

DIGGING DEEPER – HOW SOIL BIOTA DRIVE AND RESPOND TO PLANT INVASIONS

Differences in endophyte communities of introduced trees depend on the phylogenetic relatedness of the receiving forest

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Summary

1. Plant species sometimes perform extraordinarily well when introduced to new environments, through achieving higher growth rates, individual biomasses or higher densities in their receiving communities compared to their native range communities. One hypothesis proposed to explain enhanced performance in species' new environments is that their soil microbial communities may be different and provide greater benefit than microbial communities encountered in species' native environments. However, detailed descriptions of soil biota associated with species in both their native and introduced environments remain scarce.

2. We established a global network of sites in regions where the tree species *Pinus contorta* has been introduced (Chile, New Zealand, Finland, Scotland and Sweden), as well as native range sites where the introduced populations originated (Canada and USA). We conducted pyrosequencing analysis to compare the root fungal endophyte communities associated with *P. contorta* in its native environments and in introduced environments with phylogenetically similar and dissimilar tree species (i.e. *P. sylvestris* in Europe and *Nothofagus* spp. in the Southern Hemisphere).

3. Fungal communities associated with *P. contorta* consistently differed between its introduced and native environments. In Europe, *P. contorta* associated with the same community as *P. sylvestris*, where one particular species (*Piloderma sphaerosporum*) was particularly abundant relative to Canadian sites. In the Southern Hemisphere, *P. contorta* fungal communities were composed primarily of North American taxa and exhibited very little overlap with fungal communities associated with native *Nothofagus* spp.

4. Synthesis. Our work shows that plants exhibit considerable plasticity in their interaction with fungi, by associating with different fungal communities across native and introduced environments. Our work also indicates that fungal communities associated with introduced plants can assemble through different mechanisms, that is by associating with existing fungal communities of phylogenetically close species, or through reassembly of co-introduced and co-invading fungi. The identification of different fungal communities in a plant species new environment provides an important step forward in understanding how soil biota may impact growth and invasion when a species is introduced to new environments.

Key-words: ectomycorrhiza, enemy release, fungal endophytes, global study, home-versus-away comparison, introduction, invasion ecology, plant invasion, plant–microbe interaction, plant–soil (below-ground) interactions

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Introduction

Plant species are frequently introduced into new environments and sometimes achieve higher growth rates, individual plant size or higher densities within receiving communities relative to what they achieve in their native environments (Shea & Chesson 2002; Leger & Rice 2003; Hawkes 2007). The field of invasion ecology has identified many mechanisms that likely contribute to these patterns (Hierro, Maron & Callaway 2005; Gurevitch *et al.* 2011; Gundale *et al.* 2014b), and one specific mechanism receiving growing support is that plants interact with soil biota differently in their native vs. introduced environments (Klironomos 2002; Reinhart & Callaway 2006; Pringle *et al.* 2009). Soils contain an immense diversity of organisms, some of which can adversely affect plant performance (e.g. pathogens, root herbivores), while others can enhance plant performance (e.g. mycorrhizal fungi and decomposers). Non-native species could thus gain an advantage if they are able to escape antagonistic interactions with soil biota in their native environments (i.e. the Enemy Release Hypothesis), or if they are able to form novel positive associations in their new environments (i.e. the 'Enhanced Mutualism Hypothesis') (Elton 1958; Keane & Crawley 2002; Reinhart & Callaway 2006).

Evidence that soil biota contribute to the success of some exotic species has come primarily from two types of glasshouse experiments. In the first type, native and exotic species that coexist in a particular environment are grown for one or more generations in sterile and non-sterile soils derived from their local community. One of the first studies of this type showed that rare native plant species in a Canadian prairie system developed net negative feedbacks with soil biota, whereas abundant invasive plant species developed net positive feedbacks (Klironomos 2002). Several subsequent studies of this type have demonstrated similar patterns (e.g. Agrawal *et al.* 2005; van der Putten *et al.* 2007; Engelkes *et al.* 2008). In the second type of experiment, one or more exotic or invasive plant species are grown with or without soil biota from both their native or introduced environment (e.g. Reinhart *et al.* 2003; Callaway *et al.* 2004; Yang *et al.* 2013). This type of experiment can be difficult to interpret because key factors other than soil biota can also differ between native and non-native ranges (e.g. climate, soil physical properties or genetics of the focal plant species). However, several recent experiments accounting for some or all of these potentially confounding factors have provided strong evidence that introduced species can benefit from soil biotic communities in their new environments (e.g. Gundale *et al.* 2014a; Maron *et al.* 2014).

While there is increasing evidence that plant–microbe interactions can contribute to the success of introduced species, few studies have evaluated how soil biotic communities actually differ across species native vs. introduced environments, or in what types of environments introduced plants are likely to encounter advantageous or disadvantageous soil communities. One factor that could strongly influence the composition of the soil community with which a plant species interacts in

its new environment is its degree of phylogenetic similarity to the dominant native species in that environment. Many positive plant–microbe interactions have developed through long co-evolutionary histories and as a result often demonstrate some degree of host specificity (Molina & Trappe 1994; van Heijden & Sanders 2003). For example, members of several tree families, including the *Pinaceae*, are known to form genus-specific obligate ectomycorrhizal fungal (EMF) associations (Tedersoo *et al.* 2007; Dickie *et al.* 2010; Kohout *et al.* 2011). Therefore, the introduction of a plant species into environments with phylogenetically similar vegetation could increase the likelihood of encountering soil microbes with which they will positively interact. Likewise, if antagonistic soil organisms demonstrate some degree of host specificity, a plant species may be more likely to escape enemies when introduced into plant communities with phylogenetically dissimilar vegetation (Moeller *et al.* 2015); however, the absence of mutualists in these same environments may also constrain establishment and invasion (Richardson *et al.* 2000). To date, no study has compared plant–microbe associations across a species native range and a wide range of receiving environments where the phylogenetic relatedness of the native vegetation varies strongly.

In this study, we characterized root fungal endophyte communities associated with a single tree species, *Pinus contorta*, which originates from north-western North America (USA and Canada), and which has been introduced to several Southern Hemisphere countries where it has become highly invasive (e.g. Chile and New Zealand; Taylor *et al.* 2016), and across northern Europe (e.g. Scotland, Sweden, and Finland), where it typically achieves higher growth rates relative to native *Pinus sylvestris* (Varmola *et al.* 2000; Elfving, Ericsson & Rosvall 2001; Gundale *et al.* 2014b). We focused on root fungal endophytes because both EMF and pathogenic fungal interactions are well known to influence the performance of *Pinaceae* (e.g. Mallett & Volney 1999; Courty *et al.* 2010). Because *P. contorta* has been introduced into European ecosystems where phylogenetically similar species are prevalent (e.g. *P. sylvestris*), and Southern Hemisphere locations where forests are dominated by phylogenetically more distant tree species (notably *Nothofagus* spp.) and lack native *Pinaceae*, our study system provides an ideal opportunity for evaluating how an introduced plant species associates with endophytic root fungi across contrasting receiving communities.

We tested the following hypotheses: (i) *Pinus contorta* will associate with different and less species rich fungal communities in its introduced ranges; however, we expect these differences to be greater in the Southern Hemisphere because of the greater phylogenetic difference with dominant native vegetation in this region. (ii) When introduced to a new environment, *P. contorta* will associate with a different and less species rich fungal community than native species in those environments; however, we predict this difference will be most pronounced in the Southern Hemisphere where *P. contorta* is phylogenetically more dissimilar to the dominant native tree species (*Nothofagus* spp.) relative to European

introduction sites dominated by pines (*P. sylvestris*). (iii) In the Southern Hemisphere, where *P. contorta* is highly invasive, we predict that: (a) *P. contorta* individuals invading the landscape will associate with EMF communities that are more similar to the *P. contorta* source stands than to communities associated with native *Nothofagus*, indicative of fungal co-invasion; and (b) that invading trees will associate with few known fungal pathogens (i.e. indicative of enemy release). Testing these hypotheses in combination will broaden our understanding of the contexts in which soil microbial communities may enhance or constrain plants when they are introduced to new environments.

Materials and methods

STUDY SITES

The study system included 61 sampling sites in total, including 17, 16, 18 and 10 in Canada, Europe, USA and the Southern Hemisphere, respectively. Our study utilized *P. contorta* stands in two distinct source and sink populations, which we refer to as northern and southern source–sink pairs (SSP). For the northern SSP, trees were introduced from Northern British Columbia (Canada) to Northern European locations (Scotland, Sweden and Finland), while the southern SSP trees were introduced from the Pacific Northwest, USA, to two Southern Hemisphere countries (Chile and New Zealand) (Fig. 1). Trees were intentionally introduced to each region with consideration of climate and edaphic similarity, thus soils, climate and latitude are very similar across native and introduced ranges within each SSP (Gundale *et al.* 2014b). Establishment of the study system began by identifying *P. contorta* plantations between 25 and 50 years of age in Europe and the Southern Hemisphere. In each of the European countries, we utilized field experiments that were established in the early 1970s, by forest researchers for the purpose of assessing the commercial potential of exotic *P. contorta*. These trials were established with intentionally selected seed provenances, which allowed us to locate paired stands in Canada where the seed source originated. In short, 4, 6 and 6 stands (i.e. 16 in total) were selected in Scotland, Sweden and Finland, which originated near the Canadian towns of Terrace, Fort Nelson and Fort St. Johns, British Columbia, respectively. We then identified six *P. contorta* stands within a 100 km

radius of each of these Canadian towns, except for Fort St. Johns, where only five suitable sampling locations were identified (i.e. $6 + 6 + 5 = 17$ Canadian sampling sites in total).

We further identified 5 plantation stands in each of the Southern Hemisphere countries (i.e. 10 in total) that were established for the purpose of commercial wood production. The origin for introduced Southern Hemisphere stands was less precisely documented than European stands; however, it is known that three subspecies of *P. contorta* (i.e. subspecies *contorta*, *murrayana* and *latifolia*) originating from the Pacific Northwest USA were introduced (Gundale *et al.* 2014b). Thus, in order to capture the range of source populations that were likely represented among the selected Southern Hemisphere stands, we identified 18 stands in total throughout the Pacific Northwest, USA, including six for each subspecies.

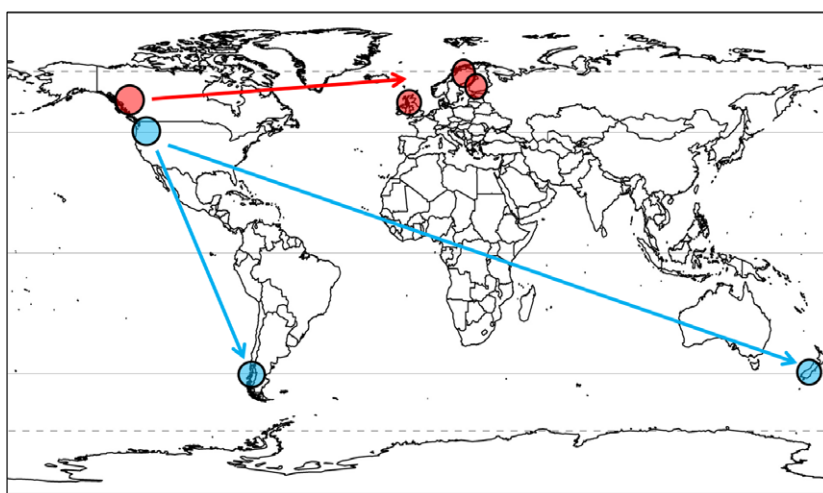
In each of the introduced regions (i.e. Europe and Southern Hemisphere), we further identified adjacent forest stands within the same age range mono-dominated by the most phylogenetically and functionally similar native tree species near the selected *P. contorta* plantations. In Europe, *P. sylvestris*, a tree species within the same genus, was selected for comparison. In the Southern Hemisphere sites, *Nothofagus* spp. were selected (i.e. *N. pumilio* in Chile, and *N. solandri* var. *cliffortioides* in New Zealand), which are also ectomycorrhizal but belong to a different phylum than *P. contorta* (i.e. the Magnoliophyta). The size of *P. contorta* stands across the study system ranged from 0.1 ha to > 10 ha, and the distance between selected *P. contorta* and paired adjacent native tree stands in both introduced regions ranged between 0.05 and 5 km.

SOIL SAMPLING

During the growing season 2012, we collected three soil cores separated by 10 m in the position of an equilateral triangle within each *P. contorta* stand (i.e. in both of its native and introduced ranges), as well as in the adjacent *P. sylvestris* and *Nothofagus* stands in European and Southern Hemisphere sites, respectively. In the Southern Hemisphere, we also collected one core beneath the 3 most distant *P. contorta* individuals invading from each plantation (0.2 to 1 km distance) at all Southern Hemisphere sites. This was not done at European sites, because we found too few invading individuals.

For all stand types, soil cores were taken beneath the outer branch margin of one tree at each of the sampling points, using a 10-cm-diameter PVC tube retrofitted with a finely serrated blade, which severed

Fig. 1. A map of two *Pinus contorta* source–sink pairs (SSP). The northern SSP is depicted in red, with native range locations occurring in northern British Columbia, Canada and corresponding introduced populations in Scotland, Sweden and Finland. The southern SSP is depicted in blue, with native range populations occurring the Pacific Northwest, USA, and corresponding introduced populations in Chile and New Zealand.



roots. Cores were collected to a depth of 10 cm. Immediately upon collection, cores were placed in a 10 cm diameter by 10-cm-depth PVC tube for structural stabilization, wrapped with cellophane and placed in a cooler. Cores were then placed in an insulated box, and express shipped from each location to the laboratory in Umeå Sweden. Shipment from non-European locations took between 4 and 6 days. In order to subject all samples to the same conditions, we kept all European collected samples at room temperature for 5 days after collection.

After samples arrived to the laboratory, we manually broke apart each core in order to separate soils and roots, to be used for chemical and DNA analysis, respectively. Northern SSP cores were primarily composed of organic horizon, whereas southern SSP soils were primarily composed of mineral soil. Thus, organic horizons were removed for analysis from all northern SSP soils and mineral soils (which we standardized to a 5 cm depth) for all southern SSP soils. Sorted soil (i.e. either mineral or organic soil) from each of the three cores from each stand were then composited, oven dried, sieved (2 or 4 mm for mineral and organic soils, respectively) and analysed for total C and N concentrations by dry combustion using an elemental analyser (LECO TruSpec CN analyzer; St. Joseph, MI, USA), and total P content by acid digestion using nitric-perchloric acid digestion and analysis by inductively coupled plasmography (Kumordzi *et al.* 2014; Maaroufi *et al.* 2015). From each core, we removed roots from the target tree species, which was possible because the study stands were mono-cultures, and no understorey species produced higher order lignified roots that could be confused with the target tree species. Thus, fine root masses attached to higher order lignified roots were removed, and from these removed, approximately ten healthy fine root segments (~5 cm long and between 3 and 5 g) per core were used for DNA extraction. Prior to DNA extraction, roots were cleaned to remove all surface-adhering soil particles in a modified version of the protocol by Gottel *et al.* (2011). Briefly, roots were placed in a 50-mL centrifuge tube and sequentially rinsed by vigorously shaking with: (i) a rhizosphere soil extraction solution (NaCl-Tris-EDTA); (ii) distilled H₂O; (iii) 1% Tween-20; (iv) dH₂O 5x consecutively; (v) 70% ethanol; (vi) dH₂O; (vii) 70% ethanol; and (viii) dH₂O. Cleaned roots were then freeze-dried and ground to a fine powder using a ball mill and then used for DNA extraction.

DNA EXTRACTION, PCR AND 454 SEQUENCING

We extracted DNA from the homogenized root samples by adding CTAB-SDS buffer (2% cetyl trimethylammonium bromide, 2% sodium dodecyl sulphate, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8), vortexing and then incubating at 65 °C for 1.5 h, followed by chloroform addition, vortexing, supernatant removal and isopropanol/ethanol precipitation. The pellet was resuspended in 50 µL of MiliQ-water (Millipore, Darmstadt, Germany) and further cleaned using Nucleospin gDNA clean-up kit (Machery-Nagel, Düren, Germany). DNA extracts from the three subsamples within each stand were then combined in equal amounts, based on nano drop DNA concentrations, to form one DNA extract for all extracted roots in a stand.

The PCR amplification of the ribosomal internal transcribed spacer (ITS) DNA was carried out for each sample in triplicate 25 µL reactions using the fungal-specific primers ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990). Each primer was elongated with adaptors required for 454 pyrosequencing (ITS1F-B adaptor and ITS4-A adaptor). The ITS4-A primer contained a sample-specific MID barcode consisting of eight bases (5'-CCATCTCATCCCTGCGTGT

CTCCGACTCAGTCCCTCCGCTTATTGATATGC-3'), which the ITS1F-B adaptor lacked (5'-CCTATCCCCTGTGTGCTTGG-CAGTCTCAGCTTGGTCATTTAGAGGAAGTAA-3'). PCR products were purified with Agencourt AMPure kit (Agencourt Bioscience Corporation, Beverly, MA, USA), and the concentration of purified PCR products was measured with the PicoGreen ds DNA Quantification Kit (Molecular Probes, Eugene, OR, USA) on a FLUOstar OPTIMA (BMG LABTECH GmbH, Ortenberg, Germany). Equal amounts of DNA from each sample were then pooled prior to 454 pyrosequencing. Sequencing (starting from the ITS4 fragment end) was performed on one quarter of an FLX 454 sequencing plate (Roche Applied Biosystems, Penzberg, Germany) using the Lib-L chemistry at the Pyrosequencing facility at Lund University, Lund, Sweden.

BIOINFORMATICS ANALYSIS

After sequencing, sequences lacking template primer sequences (more than two mismatches) or sample barcode sequences (more than one mismatch) were removed. Primer sequences were removed, and sequences < 175 bp long were excluded. Sequences were then trimmed to include only the ITS2 region with ITSx (Bengtsson-Palme *et al.* 2013), using the additional chimera removal tool and clustered using USEARCH (Edgar 2010) at 97 % sequence similarity. Clusters containing only a single read or only found in one sample (i.e. one PCR reaction) were removed. For community composition comparisons and to identify ecological roles of fungal OTUs, clusters were identified using NCBI Basic local alignment search tool (BLAST) against a trimmed UNITE+INSD sequence data base (377 000 seqs, release date 2014-02-15). Data were then purged of all sequences that did not have any taxonomic information (nearly 25% of the total), using the search terms 'unidentified', 'endophyt', 'uncultured', 'fungal sp', 's__Fungi', 'root iso' and 'root ass'. Sequences were assigned to fungal operational taxonomic units (OTUs) when there was at least 97% similarity between query sequence and top hit, given at least 90% coverage of the query sequence length. All sequences used for analysis were submitted to the International Nucleotide Sequence Database Sequence Read Archive.

Using names and taxonomy, we classified OTUs by their most current known ecological function (known EMF fungi, saprotrophic fungi, pathogenic fungi or taxa with an unknown ecology). The OTUs were considered as known EMF fungi based on the most current knowledge of the ecology of known close relatives (genera or species) according to Tedersoo, May & Smith (2010). The OTUs were considered potentially pathogenic if they belonged to the genera known as potential root pathogens in pines, which included *Armillaria*, *Fusarium* (and associated subgenera), *Heterobasidium*, *Inonotus*, *Leptographium* and *Phellinus*. The OTUs in the families Heliotiales, not identified as EMF according to Tedersoo, May & Smith (2010), were classified as having an 'unknown ecology'. All other fungal OTUs were considered saprotrophic. We acknowledge that the functional classification of fungi is constrained by an incomplete current state of knowledge and that some specific taxa identified in our study may potentially be re-classified in the future as new knowledge emerges.

After filtering, each sample was rarified to 1000 sequence reads using the 'rrarefy' function in the VEGAN package (Oksanen *et al.* 2013) in R (R Core Team 2013). For community comparisons, sequence read abundances were converted to fractional abundances, such that the read abundances for all OTUs for each sample totalled 1. The relative abundance of each functional group was defined as the total fractional abundance of all OTUs of that functional group (EMF, saprotrophs, pathogens or taxa with unknown ecology).

Richness was defined as the number of OTUs identified per sample per 1000 reads.

STATISTICAL ANALYSIS

For each of our hypotheses, forest stands served as the unit of replication. Community composition data (i.e. the relative abundance of identified fungal species) were analysed using permutational multivariate analysis of variance (PERMANOVA) in Primer 7.0 with the PERMANOVA add-on package (Primer-E Ltd, Plymouth, UK). This approach is ideal for comparing communities across the regions defined in this study because it does not require assumptions to be satisfied regarding data distributions among the diverse sites grouped together within regions (e.g. normality or homoscedasticity). Meanwhile, richness and abundance of specific fungal functional groups (i.e. EMF, saprotrophs, pathogens or taxa of unknown ecology) were analysed using analysis of variance (ANOVA) in SPSS version 22 (IBM, New York, NY, USA). For our first hypothesis, two-way PERMANOVAS and ANOVAS were analysed using two fixed factors, 'introduction status' (i.e. *P. contorta* growing in its native or introduced range) and 'source-sink pair' (i.e. European *P. contorta* originating from Canada or Southern Hemisphere *P. contorta* originating from USA). For our second hypothesis, two-way PERMANOVAS and ANOVAS were analysed using two fixed factors, 'tree type' (*P. contorta* or native tree species) and region (Europe or Southern Hemisphere). For our third hypothesis, we used one-factor PERMANOVAS and ANOVAS comparing three Southern Hemisphere tree types (*P. contorta* plantations, invading *P. contorta* or native *Nothofagus* spp.).

For all PERMANOVA analyses, when significant main or interactive effects were detected ($P < 0.05$), we conducted *post hoc* pairwise PERMANOVAS between all groups which informed us which groups were different from each other, as well as the per cent similarity within and between groups. When PERMANOVA tests showed significant differences, we also performed canonical analysis of principal coordinates (CAP), which is a constrained ordination approach used to graphically display multi-variate comparisons with *a priori* grouping designations (Primer V7). Finally, for all main or interactive effects for which PERMANOVA identified as significant, we performed 'contribution of variables to similarity' (SIMPER) analysis (Primer V7), in order to identify which fungal species contributed most to the similarity within groups and differences between groups. The PERMANOVA tests could not be performed on fungal pathogen data because a large portion of samples did not contain any pathogens.

For all ANOVA analyses, when significant main or interactive differences were detected ($P < 0.05$), *post hoc* pairwise Tukey's tests were conducted across all groups. All univariate data were assessed for adherence to parametric assumptions (i.e. normality and homoscedasticity), and this showed that only pathogen richness and abundance data across *P. contorta* native and introduced regions and between *P. contorta* and native trees (i.e. hypothesis 1 and 2) did not meet these assumptions, due to the low occurrence of pathogens in the northern SSP. Because of this, we compared pathogen richness and abundance data for the first and second hypothesis using nonparametric pairwise comparisons (Kruskal-Wallis tests, SPSS version 22.0), followed by nonparametric *post hoc* pairwise tests.

Results

We obtained a total of 769 OTUs across all sites and tree types, of which 261, 427, 150 and 7 were EMF, saprotrophs, fungi with unknown ecology and pathogens, respectively. The

number of OTUs in each of these categories for each region and tree type is reported in Table S1 (Supporting Information), and species contributing the most to the similarity within each of these groups are reported in Table S2.

PINUS CONTORTA FUNGAL COMMUNITIES ACROSS DIFFERENT REGIONS

Community composition of all fungal endophyte categories was responsive to the main effects of introduction status (IS; *P. contorta* in its native vs. introduced range), source-sink pair (northern SSP; Canada-Europe; or southern SSP, USA-Southern Hemisphere), and their interaction (Table 1; Fig. 2, see within and between group similarities in lower left of each panel). For all fungi, EMF and saprotrophs, the interactive effects occurred because the two native range communities were more similar than the two introduced ranges communities and because communities across the northern SSP were more similar than the southern SSP (Fig. 2). For

Table 1. The results from two-factor PERMANOVA or ANOVA tests evaluating *Pinus contorta* root fungal endophyte responses to introduction status (i.e. growing in its native vs. introduced range), and across two different source-sink pairs (populations from USA or Canada introduced to the Southern Hemisphere or Europe, respectively). The analysis consisted of 18, 17, 16 and 10 stands in the USA, Canada, Europe and the Southern Hemisphere, respectively. Analysis of community composition was done using PERMANOVA on a Bray-Curtis similarity matrix of fungal taxonomic units identified using 454 pyrosequencing, while richness and relative abundance were analysed using ANOVA

	Introduction Status (IS)		Source-Sink Pair (SSP)		IS × SSP	
	F-value	P-value	F-value	P-value	F-value	P-value
*Community composition						
Total Fungi	4.455	0.001	7.972	0.001	3.020	0.001
Ectomycorrhizas	4.891	0.001	8.166	0.001	2.815	0.002
Saprotrophs	3.741	0.001	5.549	0.001	2.316	0.007
Unknown ecology	4.064	0.001	13.122	0.001	2.654	0.014
Richness						
Total fungi	10.007	0.003	0.244	0.623	1.281	0.262
Ectomycorrhizas	28.813	< 0.001	5.297	0.025	5.640	0.021
Saprotrophs	2.780	0.101	10.658	0.002	0.313	0.578
Unknown ecology	0.193	0.662	4.060	0.051	0.325	0.571
Relative abundance						
Ectomycorrhizas	1.560	0.217	8.058	0.006	3.556	0.064
Saprotrophs	0.605	0.440	16.327	0.000	3.495	0.067
Unknown ecology	0.435	0.512	0.108	0.744	0.249	0.620

*For community composition data, the F-value is generated using permutations procedures and thus is referred to as a Pseudo-F-value.

The numerator, residual and total degrees of freedom for each factor is 1, 57 and 60, respectively, for all analyses.

Pathogens could not be analysed using the same statistical procedures and thus are not included in the table.

P-values in bold are significant at $P < 0.05$.

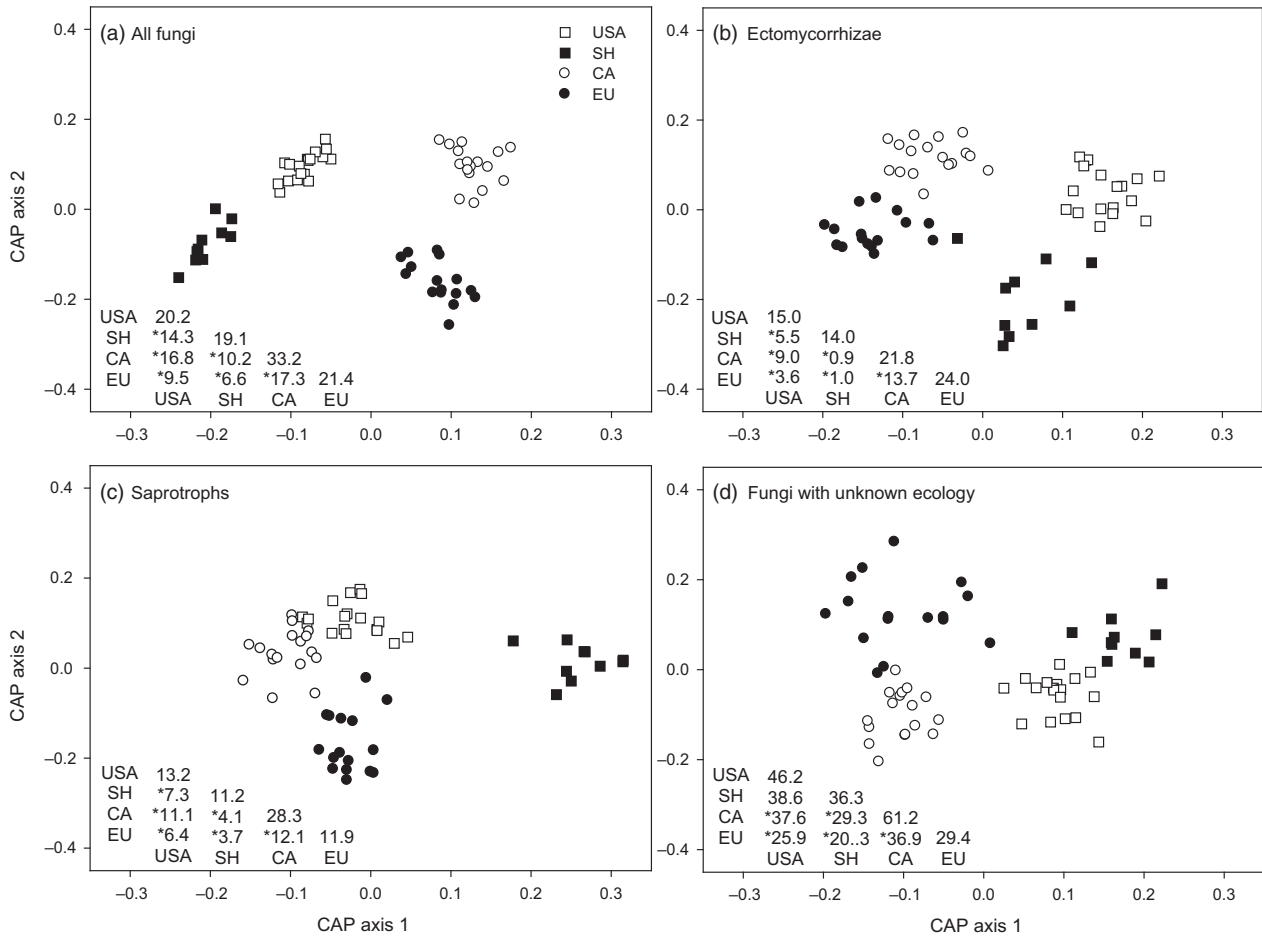


Fig. 2. A constrained ordination (canonical analysis of principal coordinates; CAP) showing the results of a PERMANOVA (permutations multivariate analysis of variance) test evaluating differences in community composition of (a) total fungal endophytes, (b) ectomycorrhizas, (c) saprotrophic fungi and (d) fungi with unknown ecology associated with *Pinus contorta* roots growing in four distinct regions (United States of America = USA, Canada = CA, Southern Hemisphere = SH, and Europe = EU). Results of the PERMANOVA are reported in Table 1. In the lower left corner of each panel, within group similarity (%) are listed in bold, while pairwise similarities between groups are non-bold. An asterisk next to pairwise similarity values indicate when *post hoc* pairwise PERMANOVA analyses indicated a significant difference ($P < 0.05$) in community composition between the two regions. *For community composition data, the F-value is generated using permutations procedures and thus is referred to as a Pseudo- F-value.

fungi with unknown ecology, the significant interaction occurred because the native and introduced range communities was significantly different within the northern SSP, but not in the southern SSP (Fig. 2d). Species contributing to the difference in the total fungal community and EMF community between native and introduced range *P. contorta* trees are reported in Tables S3, S4, S5 and S6.

For richness and abundance variables analysed with two-way ANOVAs, introduction status significantly affected richness of total fungi and EMF, while SSP affected richness of EMF and saprotrophs, as well as the relative abundance of EMF and saprotrophs (Table 1). The richness of EMF was further affected by the interaction of IS and SSP (Table 1). For total fungi and EMF, the main effect of IS occurred because richness was always lower in introduced relative to native ranges, especially within the southern SSP (Fig. 3a and 3b). The significant effect of SSP on saprotrophic fungi was due to their greater richness on average in the southern than northern

SSP. For the relative abundance data, EMF comprised a greater proportion of the root fungal community in the northern than in the southern SSP, whereas saprotrophs (Table 1) were more common in the Southern Hemisphere (Fig. 3e). For data analysed by Kruskal–Wallis tests (i.e. pathogen data), significant differences were found among the four regions for both richness and abundance (Kruskal–Wallis P -value = 0.004 and 0.003, respectively), with higher values in the Southern Hemisphere regions compared to all other regions (Fig. 3c,e).

FUNGAL COMMUNITIES OF *PINUS CONTORTA* VS. NATIVE SPECIES

All fungal community composition variables were responsive to the main effects of tree type (TT; *P. contorta* or native tree species), region (R; Europe or Southern Hemisphere) and their interaction (Table 2). These significant main effects

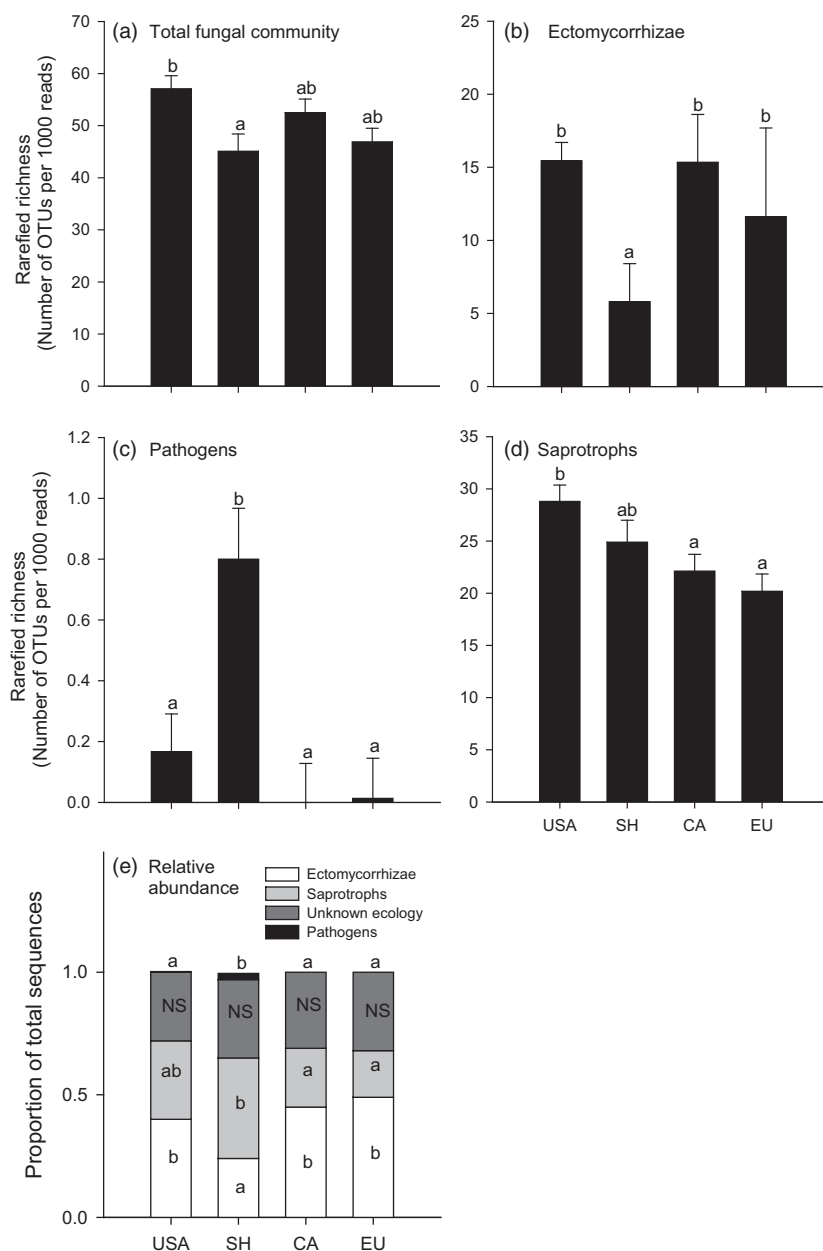


Fig. 3. Operational taxonomic unit (OTU) richness (a-d) and relative abundance (e) of fungal endophyte communities associated with *Pinus contorta* roots in two native range source populations (USA and Canada (CA)) and two sink populations (Southern Hemisphere (SH) or Europe (EU)), respectively. Richness is presented as the average rarefied richness (for 1000 sequence reads) for each sample. Results from corresponding two-way ANOVA are reported for all variables are reported in Table 1, except for pathogens which were compared using a Kruskal–Wallis test. Different letters above bars or across bar segments (a or b) with the same shade indicate significant pairwise differences determined using Tukey's *post hoc* tests, except for pathogens (e) which were compared using nonparametric *post hoc* comparisons.

occurred because on average communities were different between *P. contorta* and native species, and between European vs. Southern Hemisphere region, regardless of species (Fig. 4). The significant interactive effects for all fungal categories occurred because communities associated with *P. contorta* and the native tree species in Europe (i.e. *P. sylvestris*) were not significantly different, whereas fungal communities associated with *P. contorta* and the native *Nothofagus* species in the Southern Hemisphere significantly differed (Fig. 4; see within and between group similarities in lower left of each panel). Species contributing to the differences in the total fungal community and EMF community between introduced *P. contorta* and *Nothofagus* species are reported in Tables S7 and S8.

For all fungal richness and relative abundance variables analysed using two-way ANOVA, except for total fungal richness and

relative abundance of fungi with unknown ecology, we found a significant effect of region (Table 2). We never found a significant effect of tree type or tree type by region interaction for any fungal richness or abundance variables (Table 2), except for the relative abundance of saprotrophs (Fig. 5e). The significant effects of region on richness occurred because richness of EMF and species with unknown ecology was higher in Europe and because saprotroph richness was greater in the Southern Hemisphere (Fig. 5a–d). The significant effect of region on fungal relative abundance variables was due to greater abundance of EMF in Europe and greater abundance of saprotrophs in the Southern Hemisphere (Fig. 5e). For pathogen richness and abundance, we found significant differences among the four tree types (Kruskal–Wallis P -value = 0.008 and 0.005, respectively), with higher values for both of the Southern Hemisphere tree types (Fig. 5b,e).

Table 2. The results from two-factor PERMANOVA or ANOVA tests comparing root fungal endophyte responses between two different tree types, that is introduced *Pinus contorta* trees vs. the most similar dominant native tree (either *Pinus sylvestris* or *Nothofagus* sp.) in two different regions (Southern Hemisphere or Europe). The analysis consisted of 16 European and 10 Southern Hemisphere sites. Analysis of community composition was done using PERMANOVA on a Bray–Curtis similarity matrix of fungal taxonomic units identified using 454 pyrosequencing, while richness and relative abundance were analysed using ANOVA

	Tree Type (TT)		Region (R)		TT × R	
	F-value	P-value	F-value	P-value	F-value	P-value
Community composition*						
Total Fungi	2.5267	0.001	7.4198	0.001	2.5751	0.001
Ectomycorrhizas	2.3845	0.007	7.4607	0.001	2.3421	0.004
Saprotrophs	2.492	0.001	5.910	0.001	2.263	0.001
Unknown ecology	2.752	0.003	8.550	0.001	2.992	0.001
Richness						
Total fungi	0.496	0.485	1.295	0.261	0.401	0.530
Ectomycorrhizas	0.370	0.546	22.017	< 0.001	0.013	0.910
Saprotrophs	0.160	0.691	4.476	0.040	0.160	0.691
Unknown ecology	0.632	0.430	5.738	0.021	1.792	0.187
Relative Abundance						
Ectomycorrhizas	1.863	0.179	18.583	0.000	0.030	0.864
Saprotrophs [†]	12.062	0.001	44.837	0.000	2.439	0.125
Unknown ecology	1.340	0.253	0.741	0.394	0.975	0.328

*For community composition data, the F-value is generated using permutations procedures and thus is referred to as a Pseudo-F-value.

[†]These data could not be analysed via ANOVA because failure to meet parametric assumptions and thus were analysed using Kruskal–Wallis tests. In both cases, these tests revealed significant differences at $P < 0.05$.

The numerator, residual and total degrees of freedom for each factor is 1, 57 and 60, respectively, for all analyses.

Pathogens could not be analysed using the same statistical procedures and thus are not included in the table.

P-values in bold are significant at $P < 0.05$

FUNGAL COMMUNITIES OF INVADING *PINUS CONTORTA*

We found that community composition of all fungal variables significantly differed among the three Southern Hemisphere tree types (i.e. invading *P. contorta* individuals, source *P. contorta* plantation and native *Nothofagus* trees) (Table 3). Further, *post hoc* analysis showed that communities associated with all three tree types always differed, except for fungi with unknown ecology, where the two types of *P. contorta* did not differ (Fig. 6). All fungal community variables were more similar between plantation and invading *P. contorta* trees than between *P. contorta* and *Nothofagus* sp. (Fig. 6: see within and between group similarities in the lower left of each panel). Species contributing to the difference in the total fungal community and EMF community between invading *P. contorta* trees, *P. contorta* plantation and *Nothofagus* in the Southern Hemisphere are reported in Tables S9, S10, S11 and S12. Richness and abundance of the different fungal categories were never different among the three tree types, except for the relative abundance of saprotrophs (Table 3), which was significantly higher for *Nothofagus* than for either type of *P. contorta* (data not graphically depicted).

SOIL CHEMICAL PROPERTIES

We found no significant difference in organic soil horizon C, N or P concentrations between Canadian *P. contorta* and either European stand types (i.e. *P. contorta* and *P. sylvestris*), or in mineral soil C, N or P between USA

P. contorta and either Southern Hemisphere stand type (i.e. *P. contorta* or *P. sylvestris*) (Table S13).

Discussion

In support of our first hypothesis, the total fungal endophyte community composition of *P. contorta*, and the composition of the EMF and saprotrophic fraction of that community, was significantly different in their introduced regions (i.e. Southern Hemisphere and Europe) compared to each of their source regions (i.e. USA and Canada, respectively). For the northern SSP, many species contributed to the community differences between Canada and Europe, but one specific EMF emerged as being particularly important, *Piloderma sphaerosporum*, which was present in both regions but four times more abundant at European sites (Tables S3 and S4). Many species also contributed to the community differences across the southern SSP regions (Table S5), including several EMF species that differed in relative abundance or presence (Table S6). A majority of the EMF associated with *P. contorta* in Southern Hemisphere sites, including those that helped explain community-level differences with the USA sites, are known North American taxa, which suggests they have been co-introduced with *P. contorta* (Dickie *et al.* 2010; Hayward, Horton & Nunez 2015a). The absence of these EMF at the USA sites we sampled could be explained by the high degree of EMF beta diversity across the USA range of *P. contorta* (we observed only 20.2% similarity across the 18 USA sites that we sampled), suggesting that the communities we observed across the Southern Hemisphere

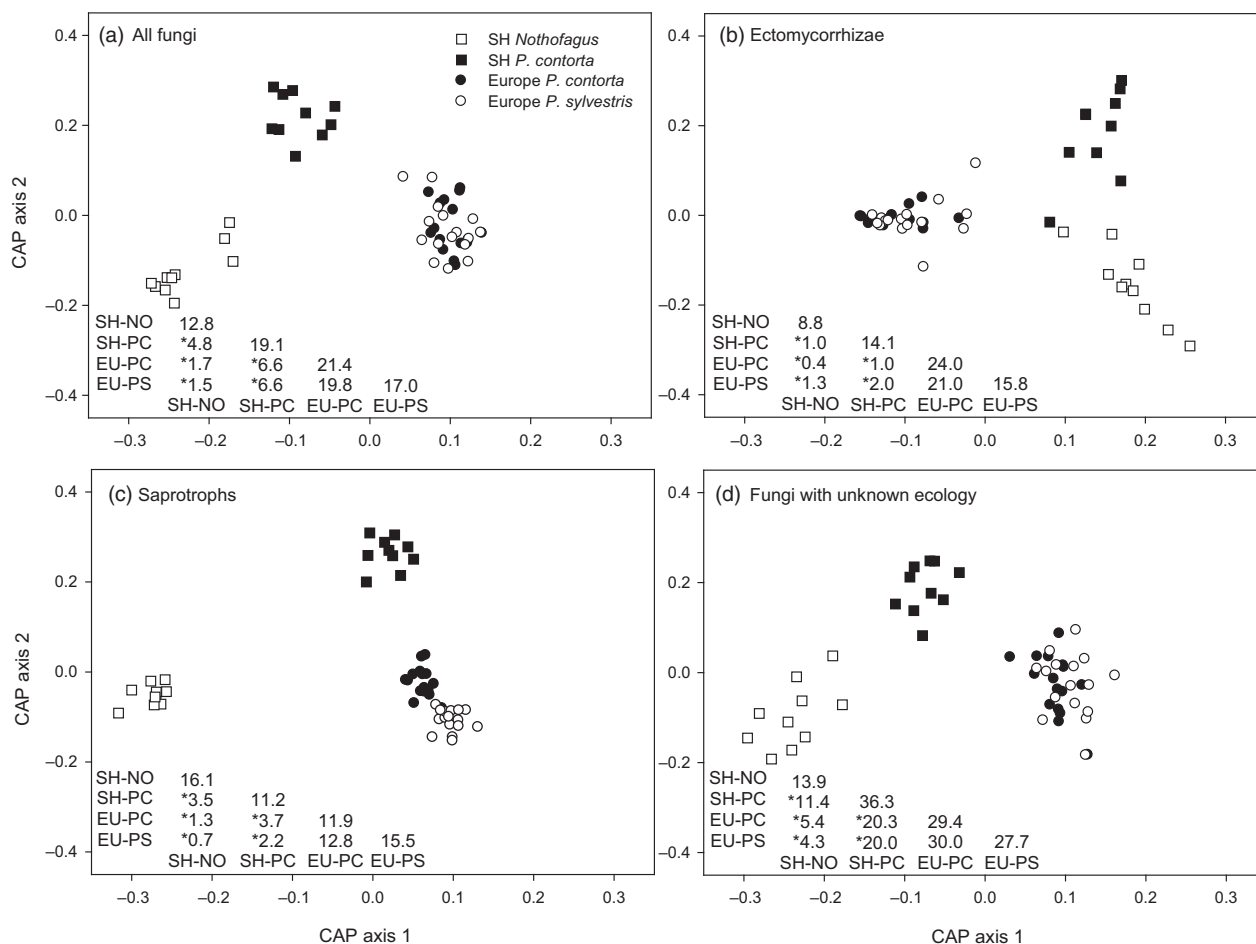


Fig. 4. A constrained ordination (canonical analysis of principal coordinates; CAP) showing the results of a PERMANOVA (permutational multivariate analysis of variance) test evaluating the effect of tree type (introduced *Pinus contorta* (PC) vs. the most similar dominant native tree species) and region (Europe = EU, or Southern Hemisphere = SH) on community composition of (a) total root fungal endophytes (b) ectomycorrhizal fungi, (c) saprotrophic fungi or (d) fungi with unknown ecology. The native trees in the Southern Hemisphere locations were *Nothofagus* sp. (NO) and *Pinus sylvestris* (PS) in European locations. In the lower left corner of the figure, similarity (%) of fungal communities across sites within each group is listed in bold, while pairwise similarities between groups are non-bolded. An asterisk next to pairwise similarity values indicates when *post hoc* pairwise PERMANOVA analyses indicated a significant difference ($P < 0.05$) in community composition. *For community composition data, the F-value is generated using permutations procedures and thus is referred to as a Pseudo- F-value.

P. contorta plantations likely resulted from tree-fungal co-introductions from multiple North American locations.

In addition to compositional differences, total fungal richness and EMF richness associated with *P. contorta* were significantly lower on average in introduced compared to native regions. Several recent studies have suggested that EMF richness associated with introduced pines is likely lower in introduced regions compared to native regions, potentially due to limited co-introduction and a lack of co-evolved host-specific specialists in the introduced environments (Dickie *et al.* 2010; Hynson *et al.* 2013; Hayward, Horton & Nunez 2015a; Hayward *et al.* 2015b). To our knowledge, this study provides the first direct comparison of fungal endophyte communities using molecular techniques across a wide array of sites in both a species native and introduced environment. Specifically, we show that introduction of *P. contorta* to a wide range of environments leads on average to reduced fungal endophyte richness at the stand level relative to in its

native range, with particularly strong responses in the Southern Hemisphere as predicted by our first hypothesis. However, this pattern did not only occur for EMF fungi, but also for saprotrophs which are not known to exhibit host specificity (van Heijden & Sanders 2003). This suggests that factors other than incomplete co-introduction of co-evolved specialists from its native range could contribute to the reduced fungal richness associated with *P. contorta* in introduced regions.

Contrary to our first hypothesis, and in contrast to the response of other fungal functional groups, pathogen richness was not lower in introduced environments. Instead, pathogens were almost completely absent for Canadian and European samples, and pathogen richness was higher in Southern Hemisphere *P. contorta* stands than in corresponding native stands in the USA. In many soil sterilization studies, enemy escape (Elton 1958) has been proposed as a key mechanism for explaining positive plant growth responses to soil biota in environments to which they have been introduced (e.g.

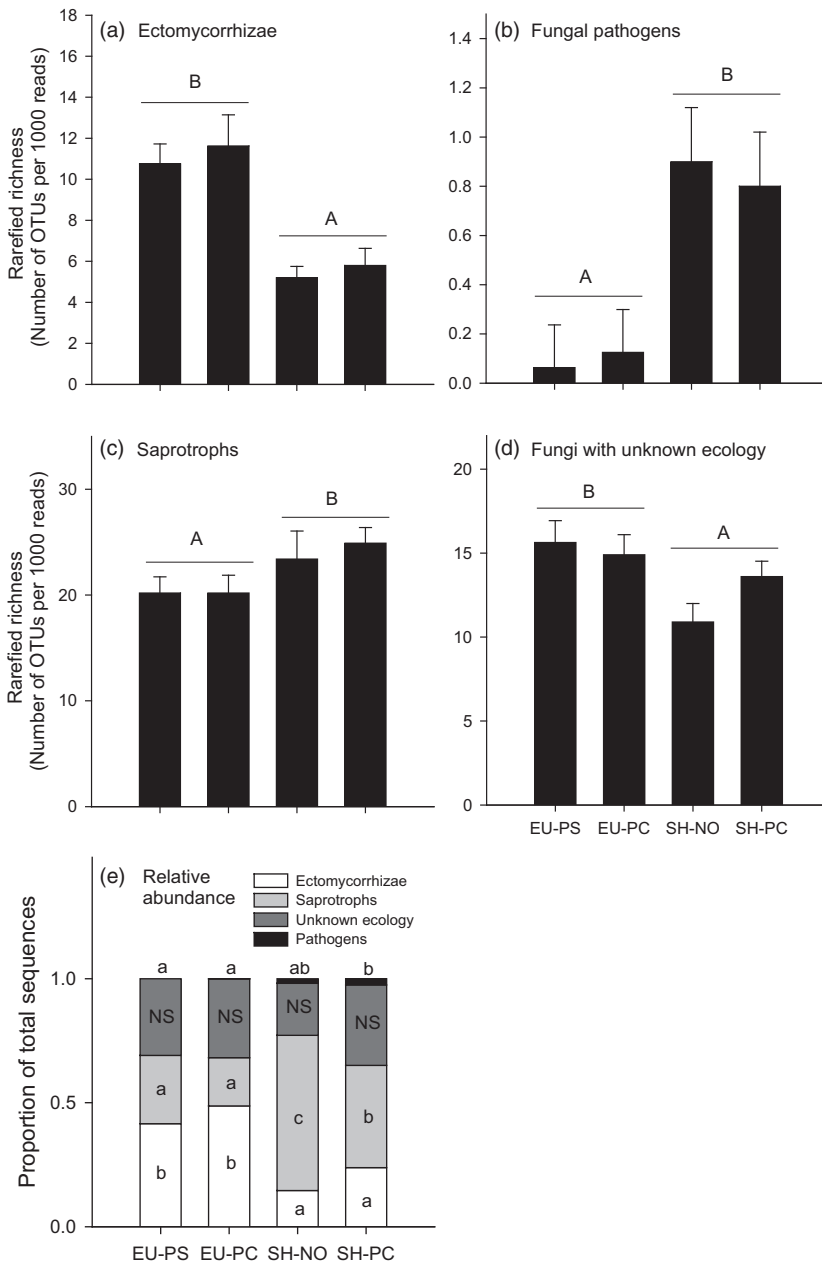


Fig. 5. Differences in operational taxonomic unit (OTU) richness (a-d) and relative abundance (e) of fungal root endophyte communities associated with either *Pinus contorta* (PC) or *Pinus sylvestris* (PS) in Europe (EU), or *P. contorta* or *Nothofagus* sp. (NO) in the Southern Hemisphere (SH). Richness is presented as the average rarefied richness (for 1000 sequence reads) for each sample. Results from a corresponding two-way ANOVA are reported in Table 2. Different letters above bars or across bar segments (a or b) with the same shade indicate significant pairwise differences determined using Tukey's *post hoc* tests, except for pathogens (e) which were compared using nonparametric *post hoc* comparisons.

Klironomos 2002; Reinhart *et al.* 2003). As such, a recent study by Gundale *et al.* (2014a) showed that *P. contorta* seedlings grew substantially better when inoculated with Swedish compared to Canadian soil microbial communities. While our pathogen data should be interpreted cautiously because plant–pathogen relationships can vary among species, genotypes or environments, our data do not suggest pathogen escape as a likely explanation for enhanced growth of *P. contorta*. Our data instead suggest a role for compositional differences between European and Canadian EMF. For instance, *P. contorta* may particularly benefit from the greater abundance of *Piloderma sphaerosporum* in Swedish soil (*sensu* the ‘enhanced mutualism hypothesis’; Reinhart & Callaway 2006). This could occur if *P. contorta* derived greater benefit from *P. sphaerosporum* than *P. sylvestris* (Bahram *et al.*

2013) or if *P. sphaerosporum* performed more optimally in Swedish compared to Canadian soil.

We also found support for the second part of our first hypothesis that differences between native and introduced range fungal community composition would be greater in environments with phylogenetically more distant native vegetation. Both EMF and saprotroph composition were more similar within the northern SSP (13.7 and 12.1 similarity, respectively) compared to the southern SSP (5.5 and 7.3% similarity, respectively). Further, the richness and abundance of EMF and pathogenic fungi were similar across the northern SSP, but showed very large changes across the southern SSP. The particularly large differences in fungal communities across the southern SSP cannot be easily explained by differences in abiotic factors across native and introduced ranges,

Table 3. The results from two-factor PERMANOVA or ANOVA tests comparing root fungal endophyte responses between three tree types in the Southern Hemisphere (*Pinus contorta* plantations, invading *P. contorta* individuals and native *Nothofagus* trees), which were sampled at 10 Southern Hemisphere sites. Analysis of community composition was done using PERMANOVA on a Bray–Curtis similarity matrix of fungal taxonomic units identified using 454 pyrosequencing, while richness and relative abundance were analysed using ANOVA

	†F-value	P-value
Composition		
Total fungi	3.0057	0.001
Ectomycorrhizas	2.977	0.001
Saprotrophs	2.411	0.001
Unknown ecology	2.648	0.001
Richness		
Total fungi	0.646	0.532
Ectomycorrhizas	2.086	0.144
Pathogens	0.618	0.547
Saprotrophs	1.917	0.163
Unknown ecology	1.899	0.169
Relative abundance		
Ectomycorrhizas	0.681	0.514
Pathogens	0.144	0.867
Saprotrophs	7.347	0.003
Unknown ecology	2.750	0.082

The between, within and total degrees of freedom for each analysis were 2, 33 and 35, respectively.

†For community composition, the *F*-value is generated using permutations procedures and thus is referred to as a Pseudo-*F*-value. Pathogens are not included in this section of the table because they were present in too few samples to compute Bray–Curtis matrices.

P-values in bold are significant at *P* < 0.05

such as latitude and climate (Gundale *et al.* 2014b), or edaphic factors (Table S13). In addition to the greater phylogenetic similarity of *P. contorta* with *P. sylvestris*, several additional factors could contribute to the greater similarities in communities across the northern vs. southern SSP, such as greater spatial ranges of taxa at higher latitudes (e.g. Rapoport's rule; Stevens 1989; Tedersoo *et al.* 2014), or inherently greater similarity of North American fungal communities with those in Europe compared to the Southern Hemisphere locations (Tedersoo *et al.* 2014).

We found strong evidence for our second hypothesis which proposed that the phylogenetic relatedness of trees was a key driver of fungal communities associated with introduced *P. contorta*. In Europe, total root endophyte community composition, as well as EMF, saprotrophs and fungi with unknown ecology never differed between the congeneric *P. contorta* and *P. sylvestris*, indicating that *Pinus*-fungal associations are relatively plastic, and show little host specificity at the subgenus level. This finding is consistent with a variety of local scale studies indicating little intrageneric host specificity of EMF within the *Pinaceae* (Molina & Trappe 1994; Tedersoo *et al.* 2007), but contrasts with a study by Kohout *et al.* (2011) which found host specificity of some EMF between European *P. sylvestris* (a two needle pine) and

Pinus strobus (a less closely related invasive five needle pine from North America). In contrast to the northern SSP, the communities of all fungal groups differed between phylogenetically distant *P. contorta* and *Nothofagus* in Southern Hemisphere locations, with many fungal species only present on one species or the other (Tables S7 and S8). Of the 10 most abundant EMF taxa found in Southern Hemisphere *P. contorta* stands (comprising 94% of all sequence reads), only two were also found in adjacent *Nothofagus* forests (*Cadophora finlandica* and *Melinomyces bicolor*); in contrast, 8 of these EMF were also observed in the native US stands. These findings strongly suggest that a majority of fungi (and notably EMF) associated with Southern Hemisphere *P. contorta* have been co-introduced from North America (Dickie *et al.* 2010; Hayward, Horton & Nunez 2015a).

Despite the likely dependence of *P. contorta* on co-introduced EMF in the Southern Hemisphere, we did not find any differences in richness or abundance of fungal endophytes (other than saprotrophs) between *P. contorta* and the native trees in either of the introduced regions (except for saprotrophs), which is inconsistent with our second hypothesis, but consistent with some other studies comparing native and introduced *Pinaceae* (Kohout *et al.* 2011; O'Hanlon & Harrington 2012; Bahram *et al.* 2013). This suggests that reduced richness associated with *P. contorta* in introduced compared to native regions may not be entirely due to incomplete co-introduction (Dunstan, Dell & Malajczuk 1998; Tedersoo *et al.* 2007), but that additional geographic or local factors may limit the richness of the fungal endophyte community in the particular introduced regions we evaluated, regardless of whether a species is native or exotic (Tedersoo *et al.* 2014). Factors such as dispersal, competition and mortality that are well known to influence plant community richness (Grime 1973; Hubbell 2001) could potentially also influence fungal richness at specific sites and at regional scales, although this remains poorly understood for fungal communities at the geographical scale of our study (Tedersoo *et al.* 2014).

Contrary to our third hypothesis, we found no evidence that invading *P. contorta* individuals in the Southern Hemisphere encountered fewer pathogens; instead, they had similarly high pathogen abundance and richness values to the other Southern Hemisphere tree types (i.e. *P. contorta* plantations and *Nothofagus* spp.). However, in support of our third hypothesis, we found strong evidence that invading *P. contorta* individuals developed unique fungal communities relative to both established *P. contorta* plantation and *Nothofagus* stands (Tables S9, S10, S11 and S12). Although several fungal species were shared between plantations and invading trees, other species that were rare in the plantations were substantially more abundant in roots of invading trees (Table S10). Several previous studies that have focused on invading conifers, and specifically *P. contorta*, have identified one specific Northern Hemisphere EMF (i.e. *Suillus luteus*), as an important co-invader in Southern Hemisphere habitats (Hynson *et al.* 2013; Hayward, Horton & Nunez 2015a; Hayward *et al.* 2015b), and this is clearly supported by our data (Table S10). A few recent studies focused on *Pinus* invasions have suggested that

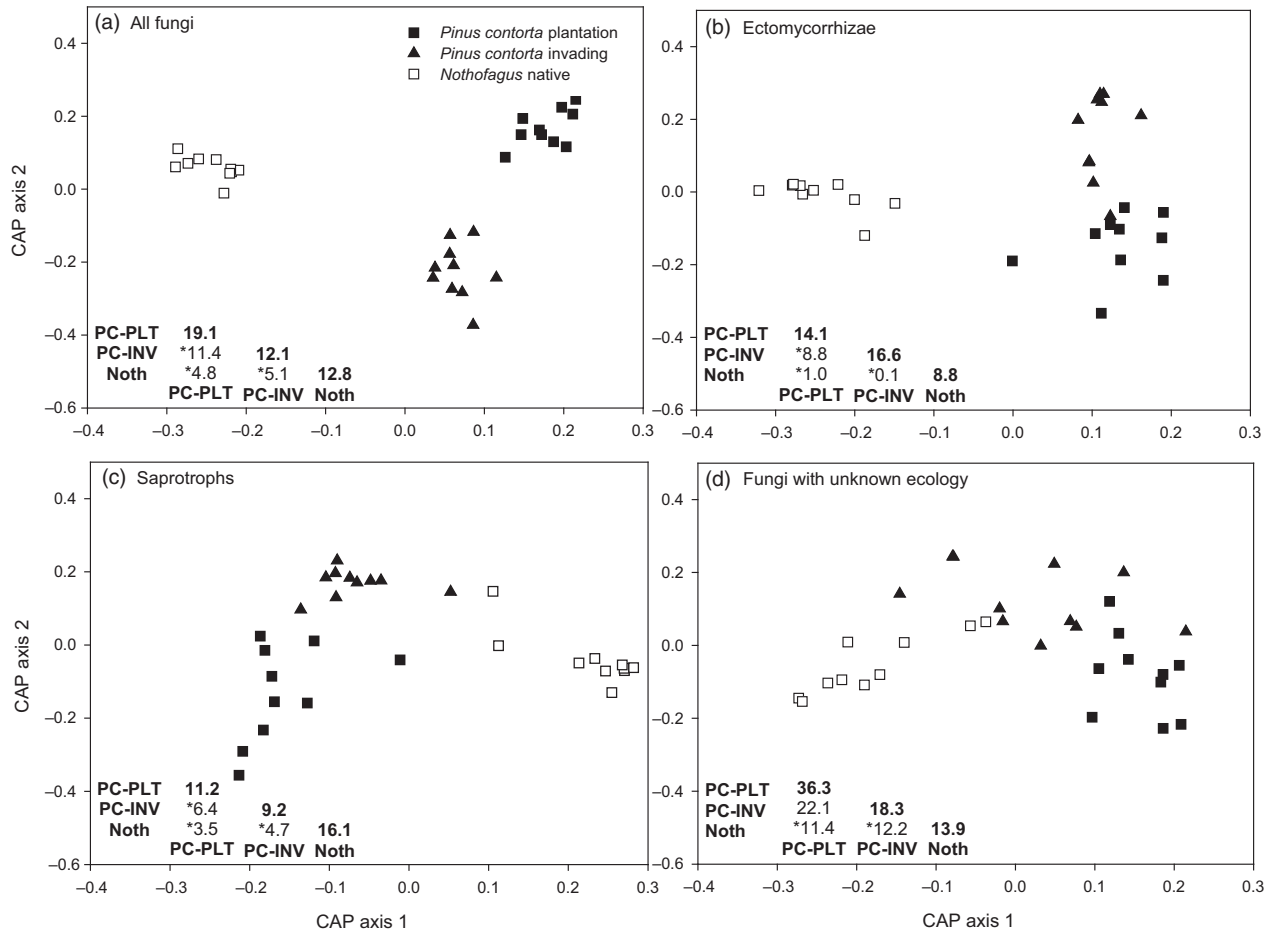


Fig. 6. A constrained ordination (canonical analysis of principal coordinates; CAP) showing the results of a PERMANOVA (permutational multivariate analysis of variance) test evaluating the community composition of (a) total fungal root endophytes, (b) ectomycorrhizal fungi, (c) saprotrophic fungi and (d) fungi with unknown ecology associated within the roots of three Southern Hemisphere tree types, source *Pinus contorta* plantations (PC-PLT), invading *P. contorta* individuals (PC-I) and native *Nothofagus* trees (Noth). In the lower left corner of each panel, the similarity (%) of fungal communities among sites within each group is listed in bold, whereas pairwise similarities between groups are non-bolded. An asterisk next to pairwise similarity values indicate when *post hoc* pairwise PERMANOVA analyses indicated a significant differences (at $P < 0.05$) in community composition. *For community composition data, the F-value is generated using permutations procedures and thus is referred to as a Pseudo- F-value.

plantation establishment could be done with a lower risk of invasion in some introduced ranges if co-introduction of mycorrhiza species such as *S. luteus* could be reduced (Hynson *et al.* 2013; Hayward, Horton & Nunez 2015a). While our data support the importance of *S. luteus* for invading *P. contorta*, they also suggest that a number of other species are effective co-invaders (e.g. *Atheliales* sp. #SH229 868.06FU, *Sistotrema* sp. #SH211497.06FU and *Wilcoxina mikolae*). Further, our data suggest that EMF communities associated with invading *P. contorta* individuals are highly variable across the broad geographic range of study sites that we evaluated (only 16% average similarity across sites), and equally rich as communities associated with *P. contorta* plantations or *Nothofagus* forests. These patterns suggest that *Pinus* invasion would likely occur in these environments even in the absence of *S. luteus*, as long as other suitable EMF co-invaders are present (Hayward *et al.* 2015b).

Conclusions

This study provides several new insights into the likely role of fungal interactions in explaining the high productivity of *P. contorta* in some European locations (Elfving, Ericsson & Rosvall 2001; Gundale *et al.* 2014b) and its successful establishment and invasion in Southern Hemisphere locations (Ledgard 2001; Langdon, Pauchard & Aguayo 2010; Gundale *et al.* 2014b). First, our data indicate that escape from below-ground fungal pathogens is probably not an important contributor to the strong performance of *P. contorta* in these environments. Instead, invading *P. contorta* individuals in the Southern Hemisphere encountered the greatest abundance and richness of pathogens across our whole study system, suggesting that invasion occurs in these regions despite greater pathogen abundance. Secondly, our data indicate that *P. contorta* associates with different fungal communities in all

environments to which it is introduced compared to its native range, but suggests the mechanism controlling these communities differs among environments. In environments with phylogenetically similar vegetation, *P. contorta* readily associates with fungal taxa already present, which may contribute to its enhanced growth in these regions. Meanwhile, in environments with phylogenetically distant vegetation, it becomes associated with a unique fungal community that appears to have originated from multiple co-introduction events of native range taxa that re-assembled to form fungal communities with unique composition. Thirdly, our study suggests that fungal co-introduction is necessary for initial establishment of ECM species in environments with phylogenetically distant vegetation. However, our study also suggests that once fungal co-introduction has occurred, invasion of *P. contorta* is not likely constrained by fungal co-invasion, as suggested by the considerable EMF diversity associated with *P. contorta* in all environments we evaluated, including invading *P. contorta* individuals as far as 1 km from source plantations. This suggests that invasion of EMF plants is likely to be limited by fungal co-invasion only under the most extreme situations, that is where native vegetation is phylogenetically distant, and no fungal co-introduction has occurred.

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Data accessibility

Data are accessible via Dryad at <http://dx.doi.org/10.5061/dryad.p513f> (Gundale *et al.*, 2016). Data files: Raw Data Summary

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Supporting Information

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

Table S1. Total number of OTUs found in each stand type.

Table S2. OTUs contributing to within group similarity of different stand types.

Table S3. OTUs contributing to fungal community differences between Canadian and European *Pinus contorta*.

Table S4. OTUs contributing to EMF community differences between Canadian and European *Pinus contorta*.

Table S5. OTUs contributing to fungal community differences between USA and Southern Hemisphere *Pinus contorta*.

Table S6. OTUs contributing to EMF community differences between USA and Southern Hemisphere *Pinus contorta*.

Table S7. OTUs contributing to fungal community differences between *Pinus contorta* and *Nothofagus* spp.

Table S8. OTUs contributing to EMF community differences between *Pinus contorta* and *Nothofagus* spp.

Table S9. OTUs contributing to fungal community differences between plantation and invading *Pinus contorta*.

Table S10. OTUs contributing to EMF community differences between plantation and invading *Pinus contorta*.

Table S11. OTUs contributing to fungal community differences between invading *Pinus contorta* and *Nothofagus* spp.

Table S12. OTUs contributing to EMF community differences between invading *Pinus contorta* and *Nothofagus* spp.

Table S13. A comparison of soil C, N, and P concentrations within each SSP.