al.* 2004) but unlike ectomycorrhizas, they do not form distinctive morphologies (e.g. mantle, rhizomorphs) that would allow for morphotype characterization. Both partners in the symbiosis may derive an equal level of benefit from the association, however, the sharing of costs and benefits of a mutualism can vary between partners (Brundrett 2004).

Ericoid fungi can be cultivated from roots on nutrient media. Not all fungi found in ericaceous plant roots are mycorrhizal, as dark septate fungi and numerous other root-associated fungi also occur (Berch *et al.* 2002). *Rhizoscyphus* (syn. *Hymenoscyphus*) *ericae* is a widely recognized ericoid fungus frequently isolated from ericaceous host roots (Read 1996; Kerley & Read 1998; Berch *et al.* 2002; Read & Perez-Moreno 2003). This fungus has been shown to facilitate host nitrogen uptake (Kerley & Read 1998; Kosola *et al.* 2007), although the nitrogen source may vary depending on the *R. ericae* isolate tested (Cairney *et al.* 2000). Other recognized ericoid fungi include *Cadophora finlandia*, several *Oidiodendron* species (Mitchell & Gibson 2006), as well as *Geomyces pannorum* (Vohnik *et al.* 2007). Many of these fungi are closely related genetically (Egger & Sigler 1993; Hambleton & Sigler 2005) and their taxonomy remains an issue of interest. They are generally assumed to facilitate the uptake of complex forms of nitrogen and phosphorus in highly organic and recalcitrant environments (Mitchell & Gibson 2006). While many of these fungi have been studied as isolates, we know much less of their community structure in natural habitats.

In the present study, we examined ericoid mycorrhizal community structure across an elevation gradient. Close proximity of sampling sites allowed us to compare fungi within an ecosystem but under very different environmental conditions. With warming in a climate change scenario, it is generally accepted that species will migrate upward in elevation and north in latitude (Hamann & Wang 2006; Parmesan 2006; Aitken *et al.* 2008), thus our sampling was intended as a snapshot in time of communities under potentially changing conditions. Understanding how mycorrhizal community structure responds to environmental conditions becomes critical as soil ecology impacts aboveground plant composition (Kardol *et al.* 2010) and mediates responses to climate change (Pendall *et al.* 2008). It is becoming evident that, in British Columbia, predicted changes in temperature and moisture regimes are more likely to follow the worst-case scenario outlined in the 2007 IPCC report (Pojar 2010). Now more than ever, it is important to decode the below ground interactions that are so crucial to our ecosystems.

V. membranaceum shrubs occur across the elevation gradient at McBride Peak. We were interested in determining if the root-associated fungal communities of *V. membranaceum* would differ between elevations. Using a combination of fungal DNA analysis and fungal culturing, the objectives of our study were to: (1) document the fungi associated with *V. membranaceum* roots along an elevation gradient, and (2) determine if differences occurred in fungal species

between elevations, and if these were correlated to soil properties or other variables. The null hypothesis for the study was that the community structure of *V. membranaceum* root-associated fungi would remain the same across the elevation gain on McBride Peak.

Methods

Study site

McBride Peak (53°20 N, 120°07 W) is located outside the town of McBride on the western side of the Canadian Rocky Mountains in east-central British Columbia. The southwest facing slope represents four elevation zones: alpine, subalpine (Engelmann Spruce-Subalpine Fir), mid-elevation (Interior Cedar Hemlock) and low elevation (Sub-Boreal Spruce). Transects were established perpendicular to the slope at approximately the midpoint of each zone, with the exception of the alpine, which was located about 50 m above the treeline. Climate BC (Wang *et al.* 2007) was used to calculate mean annual temperature (MAT), mean annual precipitation (MAP), mean annual summer (May–Sep.) precipitation (MSP) and the number of frost free days (NFFD) based on GPS points taken at transect locations. The zone referred to as 'alpine' in this study (1 923 ± 10 m, MAT = 0.5 °C, MAP = 1 262 mm, MSP = 587 mm, NFFD = 129) was the lower range of the true alpine-tundra biogeoclimatic zone. Alpine climate variables were compared to subalpine (1 801 ± 7.5 m, MAT = 0.5 °C, MAP = 1 229 mm, MSP = 586 mm, NFFD = 129), mid-elevation (1 224 ± 10.4 m, MAT = 0.4 °C, MAP = 1 491 mm, MSP = 558 mm, NFFD = 130) and low elevation (875 ± 11.2 m, MAT = 0.1 °C, MAP = 1 353 mm, MSP = 600 mm, NFFD = 125) zones.

Sampling

In mid-Jul. of 2006, a 100 m transect was established perpendicular to the slope of McBride peak in each of the four zones. At 10 m intervals along each transect, we harvested a single huckleberry plant (the closest to each 10 m marker), with roots and surrounding soil, for a total of 40 plants (10 per transect). Plants were double-bagged, kept in coolers during transport to the University of Northern BC, and subsequently stored at 4 °C. Processing for analyses took place over a period of 14 weeks. For each plant, all lateral roots with fine hair roots were harvested, randomly divided into three equal-size subsamples, and processed as follows: (1) frozen at −20 °C for later DNA extraction and ARISA analysis; (2) roots surface sterilized immediately for culturing and DNA sequencing; and (3) preserved in 50 % ethanol for microscopic analyses.

ARISA (automated ribosomal intergenic spacer analysis)

Originally developed to evaluate bacterial diversity, ARISA exploits internal transcribed spacer (ITS) length variability between fungal species to generate a community profile (Fisher & Triplett 1999). Fluorescent labelled primers amplify the ITS region in a PCR reaction which is then analyzed electrophoretically with an automated sequencer. The profile of peaks generated from a community is a fingerprint that

represents the diversity of fungi within that community (Ranjard *et al.* 2001). It has been used to effectively compare fungal communities (Ranjard *et al.* 2001; Lejon *et al.* 2005) but may, however, fail to detect rare species (Bent *et al.* 2007). DNA was extracted in duplicate from a subsample of hair roots from each *V. membranaceum* plant, using the UltraClean Soil DNA extraction (MoBio Laboratories Inc.) kit following the alternative protocol for maximum yields. A total of 80 samples (two per plant) were amplified with the forward primer ITS3 (White *et al.* 1990), and the green dye (D3) labeled reverse primer NLB4 (Martin & Rygiewicz 2005) targeting the ITS2 region of fungal ribosomal DNA. Each 30 µl PCR reaction consisted of 1 µl template, 1× PCR buffer, 0.2 mM dNTP's, 1 mM MgCl$_2$, 3 µM of each primer and 1 U of Platinum Taq DNA Polymerase (Invitrogen). Thermocycler conditions were 4 min denaturation at 94 °C followed by 35 cycles of denaturing, annealing and extension for 35 s, 52 °C for 1 min and 72 °C for 1.5 min, respectively. The final extension was 5 min at 72 °C. Fragment analysis was done with the Beckman Coulter CEQ™ 8000 Fragment Analysis System (Beckman−Coulter Inc.). The Variable Percentage Threshold (VPT) method (Osborne *et al.* 2006) was used to score peaks in ARISA profiles. Each of the 80 samples was subjected to ARISA. Duplicate profiles generated for every *V. membranaceum* plant were additively combined for a total of 40 profiles. Each profile was linked to the original plant to generate the data matrices that were then used in ordination analysis.

Cultures and sequencing

Segments of hair roots (1−6 cm in length) were surface sterilized and placed on full strength Potato Dextrose Agar (PDA) following the protocol of Berch *et al.* (2002); these were incubated at room temperature for up to 6 months. In an attempt to capture all possible culturable fungal species, 1/3 of all root segments harvested for each *V. membranaceum* shrub were prepared for culturing. From cultures that grew, a total of 298 were successfully identified by sequencing the ITS1-5.8S-ITS2 region of ribosomal genes using the following protocol: DNA was extracted using the Nucleospin multi-96 plant kit (MJS BioLynx), according to the manufacturer's protocols. Six positive controls (*Cladosporium* sp., *Endoconidioma* sp., *G. pannorum*, *Knufia* sp., *Sclerotinia sclerotiorum*, *Tilletia barclayana*) were used per 96-well plate. Extracted DNA was amplified using the forward primer ITS5 (White *et al.* 1990) and reverse primer LR6 (Vilgalys & Hester 1990). In a 10 µl reaction, consisting of 0.1 mM of dNTP's, 0.8 µM of each primer and 1× Titanium Taq™ buffer (including 3.2 mM MgCl$_2$), and 0.1 ng Titanium Taq™ Polymerase, 1 µl of template was added. The thermocycler conditions were 95 °C initial denaturation, then 95 °C for 1 min followed by annealing at 58 °C for 30 s and a 72 °C extension for 2 min for 40 cycles. A final extension of 72 °C for 8 min was included before being held at 10 °C. Bands were visualized using the E-gel® 96 high-throughput agarose electrophoresis system (Invitrogen).

PCR products were direct-sequenced using BigDye™ Terminator v. 3.1 Cycle Sequencing Reaction Kit (ABI Prism/Applied Biosystems, Streetsville, ON) in 10 µl sequencing reactions using the forward primers ITS1 and ITS5, and reverse ITS4 (White *et al.* 1990). The reaction mix included 1.75 µl 5× buffer, 0.5 µl 2.5× BDT sequencing mix and 1.6 pmol primer. Amplicons were purified by ethanol/sodium acetate precipitation and analyzed by Applied Biosystems 3130x1 Genetic Analyzer (Applied Biosystems, Streetsville, ON). Sequences were edited, and forward and reverse sequences compiled using Sequencher 4.2.2 (Gene codes corp, Ann Arbor, MI). Edited sequences were compared to GenBank using BLAST searches (www.ncbi.nlm.nih.gov) to find the closest named fungus.

Percent colonization

Approximately 30 lateral hair root segments, 3−6 cm long, were selected from each *V. membranaceum* plant, stained and mounted on microscope slides as follows: segments were first rinsed 3× in sterile dH$_2$O and then cleared in 5 % KOH at 60 °C for 12 hr. To remove the KOH, roots were again rinsed 3× in sterile dH$_2$O and then stained with 0.03 % Chlorazol Black E (Sigma C-1144) in 1:1 lacto-glycerol at 60 °C for 3 hr. Roots were mounted in 1:1 lacto-glycerol. Visual examination was made following the magnified intersections method (McGonigle *et al.* 1990), using 400× magnification and scoring within two categories (ericoid and dark septate) for a total of 100 counts per sample.

Soil analysis

Mineral and organic fractions from soil associated with each plant were collected and sieved, for a total of 80 samples. Soils were ground and pH, total C and N determined. The pH measurements were performed in duplicate using an Orion 710A pH meter, diluting the organic soil 2:1 in dH$_2$O and 1:1 for the mineral soil. Total organic C and total N content of both soil fractions were determined using a Fison NC 1500 Elemental Analyzer. Soil moisture content associated with each harvested plant was measured *in situ* at time of sampling using a 12 cm TDR probe (the mean of four measurements was reported).

Plant metrics

Ten fresh leaves per plant (randomly collected) were scanned using a LI-3100 Area meter (LI-COR, Lincoln, NE) and dried to a constant weight at 105 °C. Leaf mass to area ratio (LMA) was expressed as mean area divided by mean mass. *V. membranaceum* is a multi-stemmed shrub. All large stems attached to the same root system were cross-sectioned by hand using razor blades and stained for 1 min in 10 % potassium iodine solution, mounted on slides in lacto-glycerol, and examined with an Olympus CH30 light microscope. Rings, defined as the change in vessel dimension from early wood to late wood, were counted to determine plant age. The oldest shoot was considered to be the age of the plant (Schweingruber & Poschold 2005).

Data analyses

Ordinations of data set matrices, generated for culture-dependent sequencing and ARISA, were calculated based on Sorensen's distance and performed using PC-ORD v5.0 (McCune & Grace 2002) run on autopilot in the 'slow and thorough' mode, using random starting coordinates. Monte Carlo tests were run for each matrix, with 250 randomized runs. No solutions above a final stress of 20 or above an

instability value of 0.03 were considered. Solutions for ecological community data with stress values between 10 and 20 are common; the lower the value, the more reliable the solution (McCune & Grace 2002). Differences between the four elevations, based on composition of fragments generated by ARISA, and cultures identified by sequencing, were determined using a non-parametric multivariate ANOVA (perMANOVA). Results were deemed significant when $P \leq 0.05$.

Beta diversity (β_W, Whittaker 1960) is reported as the ratio of gamma to alpha diversity, where alpha diversity is the mean number of fragments (or cultures) per plant, and gamma diversity is the richness value for each elevation. In addition, beta sorensens and beta sim (Koleff *et al.* 2003) are included. Beta Sorensens is a broad-sense measure that emphasizes species in common and differences in richness between the fungal communities. Beta sim is a narrow-sense measure that emphasizes gains and losses of species between sites and is more sensitive to composition changes than beta Sorensens. With both measures, high values represent low beta diversity (high similarity) and, conversely, low values represent high beta diversity (low similarity between root-associated communities). Both culture and ARISA data sets were tested by the Mao Tau statistic to estimate the number of expected species with a 95 % confidence interval with 500 Monte Carlo iterations using EstimateS (Colwell 2009).

Indicator species analysis was applied to the fungal community data to assess the value of a species (or fragment) to indicate the conditions at a particular elevation. This intuitive measure, ranging from 0 to 100, applies a value of 100 to a perfect indicator. A perfect indicator species or fragment would always be present at a particular elevation (faithful) and would never occur at another elevation (exclusive) (McCune & Grace 2002). Relative abundance of a species or fragment within an elevation compared to other elevations are determined and multiplied by the relative frequency (proportion of species or fragments in each elevation that contains that species or fragment). Both values must be high to result in a high indicator species value. A Monte Carlo randomization was used to determine statistical significance (McCune & Grace 2002).

C-score analysis

C-score analysis was performed using Ecosim v.7 (Gotelli & Entsminger 2001). The C-score uses species presence/absence data to determine whether species occur together, at a site,

more or less often than expected by chance (Stone & Roberts 1990). Each V. *membranaceum* root system was considered a site. The C-score was calculated using the fixed-equiprobable null model because it is the most stringent (Gotelli & Entsminger 2001), and the data were randomized to produce a normal distribution to which the calculated C-score was compared. A significant result occurs if the calculated C-score is two or more standard errors of one of the tails of the distribution. A low C-score indicates that the two species in question co-occur more often than would be expected by chance, whereas a high score indicates species that tend not to co-occur. The analysis also produces pair-wise C-scores which correspond to the specific level of co-occurrence of the two species.

ANOVA

All univariate plant, soil and fungal variables were tested for differences between elevations using one-way ANOVA. A *post hoc* Tukey's HSD test was applied (Zar 1999). Variables assessed included plant (age and leaf mass to area ratio), soil (water content, pH [organic and mineral], C:N ratio [organic and mineral]) and fungi (percent colonization, richness of fragments and cultures). A $P \leq 0.05$ was considered significant.

Results

ARISA

Using ARISA, a total of 57 fragments (ranging from 310 to 681 bp) were generated from the 40 root samples. The Mao Tao 95 % confidence interval determined that between 50.51 and 63.48 fragments could be expected to be found using this species detection method. The alpine roots generated 20 fragments, the subalpine 27, the mid-elevation 36 and the low elevation 29. The mean number of fragments isolated per plant increased with a decrease in elevation, from 4.4 to 8.3 (Table 1), however, this trend was not statistically significant ($P > 0.05$). ARISA fragment patterns were not autocorrelated along the individual transects, nor between them. Autocorrelation was tested using Spatial Analysis for Macroecology (Rangel *et al.* 2010).

Fungal cultures

The number of roots recovered per plant was highly variable, possibly due to differences in root morphology or losses

Table 1 — Mean (SD) values for ARISA and culture data showing fragment and species richness per plant (α), beta ($\beta = \gamma/\alpha$) and gamma (γ = total species richness per elevation) diversity. Unique values indicate the number of fragments (or cultures) that were only found at that elevation

Elevation (N = 10)	ARISA				Cultures			
	α	β	γ	Unique	α	β	γ	Unique
Alpine	4.4 (3.75)	4.5	20	3	3.5 (1.35)	3.1	11	0
Subalpine	6.7 (4.88)	4.0	27	5	3.2 (1.40)	3.1	10	0
Mid-elevation	7.0 (5.75)	5.3	36	10	2.7 (0.82)	3.3	9	0
Low elevation	8.3 (1.89)	3.5	29	4	2.4 (0.97)	3.75	9	0

despite careful harvesting and processing. This resulted in variable numbers of cultured roots per plant. In general, root segments from the alpine produced fewer viable fungal cultures than low elevation plants, which always yielded fungi (data not shown). Nevertheless, in contrast to ARISA, alpha diversity decreased with decreasing elevation (Table 1); mean differences were again not significant ($P > 0.05$). Representative sequences generated from cultures are listed in Table 2. Between one and six distinct taxa were isolated from each root system. Overall, 10 taxa were identified from 367 cultures. The Mao Tao 95 % confidence interval determined that between 8.66 and 11.33 unique cultures could be expected to be found. The culture data was also checked for autocorrelation within and between transects; no samples were found to be autocorrelated.

Phialocephala fortinii was the most commonly isolated fungus, representing 53 % of all cultures and found at all four elevations. *Meliniomyces* sp. and *Cryptosporiopsis* sp. also occurred at all elevations. Several *Meliniomyces* sp. were found in the subalpine and these represented 83 % of the cultures isolated at that elevation. *Cryptosporiopsis* sp. occurred most frequently at the low elevation, representing 49 % of all isolates at this elevation. *R. ericae* was found most frequently in the alpine, representing 65 % of the isolates from that elevation. This species was not found in the low elevation.

Diversity measures

The alpha, gamma and beta diversity for species and fragments identified at each elevation are presented in Table 1. The diversity indices for beta sorensens and beta sim (Table 3) were used to compare elevations using both ARISA and culture data. Identical sites generate a value of 1, whereas sites with no co-occurring species generate a value of zero. Alpine and low elevation sites were most different from each other. The subalpine and alpine sites were most similar. Other comparisons were less clear and often varied depending on the diversity index as well as the method used to assess community structure.

Plant, soil and fungus characteristics

Six out of eight plant, soil and fungus characteristics differed significantly between elevations (Table 4). Alpine and low elevations always differed significantly, with subalpine and mid-elevations most often having values shared by higher or lower elevations. Alpine and subalpine *V. membranaceum* plants were significantly ($P \leq 0.05$) older than their mid- and low elevation counterparts. Leaf mass to area ratios were highest in the alpine. Overall, the alpine and subalpine soils were most similar, compared to mid- and low elevations, which also shared some similarities. Alpine and subalpine soils were significantly wetter compared to mid- and low elevation soils, and had lower pH (both mineral and organic) levels than low elevation soils. Mid-elevation pH levels were also lower than those for the low elevation. Mineral soil C:N ratios were lower for alpine compared to low elevation soils, with intermediate values occurring at subalpine and mid-elevations. Significant differences were not found for organic soil C:N ratios (values ranged from 16.5 to 74.8). Percentage of roots colonized by both ericoid and dark septate fungi suggested alpine (mean 46.3 ± 15.2 %) and mid-elevation (mean 45.1 ± 13.8 %) were more highly colonized (though not significant) than the subalpine (mean 34.2 ± 13.1 %) and low elevation (mean 34.6 ± 14.9 %). No correlations were identified with respect to the number of fungi cultured, either for the dark septate or ericoid hyphal complexes, or both.

Multivariate analyses

Table 5 shows significant differences in fungal community structure for ARISA and culture results between elevations, as determined by perMANOVA. In both cases, elevations furthest apart (e.g. alpine versus low elevation) are significantly

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Table 2 — Cultured and sequenced fungi isolated from V. *membranaceum* roots collected at four elevations. Values represent the number of cultures at each elevation. Overlap indicates number of base pairs matched to a sequence in 'Best match'. Identity indicates the quality of the match. Accession numbers are included for species submitted to GenBank as well as 'Best match'

Species ID	Accession number	Number of cultures at each elevation					Best match	Overlap	Identity
		Alpine	Subalpine	Mid-elevation	Low elevation	Total			
Phialocephala fortinii	HQ873697	29	65	75	27	196	*Phialocephala fortinii* (AY394905)	496/1 171	99 %
Rhizoscyphus ericae	HQ873698	15	7	1	0	23	*Rhizoscyphus ericae* (DQ658887)	484/545	99 %
Cryptosporiopsis sp.	HQ873699	11	8	19	37	75	*Cryptosporiopsis ericae* (FJ205458)	491/541	99 %
Meliniomyces sp.	HQ873700	2	25	2	1	30	Uncultured *Meliniomyces* (FJ152523)	502/1 126	100 %
Oidiodendron sp.	HQ873701	2	9	3	0	14	*Oidiodendron maius* (AY624308)	492/498	100 %
Neonectria radicicola	HQ873702	0	2	0	8	10	*Neonectria radicicola* (FJ430730)	474/517	99 %
Lachnum sp.	HQ873703	0	4	2	1	7	*Lachnum pygmeum* (AJ430219)	474/476	99 %
Epacris microphylla root-fungi	HQ873704	3	1	0	1	5	*Epacris microphylla* root-fungi (AY268217)	494/524	99 %
Salal root UBCtra153	HQ873705	3	2	0	0	5	Salal root UBCtra153 (AF149078)	471/492	99 %
Geomyces sp.	HQ873706	0	0	2	0	2	*Geomyces* sp. (AJ608960)	499/566	100 %

Elevation	ARISA		Culture	
	Beta sorensens	Beta sim	Beta sorensens	Beta sim
Alpine vs Subalpine	0.667	0.700	0.609	0.737
Subalpine vs Mid-elevation	0.632	0.667	0.459	0.538
Mid-elevation vs Low elevation	0.444	0.444	0.594	0.655
Alpine vs Mid-elevation	0.500	0.556	0.444	0.632
Alpine vs Low elevation	0.400	0.444	0.375	0.474
Subalpine vs Low elevation	0.632	0.667	0.464	0.481

different from each other, with some overlap as one moves across the elevation gradient. The NMS graph of fungal cultures (Fig 1) shows grouping of low elevation cultures with some overlap with the other elevations. Vectors indicate Pearson and Kendall correlations to measured characteristics. This analysis yielded a two-dimensional solution that had a final stress of 19.53 and instability of 0.00002. The NMS graph of ARISA data did not show clear groupings and is not shown here.

Indicator species

Analysis of the ARISA data resulted in two species of particular interest: fragment 496 (subalpine) was identified as an indicator species at this elevation, and fragment 612 was considered closely associated, although not significant ($P > 0.05$), with the low elevation. From the culture data set, *R. ericae* was found to be a significant ($P \leq 0.05$) indicator of the alpine.

C-score

Both ARISA and culture data sets analyzed by C-score showed that communities associated with *V. membranaceum* hosts were composed of fungal species that co-occurred more often than would be expected by chance (Table 6). This result was true for fungal—plant communities within all elevations except the low elevation (Table 6). Species pair scores for those that always occur together (0 value), as well as species that tend not to occur together (significantly ($P \leq 0.05$) high C-scores), are listed in Table 7.

Discussion

This study examined fungal communities associated with *V. membranaceum* growing in the Canadian Rocky Mountains in habitats representing ecologically and climatically distinct elevations, from a low sub-boreal mixed deciduous-coniferous forest to an alpine ecosystem above treeline. We showed that *V. membranaceum* root-associated fungal community structure varied significantly between elevations along the elevation gradient. Differences in fungal communities were supported by both ARISA and fungal culture approaches. Adjacent sites were always more similar than those furthest apart, i.e. the low elevation was most different from the alpine with respect to their fungal communities. The strength of this finding is in the fact that a large scale culturing effort, as well as molecular fingerprinting, both demonstrate a similar pattern within the community. Culture-dependent methods have been shown to favour ascomycetes, while DNA-dependant methods tend to capture basidiomycetes (Allen *et al.* 2003). The observation that some alpine root fragments did not yield culturable fungi may indicate this community contains relatively more basidiomycetes. Selected plant and soil characteristics differed significantly between alpine and low elevations, changing over a gradient at intermediate elevations, however, strong correlations between these factors and associated fungal communities were not found. This may be due to the overlap in communities (Fig 1) and the variability in the community data. A larger sample size was unlikely to capture more species within this system according to the Chao statistic. Thus the variability detected likely represents natural variation in the system.

This is the first report of ERM community structure over an elevation gradient. Others that have looked at effects of natural gradients on mycorrhizal community structure include Bougoure *et al.* (2007), Kernaghan & Harper (2001), Mulder & de Zwart (2003), and Nilsson *et al.* (2005). Bougoure

Table 4 − Mean (SD) and ANOVA results for plant (*Vaccinium membranaceum*) and soil properties across four elevations. N = 10. Means within the same row followed by the same letter are not significantly different (*post hoc* Tukey's HSD test, P ≤ 0.05)

Plant/soil characteristic	Elevation			
	Alpine	Subalpine	Mid-elevation	Low elevation
Plant age (years)	12.2 (3.3)a	9.5 (3.50)a	6.2 (2.1)b	5.8 (2.8)b
Leaf mass to area ratio (g/m²)	58.2 (5.4)a	47.9 (4.8)b	31.2 (7.3)c	29.2 (2.3)c
Soil water content (%) at harvest	12.1 (2.36)a	11.5 (4.06)a	8.2 (1.83)b	5.8 (1.18)b
pH organic soil	3.74 (0.29)a	3.73 (0.20)a	4.46 (0.65)b	5.40 (0.50)c
pH mineral soil	4.00 (0.27)a	3.97 (0.33)a	4.17 (0.29)a	4.96 (0.49)b
C:N ratio (mineral soil)	19.06 (5.19)a	24.41 (5.78)ab	25.08 (2.65)ab	26.78 (6.56)b

Table 5 – One-way perMANOVA results comparing root-associated fungal communities of *V. membranaceum* across four elevations as assessed by ARISA and culture methods. Within columns, elevations sharing the same letter are not significantly different ($P \leq 0.05$)

Elevation	ARISA	Culture
Alpine	a	ab
Subalpine	a	bc
Mid-elevation	ab	cd
Low elevation	b	d

et al. (2007) examined both *Calluna vulgaris* and *Vaccinium myrtillus* root-associated fungal communities along a heath to forest gradient in Scotland. They found that the fungal community composition did not differ for *V. myrtillus* between their sites, whereas it did for *C. vulgaris*. Both hosts had distinct fungal profiles and high fungal diversity associated with their roots. Kernaghan & Harper (2001) found that ectomycorrhiza (ECM) species richness and diversity decreased with increases in elevation along a gradient similar to ours in the Canadian Rocky Mountains. Host-specific ECM were mainly identified from the subalpine forest whereas, in the alpine, ECM fungi tended to be generalists. Similarly, in our
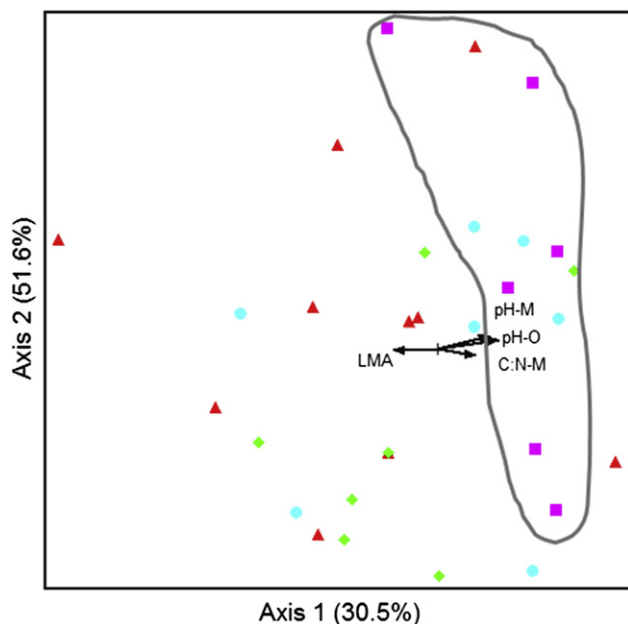

Fig 1 – NMS ordination of presence/absence data for root-associated fungal cultures isolated from *Vaccinium membranaceum* occurring at four distinct elevations: alpine (▲), subalpine (♦), mid-elevation (●) and low elevation (■). Associated primarily with Axis 1, Pearson and Kendall correlations for environmental variables are pH of organic soil ($r^2 = 0.221$), pH of mineral soil ($r^2 = 0.185$), C:N ratio of mineral soil ($r^2 = 0.135$) and LMA ($r^2 = 0.157$). Percentages denote variability explained by each axis. Arrows are vectors denoting direction and size of environmental variable change associated with groupings of fungal communities.

study, known ERM fungi (i.e. *R. ericae*) were found predominately in habitats with an abundance of ericaceous plants.

Overall, the number of roots recovered per plant was highly variable. Differences, whether intrinsic or inevitable due to losses despite careful processing, were considered random and equal for all plants at all elevations, minimizing the influence on analytical outcomes. In addition, all roots were evenly and randomly assigned to one of the three analyses as they entered the downstream processes. This sampling decision reduced bias between the three methods applied, however, any initial imbalance in numbers of roots was passed along. Other sources of possible bias could have come from culturing (e.g. loss of uncultured species) as well as PCR and peak height measures in ARISA. Focusing on presence–absence data rather than abundance, the Mao Tau statistic indicated that we should be reasonably confident that we identified most of the taxa we could expect by using these methods.

Co-occurrence analysis

Diamond (1975) posited that species in competition will exclude each other and thus will not occur together. Gotelli & McCabe (2002) found, in a meta-analysis of studies considering differing scales of presence/absence data that, in fact, non-random exclusion of species is the rule rather than the exception. Our study found that ericoid mycorrhizal fungi did not follow this trend. Fungi co-occurred on each plant more often than would be expected by chance (Table 6), although there were some exceptions (Table 7). This could be indicative of fungal colonization facilitation by the host. Koide *et al.* (2005) examined ECM systems in a red pine (*Pinus resinosa*) plantation using this approach and found less co-occurrence of species than would be expected by chance, although some species pairs did co-occur. Differences in study results may relate to differences between ecto- and ericoid mycorrhizal symbioses, or to the scale at which the systems were assessed. Koide *et al.* (2005) found evidence for competition in ECM fungi at the level of an individual root, whereas our study determined that competition did not appear to be occurring at the level of whole root systems of *V. membranaceum*.

There exists a strong possibility that the symbiotic mycorrhizal relationship influences the interspecies dynamics of associated fungi. While many studies have tested Diamond's (1975) assembly rules (e.g. Gotelli & McCabe 2002; Arrington *et al.* 2005; Luiselli *et al.* 2007), mycorrhizal systems have not been thoroughly explored from this perspective, probably because large mycorrhizal data sets are needed. In the study by Saari *et al.* (2005), Scots pine were linked to their mycorrhizal root tips *via* microsatellites. We conducted a C-score analysis (as for our study) on the raw data provided by Saari *et al.* (2005), for the presence/absence of fungi identified for each tree. Our analysis found that Scots pine ECM fungal communities were not competitively structured, that is, the C-score (0.33) was significantly ($P < 0.002$) smaller than a randomized mean score (0.64). Thus, preliminary analysis of available data demonstrates that, when considered at the same scale, these ECM communities may follow similar structuring to the ericaceous root-associated fungi in the present study. At the very least, it can be said that these fungal

Table 6 – C-scores and P-values for data resulting from ARISA and culture analysis comparing root-associated fungal communities of *V. membranaceum* across four elevations. Culture results did not show significant C-score values for elevation (data not shown). Not significant (NS)

Method	Elevation	C-score observed	C-score expected	P-value	Standardized effect size
ARISA		6.769	8.869	0.000	−11.490
	Alpine	1.200	1.974	0.000	−6.087
	Subalpine	1.154	2.149	0.000	−8.899
	Mid-elevation	1.313	1.742	0.000	−7.831
	Low elevation	2.448	2.413	NS	
Cultures		10.679	16.173	0.000	−7.035

species do colonize in a non-random pattern. Saari *et al.* (2005), because of the complexity of identifying trees associated with fungi using molecular methods, used a smaller sample size that may have impacted the results of the C-score analysis. Regardless of the limitations, it appears that mycorrhizal fungi are organized in a fundamentally different manner than most other organisms tested thus far.

The high C-scores reported for some pairs of fungi indicate that these are unlikely to occur together. The differences we see between high and low elevations (Tables 2 and 5) are with the same fungi that show high C-scores (Table 7). Ericoid fungi are not obligate symbionts. They may be present in the soil *a priori*, *via* alternate hosts. For example, *R. ericae* inoculum may already be present in high elevation soils due to other ericaceous hosts that do not occur at lower elevations. The high C-score with *Cryptosporiopsis* sp. and *R. ericae* could simply reflect a lack of hosts at their respective elevations. It has been shown that ECM fungi associated with a Scots pine plantation exhibit patchiness and inter-specific competition at a plot scale (Pickles *et al.* 2010); if *Vaccinium*-associated fungi display similar distribution patterns, their non-randomness may be due to a heterogeneous distribution in the soil rather than actual competition or facilitation. Alternatively, high C-scores could also imply that the host is exerting some control over its symbiotic fungi, as documented in other systems (Walker *et al.* 1986; Tonkin *et al.* 1989; Rosado *et al.*

1994). In contrast to the study by Pickles *et al.* (2010), the present study was conducted in a mostly undisturbed ecosystem. Mixed plant communities are expected to contribute to patchiness. Thus, the high C-scores reported here may be an artefact and are not necessarily indicative of competition. Nonetheless, the high C-scores are an exception and, at the scale of the individual host plant, facilitation may play a more important role than competition in shaping fungal communities in these ecosystems.

Under warming climate scenarios, sites that represent elevation gradients such as those chosen for this study might be expected to experience upward changes in the climatic envelopes as well as accompanying changes in plant and fungal communities. Parmesan (2006) suggested that alpine plants (and presumably also their fungal partners) will become more isolated as the treeline moves higher. With respect to *V. membranaceum*, we have shown that this host shares at least some fungal associates between plants occurring at adjacent elevations, suggesting impacts due to climate warming may be less than that for other plant (or fungal) species that currently occur at only one, or disjoint elevations, and hence lack the connectedness of associated fungi between elevations. If warming trends continue, low elevation sites might be more at risk of losses of *V. membranaceum* than higher ericaceous-rich alpine habitats. Low elevation *Vaccinium* primarily associated with *Cryptosporiopsis* sp. and *P. fortinii* but these fungi were also found at higher elevations. Warming may favour these fungi over other less frequently occurring species. *P. fortinii* is a generalist endophyte that associates with many hosts (Jumpponen & Trappe 1998) and is unaffected by changes to moisture (Addy *et al.* 2000). Although it seems likely that these fungi will survive, changing precipitation regimes and warming may impact neighbour tree and understorey plant dynamics, including the introduction of new or invasive species across elevations.

Mycorrhizas act as gatekeepers of carbon flow between the soil and atmosphere (Simard & Austin 2010). They are intimately involved with and responsive to changes in carbon flux between above and below ground, and mitigate movement of carbon from the plant to the soil and of nutrients from the soil to the plant. Northern temperate forests are expected to shift from being a carbon sink to a source. Should this happen, terrestrial ecosystems are expected to become larger contributors to CO_2 outputs compared to anthropogenic sources. Mycorrhizas and root-associated fungi stabilize forest soils and thus impact the global carbon budget (Simard & Austin 2010).

Table 7 – *Vaccinium membranaceum*-associated fungal species pairs showing C-scores that represent significant distances between each pair. Species with zero distance between them, indicate that these were always found together

Species pairs	C-score[a]
Cryptosporiopsis−Rhizoscyphus ericae	136
Cryptosporiopsis−Meliniomyces sp.	88
Lachnum sp.−*Cryptosporiopsis*	66
Neonectria radicicola−Rhizoscyphus ericae	56
Meliniomyces sp.−*Lachnum* sp.	50
Meliniomyces sp−*Epacris microphylla* root-fungi	50
Cryptosporiopsis−Epacris microphylla root-fungi	0
Cryptosporiopsis−Geomyces pannorum	0

Standard deviation = 18.46; mean ± standard deviation = 95 % confidence interval; upper limit = 46.81; any C-score ≥47 is in upper 2.5 % of distribution.
a Mean = 9.98.

In this study, we demonstrated that parallel analysis of ericoid and endophytic mycorrhizal fungi agrees that fungal community structure changes follow an elevation gradient. Most importantly, our data showed an unexpected organization of fungi at the level of the individual *V. membranaceum* shrub. Fungi co-occurred more often than would be expected by chance, implying some level of facilitation, possibly through the host. The ubiquitous nature of the fungi identified here, such as *R. ericae,* which is known to partner with ericaceous shrubs worldwide (Kerley & Read 1998; Cairney *et al.* 2000), suggests that these plant–fungal relationships may persist, at least at mid- to higher elevations, under global warming. To further our understanding of this important plant, we should now focus on the functional contributions of *Vaccinium* root-fungal assemblages and the ecological importance of these findings in future changing landscapes.

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