New Phytologist

Research

Contrasting effects of intra- and interspecific identity and richness of ectomycorrhizal fungi on host plants, nutrient retention and multifunctionality

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Received: *11 March 2016*
Accepted: *3 August 2016*

New Phytologist (2016)
doi: 10.1111/nph.14184

Key words: CO_2 efflux, diversity, ecosystem function, ectomycorrhizal fungi, *Laccaria bicolor*, leaching, multifunctionality, productivity.

Summary

- A major gap in our understanding of biodiversity–ecosystem function relationships concerns the role of intra- and interspecific diversity of mycorrhizal fungi, which are critical for plant fitness, biogeochemical cycling and other processes. Here, we test the hypothesis that the identity and richness of ectomycorrhizal (ECM) fungi at the intra- and interspecific levels affect ecosystem multifunctionality by regulating plant and fungal productivity, soil CO_2 efflux and nutrient retention.
- Microcosms containing Scots pine (*Pinus sylvestris*) seedlings colonized by different ECM fungal isolates, in monocultures and mixtures, enabled us to test for both intra- and interspecific identity and richness effects, and transgressive overyielding.
- Intra- and interspecific identity had modest but significant effects on plant and fungal productivity and nutrient retention, but no effect on CO_2 efflux. Intraspecific richness increased plant root productivity and ECM root tips but decreased hyphal length, whereas interspecific richness had no effects. Interspecific mixtures outperformed the most productive monocultures in only 10% of the cases, compared with 42% for the intraspecific mixtures.
- Both intra- and interspecific identity and richness of ECM fungi regulate ecosystem multifunctionality, but their effects on the direction and magnitude of individual variables differ. Transgressive overyielding suggests that positive niche complementarity effects are driving some of the responses to intraspecific richness.

Introduction

Concerns over biodiversity loss have fuelled efforts to achieve a better understanding of the relationship between biodiversity and ecosystem functioning (Vitousek *et al.*, 1997; Loreau *et al.*, 2001). Although there are important exceptions, many studies tend to support a positive effect of species richness on ecosystem functioning (Hooper *et al.*, 2005; Balvanera *et al.*, 2006; Cardinale *et al.*, 2007; Zavalete *et al.*, 2010; Reich *et al.*, 2012).

However, biodiversity–ecosystem function studies have tended to focus on above-ground communities, and also rarely consider how other key components of diversity, notably intraspecific diversity, regulate ecosystem processes (Antonovics, 2003; Johnson *et al.*, 2012). It is still poorly understood whether findings from manipulations of plant diversity can be extrapolated to below-ground organisms (Prosser *et al.*, 2007), although recent evidence suggests that the diversity of soil biological communities at both local (Wagg *et al.*, 2014) and global (Delgado-Baquerizo *et al.*, 2016) scales affects key ecosystem functions and multifunctionality. Yet our understanding of the importance of the diversity of mycorrhizal fungi, in particular, remains poor, despite the importance of these organisms in regulating biogeochemical cycling, biodiversity and interactions of above- and below-ground organisms (Smith & Read, 2008). This lack of knowledge is particularly apparent for ectomycorrhizal (ECM) fungi, which are major components of forest soil microbial biomass and have critical roles in nutrient acquisition and transport. Moreover, several studies have shown that physiological heterogeneity both among and within species of ECM fungi can be considerable (Cairney, 1999). For example, strains of the ECM fungus *Laccaria bicolor* vary in their extent of colonization on pine (Kropp *et al.*, 1987; Kropp & Fortin, 1988; Wong *et al.*, 1989, 1990a,b; de la Bastide *et al.*, 1995a), mycelial growth rates and solubilization of inorganic phosphorus sources in culture (Nguyen *et al.*, 1992; de la Bastide *et al.*, 1995b). The high degree of local ECM fungal genetic diversity (e.g. Bahram *et al.*, 2011) and its associated physiological heterogeneity suggest that intra- and interspecific diversity of ECM fungi could have a significant direct role in influencing ecosystem functioning (Johnson *et al.*, 2012).

To date there have only been two studies published that have explicitly tested if ECM fungal species richness directly affects the productivity of their host plants (Baxter & Dighton, 2001;

Jonsson *et al.*, 2001). These findings showed that fungal species richness positively affected some measures of plant productivity in the laboratory, but the effects depended on soil nutrient conditions (Jonsson *et al.*, 2001). In addition, fungal biomass and CO_2 production have been shown to increase significantly with increasing species richness of pure cultures of ECM fungi (Wilkinson *et al.*, 2011). Evidence from ECM fungal pure culture experiments also suggests that intraspecific diversity can have significant effects on fungal performance. However, findings have been contradictory; for example, fungal biomass and CO_2 production increased when intraspecific diversity was manipulated on its own (Wilkinson *et al.*, 2010) but decreased when both intra- and interspecific diversity were manipulated alongside each other (Wilkinson *et al.*, 2012). Furthermore, a criticism of this past work is that the fungi were grown without a host plant, and so there is a clear need to investigate in parallel the relationships between ECM fungal intra- and interspecific richness and measures of ecosystem functioning when the fungi are in symbiosis.

To better understand the patterns revealed by biodiversity–ecosystem function experiments, it is imperative for experimental designs to incorporate monoculture and mixture treatments alongside a richness gradient (Hooper *et al.*, 2005). Here, we use such an approach to test the hypothesis that both intra- and interspecific identity and richness of ECM fungi regulate ecosystem multifunctionality through their effects on plant and fungal productivity, soil CO_2 efflux and soil nutrient retention. Two separate experiments were performed under near-identical conditions that consisted of microcosms planted with pine seedlings individually precolonized by one of four specific isolates of *L. bicolor*, or one of four ECM species. The seedlings were combined to create gradients of either intra- or interspecific richness within microcosms. Using individually precolonized seedlings avoids the confounding factors associated with colonization differences between fungal isolates and unequal inoculum dispersal on seedling root systems that have led to criticism of previous ECM species diversity–function studies (Leake, 2001). We tested the hypothesis that seedlings colonized by different ECM fungal isolates within the same microcosm would grow more and support greater fungal biomass than microcosms containing identical ECM fungal isolates, and that soil CO_2 efflux would concomitantly increase and soil nutrient loss would decrease as a result of improved nutrient retention. Specifically, based on past work in pure culture (Wilkinson *et al.*, 2010, 2011, 2012), we predicted that positive effects of both intra- and interspecific richness would be of similar magnitude and direction.

Materials and Methods

Colonization of Scots pine seedlings by ECM fungi

Four strains of *Laccaria bicolor* (LbA, LbB, LbC, LbD), and one strain each of *Suillus variegatus* (Sv), *Paxillus involutus* (Pi) and *Amanita rubescens* (Ar) were obtained for the experiments. Cultures were derived from fruit bodies collected in Scots pine forests in Aberdeenshire, Scotland, in 2010 and are part of the fungal culture collection at the University of Aberdeen, UK. Cultures were maintained on modified Melin–Norkrans (MMN) agar at 22°C in the dark. These ECM strains were chosen because of their ability to colonize pine *in vitro*, and to represent the different extramatrical mycelium exploration types found in nature (with the exception of the contact type) (Agerer, 2001).

Under sterile conditions, four inoculum plugs of one of the four *L. bicolor* isolates or the different species isolates were placed along a row on large (15 cm diameter) Petri plates containing 1.0 g l^{-1} glucose MMN overlaid with cellophane (Type 325P) (Cannings Packaging Ltd, Bristol, UK). The cultures were grown at 22°C in the dark for 2–4 wk until the mycelium covered the bottom half of the plate. Four sterile germinated Scots pine (*Pinus sylvestris* L.) seedlings were positioned along a row onto each plate such that the roots were in direct contact with the fungus. The Scots pine seeds (lot 08RP20SI, Forestry Commission, UK) were surfaced-sterilized in 30% w/v hydrogen peroxide for 40 min, rinsed in sterile water, placed onto water agar plates and germinated after 2 wk in a growth chamber under controlled conditions (16 : 8 h light : dark cycle at 180 mmol photons m^{-2} s^{-1} and constant temperature of 18°C). The seedling-fungal plates were sealed with Parafilm and partially covered with aluminium foil to prevent light exposure to the pine roots and fungi, and incubated in a growth chamber under controlled conditions (as described earlier). After 2 months, pine seedlings that had formed mycorrhizal root tips were retained for the experiment. To take into account initial growth differences between seedlings colonized by different species and *L. bicolor* isolates, seedling biomass (FW) and root length, and the total number of ECM root tips were measured for each seedling before planting (Supporting Information Table S1).

Microcosms and experimental design

The colonized pine seedlings were planted into 17 cm × 12 cm pots (LBS Worldwide Ltd, Colne, UK) filled with a soil substrate composed of 380 g of homogenized sterile (2× autoclaved at 121°C for 1 h) sieved peat (Shamrock Irish moss peat; Bord Na Mona Horticulture, Ireland) and vermiculite (Vermiculite V3 medium 2.0–5.0 mm; William Sinclair Horticulture Ltd, Lincoln, UK) with 1.0 g l^{-1} glucose MMN added at the ratio of 1 : 5 : 3. The MMN medium was chosen as the nutrient source in order to be consistent with the initial growth conditions that the fungi and seedlings were under, and this nutrient solution was shown in pre-experimental tests to produce healthy active plant–fungal microcosm systems. Each pot contained four seedlings, and each seedling was randomly designated a position within the pot, with the positions equidistant from each other. The seedlings were planted to create two experiments, each with four monocultures, three random mixtures of two isolates and one four-isolate mixture (Table 1). The only difference was that one experiment comprised isolates of the same species (LbA-D), while the other comprised isolates of different species (LbA, Sv, Pi and Ar). Each intra- and interspecific treatment was replicated six times, resulting in 384 seedlings (eight intra- and interspecific treatments, each with six replicates × four seedlings per pot). Nonmycorrhizal pine seedlings (NM control) were used as a control to test

Table 1 Experimental design consisting of ectomycorrhizal intra- and interspecific monoculture and mixture treatments across a richness gradient

Fungal isolates		
Intraspecific	Interspecific	Richness
LbA (*Laccaria bicolor*)	LbA (*Laccaria bicolor*)	1
LbB	Sv (*Suillus variegatus*)	1
LbC	Pi (*Paxillus involutus*)	1
LbD	Ar (*Amanita rubescens*)	1
LbA + LbB	LbA + Sv	2
LbC + LbD	Pi + Ar	2
LbA + LbD	Sv + Pi	2
LbA + LbB + LbC + LbD	LbA + Sv + Pi + Ar	4

for extraneous fungal contamination, which was not detected, and to test the overall effects of mycorrhizal colonization on ecosystem responses. The microcosms were placed randomly within a growth chamber and incubated under controlled conditions (same as described earlier) and watered with 75 ml of dH_2O three times a week. After 6 months, the microcosms were harvested, and the soil substrate was stored at −20°C until used. The root systems were cleaned by gently washing them in water, and then the seedlings were placed into clear trays with the roots spread out and submerged in water. The ECM root tips on each seedling from the interspecific mixture microcosms were morphotyped (Agerer, 2001) under a dissecting microscope to quantify the abundance of each species (Table S2).

Plant and fungal productivity measurements

Measures of plant productivity included seedling shoot height, shoot biomass, root biomass, root length and shoot nitrogen (N) and phosphorus (P) content. Seedling shoot heights were measured and shoot and root biomass was determined after oven drying at 60°C for 48 h. The root length and the number of root tips were measured by scanning the root systems (Epson Expression 10000XL; Epson Canada Ltd, Toronto, CA, USA) and analysis using WinRhizo Pro v.2009 (Regent Instruments Canada Inc., Quebec, CA, USA). For determination of shoot total N and P, finely ground milled (Mixer Mill MM 400, Retsch, Haan, Germany; Smith *et al.*, 2013) shoots were digested in a mix of sulphuric acid and hydrogen peroxide (Allen, 1989), and concentrations were measured on a flow injection analyser (FIAstar 5000 system with 5027 Sampler; Foss NIRSytem Inc., Hillerød, Denmark).

Measures of fungal productivity included the number of ECM root tips per root length on each seedling, and the total length of fungal hyphae in the soil of each microcosm. To calculate ECM root tips per root length, the % ECM colonization of each seedling was estimated by counting the number of root tips that were mycorrhizal out of 200 randomly selected root tips under a dissecting microscope. Then, the number of root tips (determined from root scanning) was multiplied by the % ECM colonization, and divided by the root length (determined from root scanning).

Total length of fungal hyphae was measured on extracted hyphae using the membrane filter technique modified after Hanssen *et al.* (1974), followed by staining the hyphae with calcofluor white M2R fluorescent brightener (Sigma-Aldrich) (Bloem *et al.*, 1995) and quantification with the grid-line intersect method (Brundrett *et al.*, 1996). Briefly, 10 g of soil in 95 ml of dH_2O was blended at high speed in a blender for 1 min, and 5 ml of the slurry was diluted with 5 ml of dH_2O, and then 1 ml of the diluted slurry was added to 1 ml of formalin, 1 ml of stain ($0.002\ g\ ml^{-1}$) and 7 ml of dH_2O. Samples were incubated in the dark at room temperature for 2 h and stored at 4°C until further processing. Samples were filtered through a 25-mm-diameter black 1 µm pore size polycarbonate filter (Osmonics Inc., Minnetonka, MN, USA) and rinsed three times with 3 ml of dH_2O. The filters were placed on slides with immersion oil, and five transects across the filter, for a total of 25 gridded fields ($100\ mm^2$ grid area), at ×200 magnification were viewed under a UV-illuminated microscope (Olympus BX61; Olympus Co., Tokyo, Japan). Hyphal length (H) was calculated using the equation $H = (I\pi A)/(2L)$, where I is the average number of intersections per grid, A is the grid area, and L is the total length of the grid lines. Then, the total length of fungal hyphae (F) ($m\ g^{-1}$of soil) was calculated using the equation $F = H \times 10^{-6}(A/B)(1/S)$, where A is the area of the filter, B is the grid area, and S is the amount of soil filtered (Bloem *et al.*, 1995).

Nutrient retention ability of microcosms

At 1 wk before microcosm harvest, 40 ml of $1.0\ g\ l^{-1}$ glucose MMN was added to each microcosm as an assay of the ability of the systems to retain added nutrients. Before harvest, the soil moisture content was adjusted to 80%, after which 100 ml of dH_2O was evenly distributed into each microcosm, and leachate was collected into sterile plastic tubes. Immediately after collection, the leachate was passed through sterile syringe 0.45 µm pore size filters (mixed cellulose esters membrane) (EMD Millipore, Billerica, MA, USA), total leachate volume was measured and samples stored at 4°C for 3 d until nutrient content analysis. Concentrations of ammonium (NH_4), nitrate (NO_3), total N, organic N, phosphate (PO_4), total P, organic P and dissolved organic carbon (DOC) in leachates were determined from analyses on a colorimetric autoanalyzer (Skalar San^{++} Continuous Flow Analyzer; Skalar Analytical BV, Breda, the Netherlands) at the James Hutton Institute, Aberdeen, UK.

Microcosm soil CO_2 efflux measurements

Soil CO_2 efflux was measured by sealing a removable modified collar onto a microcosm such that the soil and plants were not disturbed. A single 10 cm survey chamber (Model: 8100-102) with an auxiliary probe attached to the LI-8100 portable soil CO_2 flux system (Li-Cor, Lincoln, NE, USA) was used to simultaneously measure CO_2 and soil temperature. Machine settings were as follows: 10 cm chamber offset, 45 s dead band, 1 min 30 s observation delay, 45 s purge time, and 9 cm × 9 cm soil surface area. Soil moisture content was measured using a ML2

ThetaProbe soil moisture sensor and HH2 meter (Delta-T Devices Ltd, Cambridge, UK) immediately after CO_2 measurement. Three consecutive CO_2 readings were taken for each microcosm during each of the two sampling time points; 4 wk before harvest and 1 wk before harvest.

Data analyses

Statistical analyses were carried out in R v.3.3.0 (R Development Core Team, 2016). Total values for plant and fungal productivity, nutrient concentrations in leachate and CO_2 efflux variables on a microcosm basis (i.e. across the four seedlings in a microcosm) were calculated and used untransformed in statistical models. First, we tested for significant intra- and interspecific identity effects (defined as differences among individual inoculation treatments whether they were monocultures or mixtures; Wilkinson *et al.*, 2010) on the variables using one-way ANOVA (fit by the aov function) and assuming unequal variances. When main effects were significant, *post hoc* Tukey tests were used to examine differences between individual treatments.

Second, we tested for intra- and interspecific richness effects on the variables using linear mixed-effects models (fit by REML; lme function) with intra- or interspecific identity included as a random factor to account for the variance within the richness levels. Where a significant effect of richness was identified, linear regression was used to test whether there was an increasing or decreasing trend with richness. To account for possible confounding effects as a result of growth differences in the seedlings before planting into the microcosms, corresponding variables (seedling biomass, root length and ECM root tips per root length) were kept in aov and lme models as covariables when significant. In only three cases were covariables significant and kept in models. In the CO_2 efflux models, soil temperature and moisture were included as covariables.

Third, to test the overall effect of mycorrhizal colonization, one-way ANOVA followed by *post hoc* Tukey tests was used to contrast the NM control to the monoculture treatments. Linear regression analysis was performed to examine relationships between fungal and plant productivity variables and fungal productivity and soil nutrient concentrations.

Fourth, to determine if intra- and interspecific mixtures had a positive or negative effect on plant and fungal productivity, transgressive overyielding was calculated (D_{max} = (observed yield of mixture) − (observed yield of maximally yielding monoculture)/(observed yield of maximally yielding monoculture)), such that a D_{max} > zero results when an intra- or interspecific mixture produces more than its most productive corresponding monoculture (Loreau, 1998). Significance of the D_{max} values from zero was tested using one-sample *t*-tests.

Finally, principal component analysis (PCA) was used (performed by the function PCAshiny within the package Factoshiny) to examine overall patterns of intra- and interspecific richness on multifunctionality (comprising plant and fungal productivity, soil nutrient concentrations in leachate and CO_2 efflux). Vectors showing significant correlation of the variables with the axes were included to evaluate collinearity between the variables without prejudice of the direction (positive or negative) of the responses.

Results

ECM fungal identity and richness effects on plant productivity

Intraspecific identity of ECM fungi significantly affected shoot biomass ($F = 10.28$, $P = 0.001$) and shoot N concentrations ($F = 2.66$, $P = 0.023$) of host plants (Fig. 1). There were no significant genotype identity effects on shoot height ($F = 1.29$, $P = 0.282$), shoot P concentrations ($F = 2.07$, $P = 0.069$), root length ($F = 1.91$, $P = 0.093$) and root biomass ($F = 1.00$, $P = 0.447$).

Mean shoot biomass was greatest in the mixtures containing two genotypes compared with the monocultures, with the exception of LbA (Fig. 1). Plants inoculated with LbC had significantly greater mean shoot N concentrations than those inoculated with LbA + LbB (Fig. 1). Although not significant, mean root lengths were consistently greater in the mixtures than in the monocultures (Fig. 1). Significant intraspecific richness effects for root length and root biomass were found, being greater in the mixtures than in the monocultures (Fig. 2).

In comparison, interspecific identity effects on host plants were significant for all of the productivity variables (shoot height, $F = 5.42$, $P = 0.001$; shoot biomass, $F = 11.68$, $P = 0.001$; shoot P, $F = 10.93$, $P = 0.001$; shoot N, $F = 6.93$, $P = 0.001$; root length, $F = 7.81$, $P = 0.001$; root biomass, $F = 3.20$, $P = 0.009$), with some significant differences between monocultures, between mixtures and between monocultures and mixtures (Fig. 1). In contrast to intraspecific richness, no significant interspecific richness effects were found ($P > 0.152$; Fig. 2).

ECM fungal identity and richness effects on fungal productivity

Ectomycorrhizal root tips per root length ($F = 3.49$, $P = 0.005$) and hyphal length ($F = 6.13$, $P = 0.001$) were both significantly affected by intraspecific identity (Fig. 3). The mixtures LbA + LbD and LbA + LbB + LbC + LbD had the greatest numbers of ECM root tips per root length, and were significantly different from LbA, which had the smallest number. By contrast, the LbA + LbB + LbC + LbD mixture produced the least amount of hyphae, which was almost half the length produced by LbA, and was significantly different from all but one of the monocultures (Fig. 3). Intraspecific richness effects on both ECM root tips per root length and hyphal length were significant (Fig. 2) but had opposite trends. Mean ECM root tip per root length increased with increasing intraspecific richness, while mean hyphal length decreased with increasing intraspecific richness (Figs 2, 3).

Similarly, there were significant interspecific identity effects on ECM fungal productivity, as reflected by significant effects on ECM root tips per root length ($F = 5.96$, $P = 0.001$) and hyphal length ($F = 41.38$, $P = 0.001$) (Fig. 3). For ECM root tips per

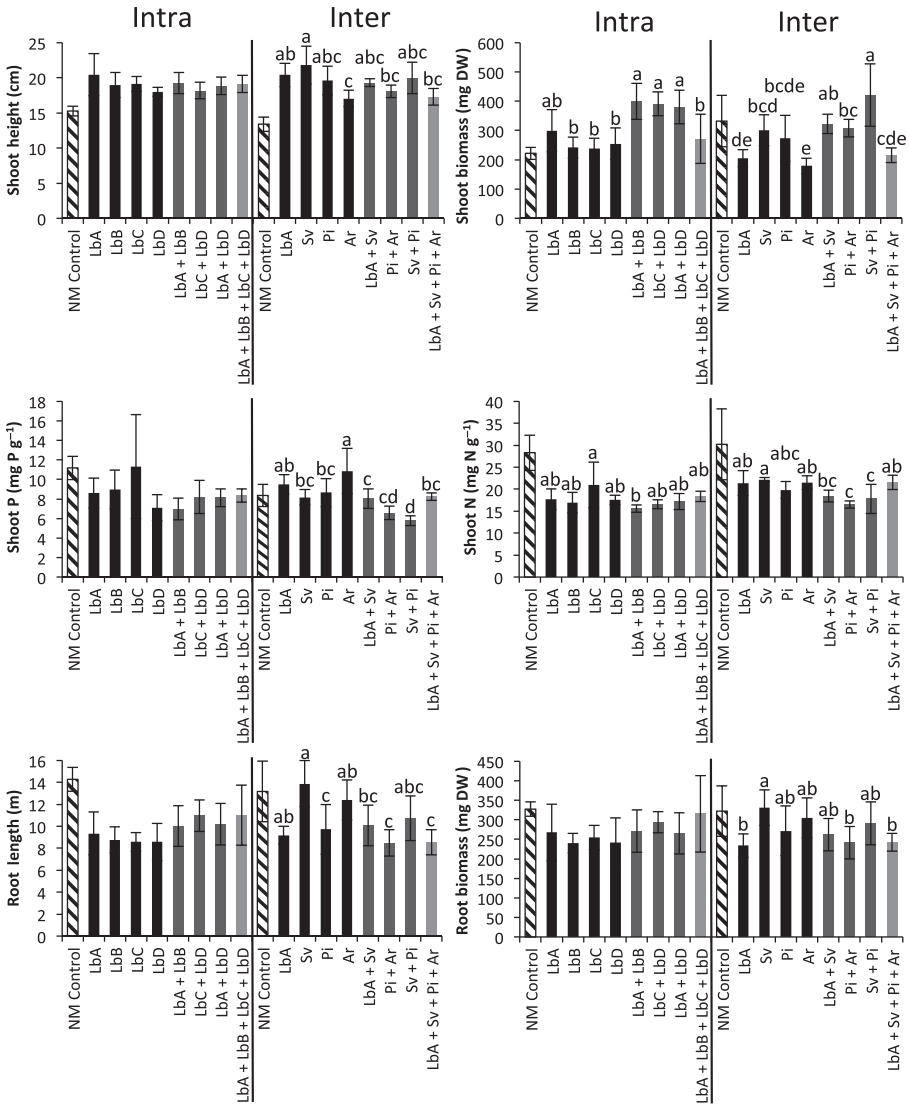

Fig. 1 Ectomycorrhizal intra- and interspecific identity effects on plant productivity of Scots pine seedlings across a richness gradient (NM control, nonmycorrhizal control; LbA, LbB, LbC, LbD, *Laccaria bicolor* isolates; Sv, *Suillus variegatus*; Pi, *Paxillus involutus*; Ar, *Amanita rubescens*). Means (± SD) with different letters are significantly different based on Tukey's *post hoc* test following one-way ANOVA (NM control was excluded from this analysis).

root length, the monocultures Sv and Ar had the smallest mean density of tips and were significantly different from the other monocultures and mixtures (Fig. 3). Hyphal length was low in all of the species treatments except for LbA and the two mixtures containing LbA, and these treatments had significantly greater mean hyphal length than the other intraspecific treatments (Fig. 3). Interspecific richness did not significantly affect fungal productivity ($P > 0.652$; Fig. 2).

ECM fungal identity and richness effects on soil nutrient retention and CO_2 efflux

Intraspecific identity significantly affected the retention of nutrients in microcosms. Specifically, intraspecific identity significantly affected the concentrations of NO_3 ($F = 3.58$, $P = 0.004$), total N ($F = 3.51$, $P = 0.005$), organic N ($F = 3.79$, $P = 0.003$), total P ($F = 2.88$, $P = 0.016$), organic P ($F = 8.07$, $P = 0.001$) and DOC ($F = 4.28$, $P = 0.001$) in leachate, but not NH_4 ($F = 1.34$, $P = 0.260$) and PO_4 ($F = 1.67$, $P = 0.087$). For NO_3, significant differences were found between the mixtures with two genotypes (Fig. 4), with LbA + LbB producing the greatest concentrations in leachate. LbA produced leachate with the greatest concentrations of total and organic N and was significantly different from the other monocultures and two of the mixtures (Fig. 4). Mean total P concentration was greatest in LbC, which was significantly different from the LbD and LbA + LbD treatment (Fig. 5). Mean organic P concentration was highest in the monocultures LbA, LbB and LbC compared with the mixtures (often significantly so); by contrast, LbD produced leachate with one of the lowest mean concentrations of organic P (Fig. 5). Mean DOC concentration was significantly different between LbA + LbD and the other mixtures with two genotypes, and LbA which produced leachate with the greatest mean concentration (Fig. 5). Intraspecific richness had no significant effects on the soil nutrient concentrations in leachate ($P > 0.199$; Table S3).

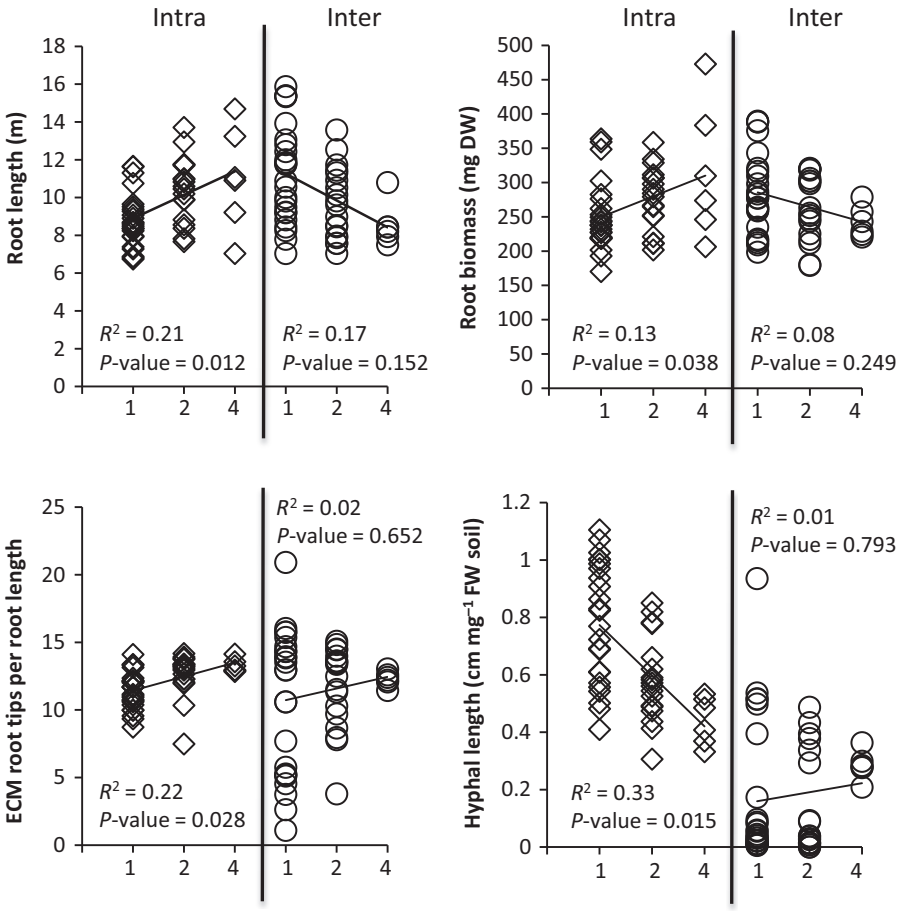

Fig. 2 Ectomycorrhizal (ECM) intra- and interspecific richness effects on Scots pine plant root and fungal productivity. Linear regression trend lines and R^2 values are shown, and linear mixed-effects *P*-values.

Interspecific identity affected concentrations of NH_4 ($F = 2.92$, $P = 0.014$), total N ($F = 9.62$, $P = 0.001$), organic N ($F = 9.93$, $P = 0.001$), PO_4 ($F = 13.67$, $P = 0.001$), total P ($F = 11.90$, $P = 0.001$), organic P ($F = 4.01$, $P = 0.002$) and DOC ($F = 6.37$, $P = 0.001$) in leachate. For both total and organic N, Sv, Pi and Sv + Pi had the highest means and were significantly different from the other treatments except for Ar and Pi + Ar (Fig. 4). Pi + Ar and Sv + Pi had significantly greater mean PO_4 and total P concentrations in leachate than the other treatments (Fig. 5). Mean organic P concentration was greatest in Sv and Sv + Pi and was significantly different from Pi + Ar treatment. LbA had the least and Pi had the greatest mean DOC concentrations, which were often significantly different from the other treatments (Fig. 5). No interspecific richness effects on soil nutrient concentrations from leachate were found ($P > 0.347$; Table S3).

There were no intra- and interspecific identity or richness effects on microcosm soil CO_2 efflux, or distinguishable trends, except that for all treatments CO_2 efflux consistently increased across the two sampling time points (Fig. S1).

Overall effect of mycorrhizal colonization on responses

Plant productivity was significantly different between the nonmycorrhizal control and the intra- and interspecific monocultures ($P < 0.022$), except for intraspecific shoot biomass and shoot P (Table S4). The nonmycorrhizal control plants had the least shoot height but the greatest shoot N concentrations, root length and root biomass (Table S4). Soil nutrient concentrations in leachate were usually significantly different between the nonmycorrhizal control and only one monoculture (Table S5). The leachate nutrient concentrations of the nonmycorrhizal control were either the least or median, except for PO_4 and total P, in comparison to the monocultures (Table S5). There were no significant differences between the monocultures and the nonmycorrhizal control for soil CO_2 efflux (Table S4).

Transgressive overyielding

Transgressive overyielding (D_{max}) (i.e. when an intra- or interspecific mixture produces more than its most productive corresponding monoculture) was determined for each of the plant and fungal productivity variables (except hyphal length) (Fig. 6). The intraspecific monoculture LbA was the most productive for shoot height, shoot biomass, root length and root biomass, LbB for ECM root tips per root length and LbC for shoot P and N concentrations. The productivity values for these monocultures were therefore used in the calculations of D_{max}. Mean values for D_{max} in the intraspecific manipulation treatments ranged from −0.274 to 0.334, with eight out of the 19 mean values positive, and three

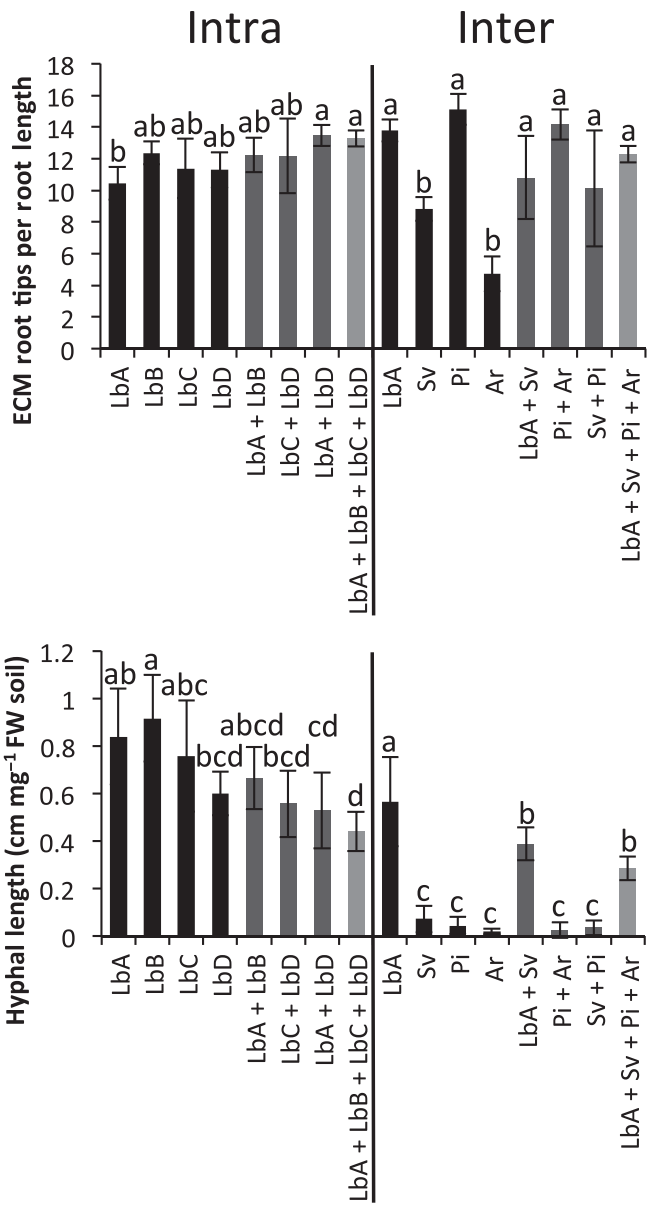

Fig. 3 Ectomycorrhizal (ECM) intra- and interspecific identity effects on fungal productivity across a richness gradient (LbA, LbB, LbC, LbD, *Laccaria bicolor* isolates; Sv, *Suillus variegatus*; Pi, *Paxillus involutus*; Ar, *Amanita rubescens*). Means (± SD) with different letters are significantly different based on Tukey's *post hoc* test following one-way ANOVA.

of the means were significantly different from zero (Fig. 6a). Seven of the 11 negative mean values were also significantly different from zero (Fig. 6a).

The interspecific monoculture Sv was the most productive for shoot height, shoot biomass, root length, root biomass, Pi for ECM root tips per root length, and Ar for shoot P and N concentrations, and the productivity values for these monocultures were used in the calculations of D_{max}. Mean D_{max} in the interspecific manipulations ranged from −0.496 to 0.399, but only two out of the 20 mean values were positive, and all but five of the mean values were significantly different from zero (Fig. 6b).

Effects of ECM identity and richness on ecosystem multifunctionality

Principal component analysis considered all measured variables and revealed some separation between the different levels of intraspecific richness (Fig. 7). The first two principal components accounted for 25.1% and 19.7% of variation, respectively. The second principal component, which corresponded to CO_2 efflux, shoot biomass, root length, root biomass, shoot N and P and hyphal length, tended to separate microcosms comprising monocultures of Lb isolates or two-isolate mixtures, whereas there was considerable overlap with the four-isolate mixtures. For interspecific richness, the first and second PCA axes accounted for 32.7% and 16.8% of variation, respectively. There was some evidence of clustering of the four-isolate mixtures, but overall there was no clear separation of the treatments. For both intra- and interspecific richness, some of the vectors for the variables were in close proximity to each other (e.g. NH_4 and Org N), suggesting collinearity between these variables (Fig. 7).

Discussion

This study used parallel manipulations of Scots pine seedlings inoculated with either different isolates of *L. bicolor*, or with *L. bicolor*, *P. involutus*, *S. variegatus* and *A. rubescens* to simultaneously test the effects of intra- and interspecific identity and richness on ecosystem multifunctionality, comprising plant and fungal productivity, soil nutrient retention and CO_2 efflux.

We tested the hypothesis that both intra- and interspecific richness and identity of ECM fungi would increase plant and fungal productivity, nutrient retention and soil CO_2. We predicted that both the direction and magnitude of intra- and interspecific richness effects would be similar, based on previous pure culture model systems, where it was found that intraspecific identity significantly affected fungal productivity and activity (CO_2 production), but that productivity and CO_2 efflux also increase alongside intraspecific richness (Wilkinson *et al.*, 2010). While our results support the view that intraspecific richness of mycorrhizal fungi is an important driver of plant performance (Johnson *et al.*, 2012), we found that interspecific richness of ECM fungi had overall weaker effects on measured variables.

We found significant positive effects of intraspecific richness on plant and fungal productivity, but no effect on soil nutrient retention and soil CO_2 efflux. Intraspecific identity had more modest effects: with four exceptions (out of 96 possible), intraspecific monocultures were not significantly different from each other with respect to the ecosystem metrics, and differences mainly lay between monocultures and mixtures. Moreover, for around half of the measured variables, we found that the mixtures outperformed the maximum yielding monocultures ($D_{max} > 0$). Taken together, these findings imply that genetic diversity within species was an independent factor regulating several key ecosystem functions.

Positive interactions could occur through physiological complementarity, for example, if different ECM isolates within a

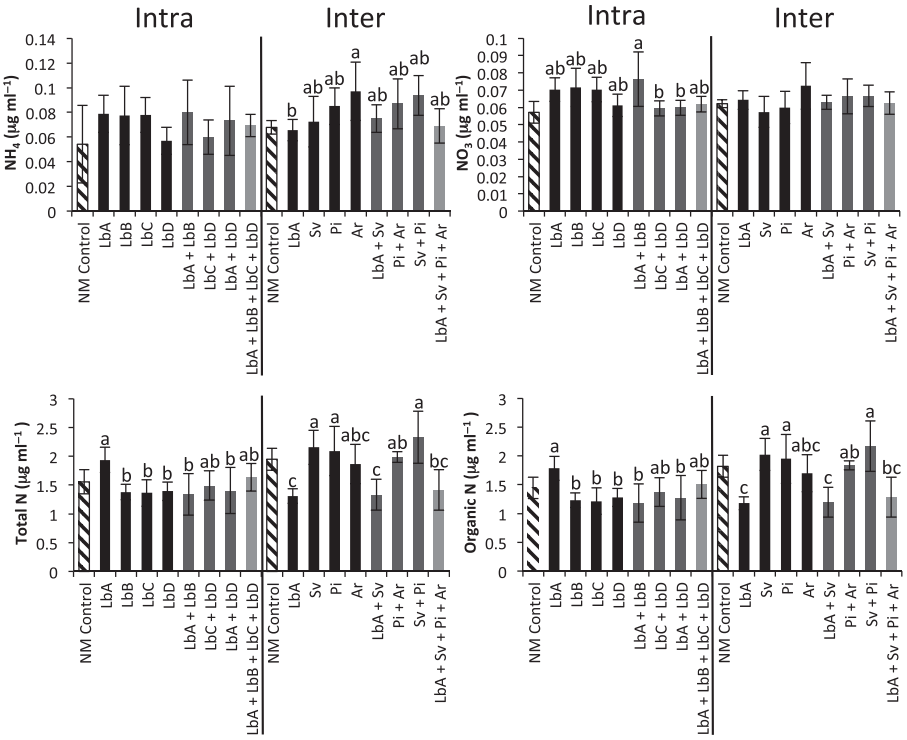

Fig. 4 Ectomycorrhizal intra- and interspecific identity effects on soil nitrogen (N) retention across a richness gradient (NM control, nonmycorrhizal control; LbA, LbB, LbC, LbD, *Laccaria bicolor* isolates; Sv, *Suillus variegatus*; Pi, *Paxillus involutus*; Ar, *Amanita rubescens*). Means (± SD) with different letters are significantly different based on Tukey's *post hoc* test following one-way ANOVA (NM control was excluded from this analysis).

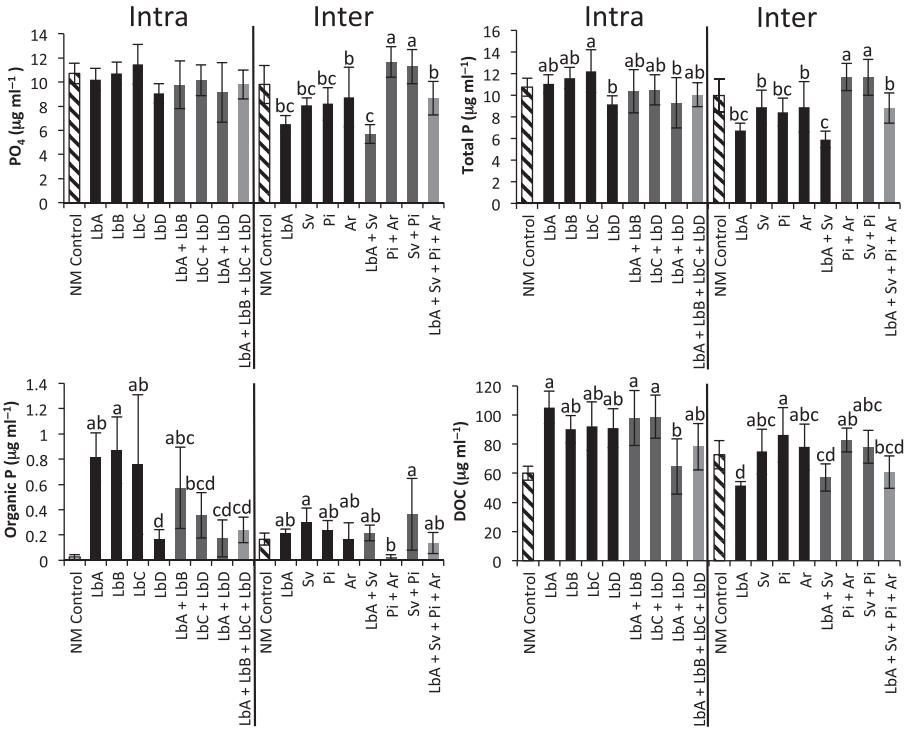

Fig. 5 Ectomycorrhizal intra- and interspecific identity effects on soil phosphorus (P) and dissolved organic carbon (DOC) retention across a richness gradient (NM control, nonmycorrhizal control; LbA, LbB, LbC, LbD, *Laccaria bicolor* isolates; Sv, *Suillus variegatus*; Pi, *Paxillus involutus*; Ar, *Amanita rubescens*). Means (± SD) with different letters are significantly different based on Tukey's *post hoc* test following one-way ANOVA (NM control was excluded from this analysis).

species are differentially superior in acquiring particular nutrients. Considerable variation in the physiological properties of ECM fungi within species has been reported previously (Cairney, 1999); *L. bicolor* has been shown to vary in its ability to solubilize inorganic phosphorus sources in culture (Nguyen *et al.*, 1992; de la Bastide *et al.*, 1995b). Negative interactions can occur through the release of extracellular enzymes, secondary metabolites and volatiles from 'nonself' recognition of mycelia that could change fungal growth rates and patterns (Malik & Vilgalys, 1999).

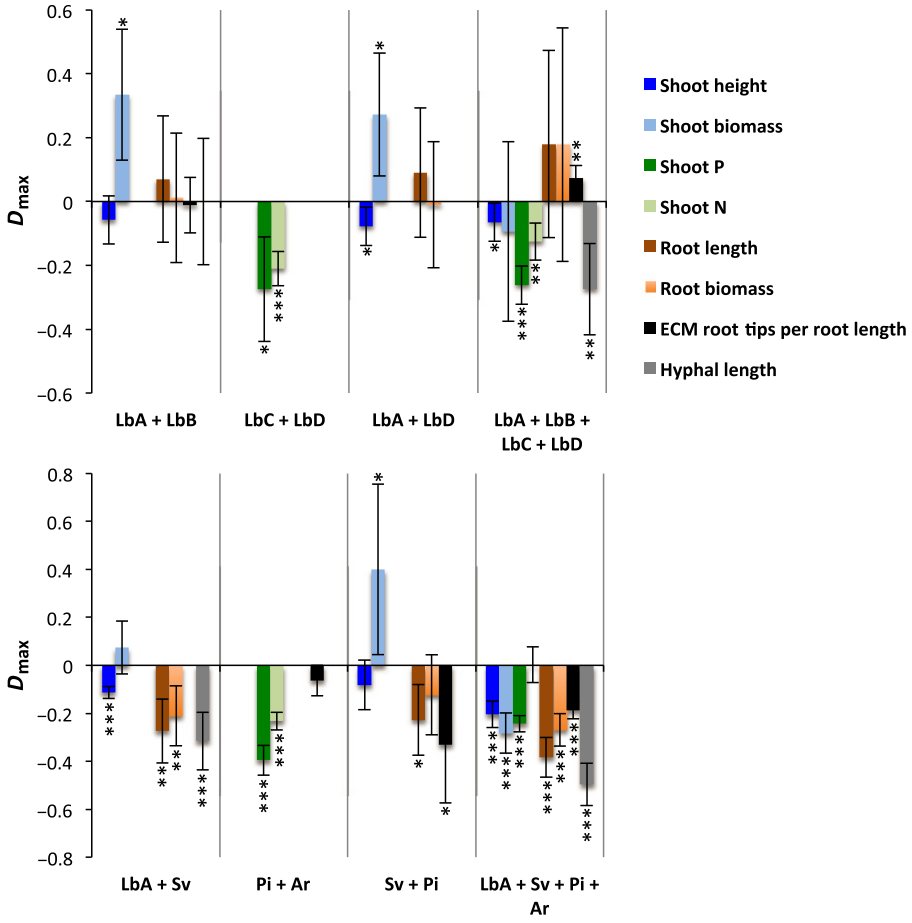

Fig. 6 Transgressive overyielding (D_{max}) of plant and fungal productivity in Scots pine microcosms in response to ectomycorrhizal (ECM) intraspecific (a) and interspecific (b) identity (LbA, LbB, LbC, LbD, *Laccaria bicolor* isolates; Sv, *Suillus variegatus*; Pi, *Paxillus involutus*; Ar, *Amanita rubescens*). Bars are absent for a productivity variable when the maximum yielding monoculture is not within a mixture. Mean ± SD is shown, and significance levels of the one-sample *t*-test for difference from zero are indicated as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

We measured significantly fewer hyphae with increasing intraspecific richness, despite there being greater ECM tips per unit root length. This observation suggests that intraspecific diversity may stimulate competition for sites of colonization on roots at the expense of extraradical hyphal production. Intriguingly, a negative linear relationship between ECM root tips per root length and nitrogen concentrations in leachate was found (Table S6). Significant variation in the utilization of nitrogen has been found in the ECM fungi *Hebeloma cylindrosporum* (Guidot *et al.*, 2005) and *Amanita muscaria* (Sawyer *et al.*, 2003), and the likelihood is that similar variation will occur in *L. bicolor*. Combining diversity–function and physiological experiments with genome comparative analyses of the fungal isolates would be a powerful approach to elucidate the importance of intraspecific variation. Resequencing the genomes of the *L. bicolor* genotypes in the experiment enabling comparative analyses of the *c.* 116 proteases involved in nitrogen utilization by *L. bicolor* (Plett & Martin, 2011) and the genes involved in nutrient transport across membranes (Lucic *et al.*, 2008) is an obvious next step.

In contrast to our hypothesis and past work (Baxter & Dighton, 2001; Jonsson *et al.*, 2001; Wilkinson *et al.*, 2011), we found that the effect of ECM fungal interspecific richness on the measured variables was less pronounced than for intraspecific richness. For example, Baxter & Dighton (2001) found shoot biomass to decrease but root biomass and plant P content to increase with increased ECM fungal diversity (up to four species) on *Betula populifolia* seedlings in axenic culture. Also in contrast to the intraspecific results, mixtures outproduced the monocultures only 10% of the time, suggesting stronger interspecific identity effects relative to richness effects on productivity. Additionally, significant identity effects on plant productivity occurred more at the interspecific than at the intraspecific level. The similarity of interspecific abundances within the mixture treatments (Table S2) further suggests that effects were driven by variation in interspecific functional traits and not simply dominance by a particular species on the root systems. Taken together, the intra- and interspecific level of ECM fungal diversity both played a role in regulating plant and fungal productivity and nutrient retention when in symbiosis with a host plant, but the effects were sometimes in opposite directions. Further, comparison of productivity between the mycorrhizal and the nonmycorrhizal systems highlight the benefits on shoot productivity, but at the expense of root productivity.

When extrapolating the experimental findings to natural forest systems, some precaution should be taken. First, we used a relatively simple system containing up to four ECM isolates or species, which is more akin to a pioneer system. In nature, the intra- and interspecific richness of ECM fungi on host trees is

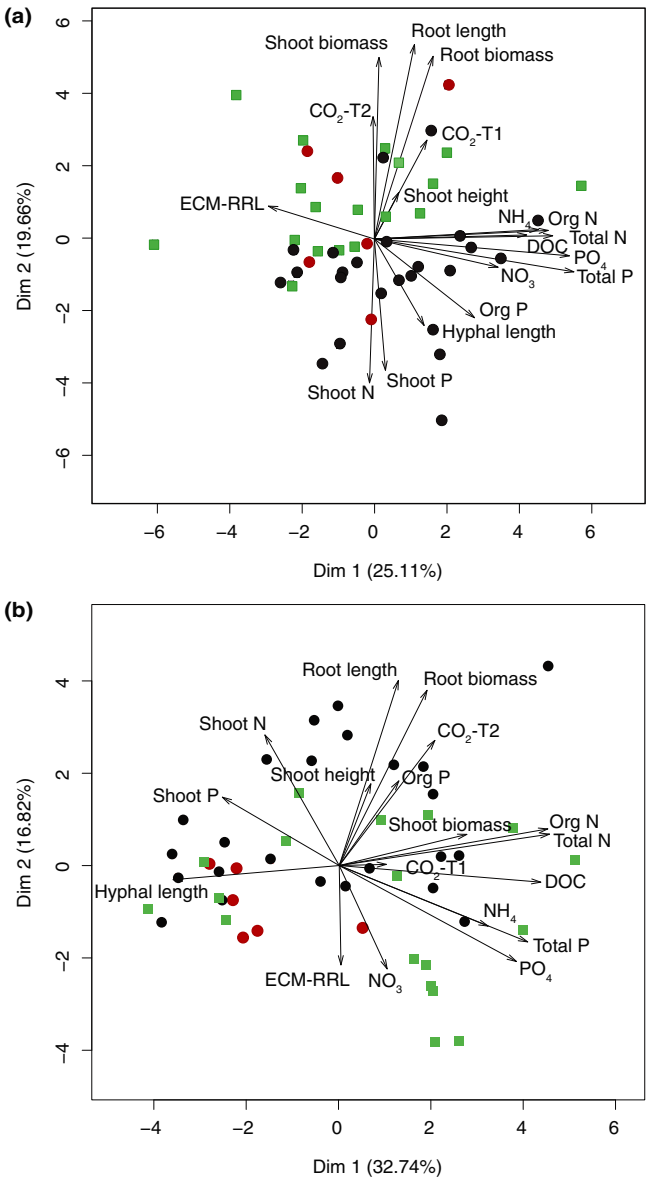

Fig. 7 Principal component analysis of intra- (a) and interspecific richness (b) (black circles, monocultures; green squares, two isolate mixtures; red circles, four isolate mixtures) of ectomycorrhizal (ECM) fungi on ecosystem multifunctionality (plant and fungal productivity (ECM-RRL, ectomycorrhizal root tips per root length), soil nutrient concentrations in leachate (Org, organic; N, nitrogen; P, phosphorus; DOC, dissolved organic carbon) and CO_2 efflux (CO_2-T1, 4 wk before harvest; CO_2-T2, 2 wk before harvest)). Vectors are shown for the variables with significant correlation to the axes (principal components). The proportion of variance explained by each axis is shown in parentheses.

considerably greater, although molecular-based estimates of the number of *L. bicolor* genotypes inhabiting individual seedlings, trees and forest soils is currently lacking. In one extreme case, 122 species and 23 *Cenococcum geophilum* internal transcribed spacer genotypes were identified on a single *Populus tremula* tree (Bahram *et al.*, 2011), while 15–19 species have been identified on individual *P. sylvestris* roots (Saari *et al.*, 2005), and several genotypes of *H. cylindrosporum* have been found on individual pine seedlings (Guidot *et al.*, 1999). Inclusion of other species at the intraspecific level in mixtures would also have allowed conclusions to be drawn from more than one fungal species, although the technical challenges of establishing hundreds of inoculated seedlings are considerable.

Second, in the experimental systems, the amount, composition and distribution of nutrients in the soil medium probably differs from many natural forest soils, where N and P concentrations are both lower and heterogeneous, which can affect the composition of ECM fungal communities (Conn & Dighton, 2000). Our microcosms were relatively nutrient-enriched, as reflected in the tissue N and P concentrations of the plants, and it has been shown that interspecific richness of ECM fungi has weaker effects on plant productivity when soil fertility is high (Jonsson *et al.*, 2001). We also found that intra- and interspecific richness had little effect on nutrient retention (as assayed through addition of nutrient-rich media to the microcosms), but there was considerable variation between individual treatments. These findings do not support the idea of complementarity in the use of organic P forms, as hypothesized in mycorrhizal systems (Turner, 2008) and based on known abilities of ECM fungi to break down inositol phosphates (Antibus *et al.*, 1992). However, such processes are more likely to be of importance under oligotrophic conditions rather than the relatively nutrient-enriched edaphic environment of our experimental systems. In addition, consideration of the balance between photosynthetic capacity and carbon availability with mineral nutrient availability may also impact plant–fungal interactions.

Third, we overcame possible 'founder effects' of using mixtures of ECM fungi inoculated onto individual plants (Leake, 2001) by assembling groupings of individual plants each inoculated with one fungus. Such an approach enables testing of hypotheses at the community (microcosm in our case) level but does not permit testing hypotheses at the level of the individual host plant.

The overall stronger effect of intraspecific richness compared with interspecific richness was reflected in the analysis of ecosystem multifunctionality. The use of correlative analyses or non-specific manipulations of soil biodiversity has shown that soil invertebrates, bacterial and fungal communities are key drivers of ecosystem multifunctionality (Wagg *et al.*, 2014; Delgado-Baquerizo *et al.*, 2016), and our study indicates that intraspecific richness of ECM fungi specifically are also important. However, biodiversity effects on ecosystem processes must ultimately consider both fungal and plant partners before general inferences to ecosystems can be made, because we know that host plant–fungal interactions can operate at both intra- and interspecific levels (Johnson *et al.*, 2012).

Conclusions

We demonstrate that the intraspecific diversity of ECM fungi affects plant and fungal productivity and nutrient retention, and supports the view that intraspecific diversity should be considered in studies of biodiversity–ecosystem functioning (Antonovics, 2003; Johnson *et al.*, 2012). Further research expanding on the mechanisms underpinning the observed effects is needed to better understand how intraspecific diversity affects plant and fungal

productivity and soil nutrient dynamics in nature. A better understanding of the links between ECM fungal traits and ecosystem functioning is required (Aguilar-Trigueros *et al.*, 2015), and may facilitate prediction of the effects of environmental change on ecosystem functioning (Koide *et al.*, 2014).

Acknowledgements

This work was funded by the Natural Environment Research Council (NE/I014527/1). We thank Prof. I. C. Anderson for scientific discussions and Dr H. Weitz for technical support. We also thank the referees for their helpful input. A.F.S.T. receives funding from the Scottish Government.

Author contributions

C.H., A.F.S.T. and D.J. planned and designed the research. C.H. performed research and data analysis. C.H., L.K. and H.D. collected data. C.H., A.F.S.T. and D.J. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Ectomycorrhizal intra- and interspecific identity effects on soil CO_2 efflux at two time points across a richness gradient.

Table S1 Plant and fungal productivity of Scots pine seedlings planted in each of the experimental microcosm treatments

Table S2 Quantification of ectomycorrhizal species on the root tips of Scots pine seedlings within the mixture microcosms based on morphotyping using a dissecting microscope

Table S3 Summary of linear mixed effects results for ectomycorrhizal intra- and interspecific richness effects on soil nutrient concentrations in leachate and CO_2 efflux

Table S4 Comparison between monocultures and nonmycorrhizal control microcosms for plant and fungal productivity, and CO_2 efflux

Table S5 Comparison between monocultures and nonmycorrhizal control microcosms for soil nutrient concentrations in leachate

Table S6 Linear regression analysis between fungal and plant productivity variables and fungal productivity and soil nutrient concentrations for ectomycorrhizal intra- and interspecific experimental data

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