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LETTER

Nitrogen availability is a primary determinant of conifer mycorrhizas across complex environmental gradients

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Abstract

Global environmental change has serious implications for functional biodiversity in temperate and boreal forests. Trees depend on mycorrhizal fungi for nutrient uptake, but predicted increases in nitrogen availability may alter fungal communities. To address a knowledge gap regarding the effects of nitrogen availability on mycorrhizal communities at large scales, we examine the relationship between nitrogen and ectomycorrhizas in part of a European biomonitoring network of pine forest plots. Our analyses show that increased nitrogen reduces fungal diversity and causes shifts in mycorrhizal community composition across plots, but we do not find strong evidence that within-plot differences in nitrogen availability affect ectomycorrhizal communities. We also carry out exploratory analyses to determine the relative importance of other environmental variables in structuring mycorrhizal communities, and discuss the potential use of indicator species to predict nitrogen-induced shifts in fungal communities.

Keywords

Deposition, fungi, indicator species, mycorrhizas, nitrogen, *Pinus,* pollution, *Russula,* symbiosis.

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INTRODUCTION

Ectomycorrhizal (ECM) fungi form symbioses with most temperate and boreal tree species, providing their hosts with soil nitrogen (N) and other nutrients, as well as increased resistance against pathogens and drought, in exchange for carbohydrates (Smith & Read 2008). These symbioses predominate in forest ecosystems where N is a major limiting nutrient (Smith & Read 2008). N deposition from anthropogenic pollutants has increased dramatically from pre-industrialized rates in many parts of the world, and this has already had major ecological impacts, including altering nutrient cycles, leaching of base cations, acidification of soils, and alterations of plant and microbial communities (Vitousek *et al.* 1997; Chung *et al.* 2007; Galloway *et al.* 2008). Even low-level N deposition can cause a significant loss of plant species diversity over several years (Clark & Tilman 2008). ECM fungal communities are known to respond to increased N availability at local scales (reviewed by Wallenda & Kottke 1998). However, there is a growing awareness that the factors influencing patterns of biodiversity are strongly determined by scale, and those variables important at local scales may not necessarily be the primary drivers of diversity at larger spatial scales (Willis & Whittaker 2002). Although factors such as competition, disturbance and nutrients are likely to be important determinants of ECM fungal communities at local scales, factors such as climate and biogeographic constraints are hypothesized to become more significant at larger scales (Lilleskov & Parrent 2007). The below-ground responses of ECM fungi to increased N availability at landscape, regional and continental scales are still unclear (Lilleskov & Parrent 2007; Smith & Read 2008).

Studies of above-ground fungal fruiting bodies have found evidence for a decrease in diversity and abundance of ECM reproductive structures over regional scale N availability gradients (Arnolds 1991), but it has been demonstrated that extrapolating these trends to below-ground responses is inaccurate (Jonsson *et al.* 2000; Peter *et al.* 2001). Most studies that specifically investigate below-ground responses of ECM fungi to N availability have been conducted at local scales, often involving single stands, or sites within a few kilometres of one another (see Table S1). Although this reduces the problem of complex gradients, which can make interpretation of results difficult, it does not

address how below-ground ECM communities respond to N deposition at larger spatial scales. Local-scale studies have sometimes shown inconsistent findings (Table S1) and the different methodological approaches used make comparing these studies extremely difficult. For example, the use of different morphological and molecular techniques for identifying fungi can lead to inconsistently defined communities (Lilleskov & Bruns 2001). In addition, studies based on N fertilization may differ from those based on long-term N deposition gradients due to the application of high levels of N over short periods of time and the absence of indirect effects such as acidification.

In this study, we use molecular techniques to assess the below-ground responses of ECM fungi to increased N availability over a geographic region of 300 000 km^2, which lies somewhere between regional and continental scales, defined here as 'intracontinental scale'. We test the hypotheses that increased N availability, assessed here through soil solution nitrate and plant tissue N concentrations, decreases ECM diversity and alters community composition both within and between forest plots. We then explore the responses of individual ECM fungi to increased N availability across plots to identify fungi that are indicators of high and low N availability. Finally, we explore the relative importance of N in comparison with other environmental parameters hypothesized to drive ECM fungal communities at intracontinental scales. Our results indicate that increased N reduces species richness and alters community composition between forest plots, but we do not find strong evidence that N affects ECM communities within plots. N availability appears to be a dominant variable influencing ECM community composition, and may be more significant than variables such as climate and soil type at the scale of this study.

METHODS

Plot descriptions

Three plots in the UK and nine plots in Germany were included in this study (Fig. 1). All plots are part of the International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests (ICP Forests); a network of *c.* 800 long-term intensively monitored plots (also known as 'Level II' plots). The forest plots included in this study are all managed, 0.25–0.3 ha, even-aged stands of mature *Pinus sylvestris* L., subject to substantial differences in N deposition levels (Table 1). We used data generated through ICP Forests monitoring activities on plot characteristics (mean temperature, precipitation, altitude, plot age, understory vegetation, throughfall N deposition), foliar chemistry (N, Ca^{2+}, Mg^{2+}, K^+, P) and soil organic layer characteristics (pH, organic carbon concentration and C : N ratio). Soil

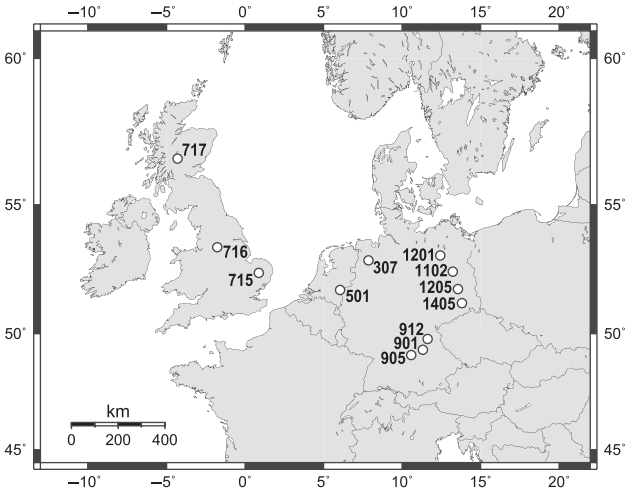


Figure 1 Locations of ICP Forests long-term monitoring plots included in the present study.

solution nitrate data were also available for 11 plots. Detailed methodology of data collection by ICP Forests is available online (http://www.icp-forests.org), where full information about the network, harmonization and quality control of data collection are also available. Details on the data are included in Table S2.

In addition to ICP Forests' data, we generated data on root N concentrations (% N content) for each transect in a plot (see below). Mean unweighted Ellenberg values were calculated for each plot as a measure of understory vegetation responses to N availability; each plant species has a value, ranging from 1 to 9, indicating the fertility conditions within which they typically occur. Original Ellenberg values were used for German plots (Ellenberg *et al.* 1992), whereas values calibrated for British vascular plants were assigned to plants in UK plots (Hill *et al.* 1999).

Sampling of mycorrhizas

Sampling of the three UK plots took place in autumn 2006, whereas the nine German plots were sampled in autumn 2008. Each tree within a Level II plot has a unique identification number. At each plot, 10 trees were randomly selected and a transect laid out to the nearest neighbouring tree. The average transect length across plots was 2.53 m, and ranged between 1.05 and 4.25 m. Along each transect, four pairs of soil cores, each 30 cm deep and 2 cm in diameter, were removed at evenly spaced intervals. Soil cores in a pair were located 10 cm apart, 5 cm to the left and right of the transect. A total of 80 soil cores were removed at each plot and all cores were analysed for ECM community composition and root N concentrations.

Table 1 Characteristics of the 12 study plots

Plot	Country	Soil type	Planting year	Precipitation total (mm^{-1} year)	Temperature mean (°C)	Altitude (m)	N deposition (kg^{-1} ha^{-1} year)
307	Germany	Haplic podzol	1949–1969	768	8.76 ± 5.96	50	28.57 ± 2.51
501	Germany	Dystric cambisol	1934	776.7	9.48 ± 5.79	50	28.48 ± 2.89
1405	Germany	Cambric arenosol	1908	646.6	8.60 ± 7.14	200	18.04 ± 0.82
716	England	Cambric podzol	1952	860.9	8.85 ± 4.99	300	17.25 ± 5.12
901	Germany	Haplic podzol	1909–1929	715.3	7.92 ± 6.96	450	16.07 ± 2.57
905	Germany	Cambric arenosol	1889–1909	774.3	7.78 ± 6.86	500	14.80 ± 1.92
1102	Germany	Dystric cambisol	1951	598	8.51 ± 7.17	100	13.81 ± 2.49
715	England	Ferralic arenosol	1967	588.4	9.58 ± 5.38	50	12.49 ± 0.70
1201	Germany	Cambric arenosol	1927	584.4	8.33 ± 6.77	100	11.22 ± 2.83
1205	Germany	Dystric cambisol	1924	548.5	8.96 ± 7.19	100	10.64 ± 0.82
912	Germany	Haplic podzol	1929–1949	700.7	7.76 ± 8.86	450	8.37 ± 0.25
717	Scotland	Gleyic podzol	1965	2351.6	5.74 ± 6.04	500	4.56 ± 1.33

Standard deviations (±) are provided for the mean daily temperature across a 30-year period, and for mean annual throughfall nitrogen (N) deposition levels between 2001 and 2006. Soil types are named according to Food and Agriculture Organization classifications.

Sample processing and DNA sequencing

Live roots were maintained at 4°C and processed within 10 days of collection to minimize degradation. Soil was removed from roots by washing in 500 μm sieves. Using a dissecting microscope, we selected the first live ectomycorrhiza encountered at the end of a severed root, from each of the four largest root fragments in each soil core, to maximize independence and minimize observer bias. From each plot, we therefore selected 320 root tips for molecular analysis. These ectomycorrhizas underwent immediate DNA extraction using 8 μL Extract-N-Amp, and following the manufacturer's protocol (Sigma, Dorset, UK).

Polymerase chain reaction (PCR) amplification was carried out using the fungal-specific primer combination ITS1f and ITS4. An aliquot of 0.5 μL of extracted DNA was combined with 4 μL of Extract-N-Amp PCR solution in an 8 μL reaction. Amplifications were performed with an initial denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 30 s, 53°C for 55 s and 72°C for 50 s, with a final extension of 72°C for 7 min. Successful PCR products were purified using ExoSAP-IT (USB, Cleveland, OH, USA). Cycle sequencing was conducted using BIGDYE v3.1 (Applied Biosystems, Foster City, CA, USA) and the resulting products were precipitated following the manufacturer's instructions for EDTA/ethanol. Sequences were analysed on an ABI Prism 3730 Genetic Analyser (Applied Biosystems) and edited with Sequencher (GeneCodes, Ann Arbor, MI, USA), before being preliminarily identified to family level using BLASTn searches on GenBank (http://www.ncbi.nlm.nih.gov/blast). Sequence alignments were subsequently generated for each family. Sequences were then assigned to a species-level grouping according to 97 or 98% sequence similarity for Basidiomycetes and Ascomycetes, respectively (Nilsson *et al.* 2008), using the furthest neighbour algorithm in DOTUR (Schloss & Handelsman 2005). Representative DNA sequences were later re-checked against the GenBank and UNITE sequence databases to assign a taxonomic name to each group, where possible.

Root N concentrations

Roots not used for molecular analysis were freeze-dried and non-ECM segments of 1–2 mm in diameter were selected under a dissecting microscope, pooled according to transect, and ground to a fine powder in a Retsch MM301 mixer mill. Ground material was subsampled and analysed for carbon and N on a Fisons NA 1500 Elemental Analyzer.

Statistical analyses

All analyses were carried out using R version 2.9.2 (R Development Core Team 2009). We carried out linear regression analyses to examine the relationship between soil solution nitrate and plant tissue N concentration, and predict soil nitrate levels in plot 501 where no soil solution data were available. Soil nitrate data were log-transformed prior to all analyses as values spanned several orders of magnitude. Relationships between N deposition levels, foliar and root N concentrations, soil nitrate, Ellenberg N and stand age were explored with Pearson's correlations, r, or when variables did not meet the normality assumptions of the test, the nonparametric Spearman's correlation coefficient, ρ.

To test whether N status influenced ECM richness, linear regressions were performed on the estimated richness of each plot against both plant tissue N concentrations and soil nitrate levels. As final sample size differed due to variable DNA sequencing success rates, total richness was estimated

using the nonparametric Abundance-based Coverage Estimator (ACE) (Chao & Lee 1992). The ACE values were log-transformed to linearize relationships with the predictor variables. We carried out global Moran's *I* tests on the residuals of the three fitted models to assess whether residuals were autocorrelated and the assumptions of the test violated. As well as testing for intracontinental-scale effects, the above analysis was repeated to test for small-scale (within-plot) effects of root N concentration on the estimated richness of transects. Regressions were also carried out to test for between-plot effects of soil nitrate and foliar and root N concentrations on Pielou's (1966) estimate of community evenness.

Partial Mantel tests, carried out in the ecodist package (Goslee & Urban 2007), were used to test for a relationship between N availability and ECM fungal communities between plots, comparing a Bray-Curtis dissimilarity matrix of fungal community composition to Euclidean distance matrices of soil nitrate, foliar N concentrations and root N concentrations. To account for differences in sample size, the Bray-Curtis matrix was based on relative abundances of each fungus (the number of root tips occupied by each fungus in a plot, divided by the total number of samples from the plot). Because the 12 plots covered a large geographic area, there is a strong possibility that, as a result of biogeographic patterns, plots located nearer to one another are more similar in ECM community composition than plots that are further away, i.e. spatially autocorrelated. In addition, stand age is thought to influence ECM communities (Termorshuizen 1991; Visser 1995). Therefore, variability caused by geographic distance and stand age were partialled out from the analysis using Euclidean distance matrices, so that the effects of N availability could be assessed independently. Mantel tests were performed to test for within-plot effects of root N concentration on the fungal community composition of transects. Distances between transects were available for six plots and here the effects of geographic distance were partialled out.

To identify the responses of different fungal taxa to N levels between plots, we carried out linear regressions between the log-relative abundances of each fungus, and both soil nitrate and plant tissue N concentrations. As we could not exclude the possibility that a fungus was absent from a plot due to dispersal barriers rather than inappropriate habitat, we only took into account abundances from plots in which a particular fungus was found to occur at least once. In addition, to provide greater statistical power and maximize the usefulness of potential indicators, we limited this analysis to include only widespread fungi, i.e. those occurring in five or more plots. To consider nonlinear relationships between ECM fungal taxa and N availability, we carried out Dufrêne–Legendre indicator species analysis (Dufrêne & Legendre 1997) to detect taxa indicative of high or low N status. Here, we again restricted our analysis to taxa occurring in five or more plots, but we included absence data. We repeated the above analyses for genus-level groups of ECM fungi.

To explore the relative importance of soil nitrate and plant tissue N concentration in structuring ECM fungal communities, we carried out unconstrained ordination with subsequent fitting of environmental variables. We used the function 'envfit' from the Vegan library (Oksanen *et al.* 2009) to identify variables that were significantly correlated with the community composition of ECM fungi. Eighteen variables relating to N availability, acidification, climate, soil type, stand age and geography were fitted as vectors or centroids (in the case of factors) to a non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis community dissimilarities between plots. The significance of correlations was assessed by comparing the r^2 fit to r^2 values generated *via* 1000 random permutations of the environmental variables. To assess the independent importance of matrices of related environmental variables with effects of other matrices removed, we performed exploratory partial Mantel analyses. This involved sequentially testing the importance of N availability (soil nitrate, foliar and root N concentrations), soil characteristics (soil-type, pH, organic carbon content), stand age, climate (mean temperature, total precipitation, altitude) and geographic distance (latitude, longitude) on ECM community composition once the effects of remaining matrices were partialled out from the analysis.

RESULTS

Mycorrhizal community structure

A total of 3840 roots were analysed, generating 3114 successful DNA sequences. Of these, 48 sequenced roots (*c.* 1.5%) yielded non-ECM fungi, and were excluded from further analyses; sequences were designated non-ECM when they matched known root endophytes or free-living soil fungi. The remaining 3066 sequences represented an average success rate of 80% across all plots, although success rate varied from 69% at plot 1205 to 84% at both plots 716 and 715. Average ECM richness within plots was 24, but actual values varied considerably between 15 fungi at 716 and 34 at 1102. Fungal accumulation curves begin to reach an asymptote at most plots indicating that sampling was generally of sufficient intensity to capture ECM diversity (Figure S1). The ACE estimates of total richness predicted that the percentage of fungi sampled from the total pool ranged from 73% at plot 901 to 100% at plots 501 and 716. Pielou's indices and rank abundance curves (data not shown) indicate a similar pattern of evenness across plots, with one or two fungi dominating, and many rare fungi. The

most dominant in terms of sample number was *Russula ochroleuca*, representing 7.7% of all samples. Two fungi that form inconspicuous hypogeous fruiting bodies, *Piloderma* sp. 1 and *Elaphomyces granulatus* were the second and third most dominant, representing 6.8 and 6.3% of all samples, respectively (see Table S3 for relative abundances of each fungus). In several German plots, *Cenococcum* sclerotia were found in abundance despite infrequent occurrence of *Cenococcum* mycorrhizas. The dominant species in plot 717 (Scotland) was an unknown Pezizalean fungus. Re-collection of live roots and sequencing of additional loci placed the fungus in a clade of Leotiomycetes not formerly known to form ectomycorrhizas. Examination of vouchers identified the fungus as the morphologically characterized 'Piceirhiza sulfo-incrustata' (Palfner *et al.* 2005; Götz Palfner, personal communication).

Relationships between soil- and plant-based measures of N

Regression analysis showed soil nitrate was positively related to both foliar and root N concentrations ($r^2 = 0.86$, $P < 0.001$ and $r^2 = 0.60$, $P = 0.005$, respectively). The two regressions predicted soil nitrate values for site 501 of 3.6 and 6.3 mg L^{-1}, respectively. As the regression between foliar N concentration and soil nitrate yielded the highest r^2 value, we present results from analyses in which only the former value is employed; however, results are similar when the alternative predicted value is used.

Relationship between N deposition and N availability

Nitrogen deposition was positively correlated with foliar N concentration ($r = 0.72$, $P = 0.008$) and soil nitrate ($r = 0.64$, $P = 0.026$), but not significantly correlated with fine root N concentration. Soil nitrate and foliar N concentration were not significantly related to stand age, but fine-root N concentration was negatively correlated with stand age ($\rho = -0.82$, $P = 0.001$). A partial correlation between N deposition levels and root N concentration, in which the effects of stand age are removed, found a strong correlation ($\rho = 0.70$, $P = 0.003$). Ellenberg N values for vascular understory plant communities were positively related to soil nitrate ($r = 0.76$, $P = 0.004$), foliar N concentration ($r = 0.67$, $P = 0.018$), and fine root N concentration ($\rho = 0.71$, $P = 0.009$).

Effect of N on mycorrhizal richness

Across plots, there was a significant negative relationship between the total fungal richness estimate (ACE) and soil nitrate and plant tissue N concentrations (Fig. 2). Moran's *I* tests for autocorrelation in the residuals of fitted models were not significant, indicating that the assumption of independence of the residuals was met. There were no significant relationships between Pielou's evenness and soil nitrate, foliar or root N concentrations. Within plots, linear regression of ACE values for transects against root N concentration showed significant patterns of increasing estimated fungal richness with increasing root N concentrations in plot 717 ($r^2 = 0.72$, $P = 0.016$). There were no significant relationships in the other 11 plots (Table S4).

Effect of N on mycorrhizal communities and individual fungal taxa

Across plots, partial Mantel tests found Euclidean distances of soil nitrate (Mantel $r = 0.51$, $P = 0.005$), root N concentration (Mantel $r = 0.45$, $P = 0.002$) and foliar N concentration (Mantel $r = 0.44$, $P = 0.004$) to be significantly correlated with Bray-Curtis community dissimilarity of ECM fungal communities when effects of stand age and geography were removed. Within plots, Mantel and partial Mantel tests (where spatial data were available) revealed no

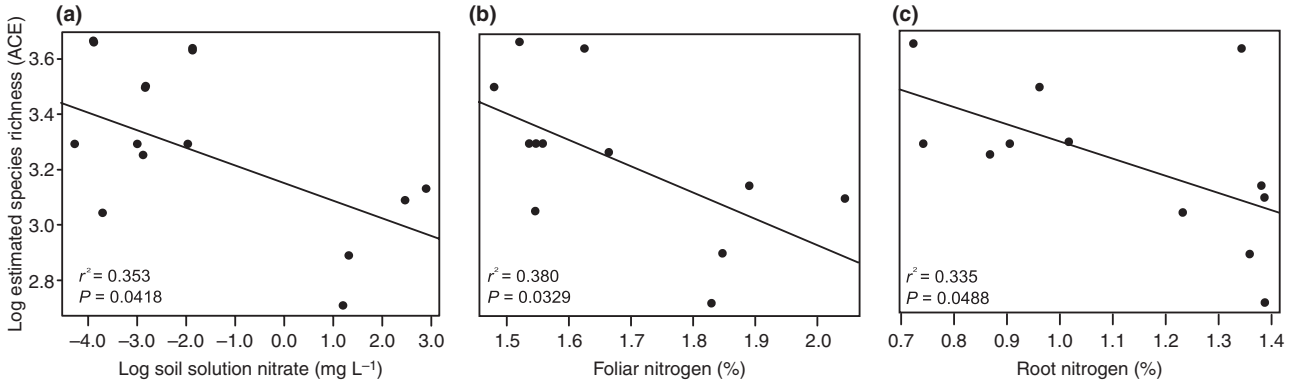


Figure 2 Regression analysis of the Abundance-based Coverage Estimates (ACE) of total fungal richness, against (a) log soil solution nitrate (mg L^{-1}), (b) foliar nitrogen (%) and (c) root nitrogen (%).

Table 2 Responses of mycorrhizal fungal abundance (log % abundances) to increasing nitrogen (N) availability

		Log soil solution nitrate		N roots		N foliar		
Response	Taxa	r^2	P	r^2	P	r^2	P	d.f.
Positive								
Species	*Russula ochroleuca*	**0.891**	**0.016**	**0.979**	**0.001**	0.731	0.065	3
	Thelephora terrestris	**0.481**	**0.038**	0.131	0.339	**0.462**	**0.044**	7
	Pezizales sp. 3	0.116	0.307	**0.424**	**0.030**	0.215	0.151	3
Genus	*Amanita*	0.216	0.207	**0.460**	**0.045**	0.172	0.268	7
	Thelephora/Tomentella	**0.646**	**0.003**	**0.402**	**0.036**	**0.368**	**0.048**	9
	Lactarius	0.356	0.053	**0.581**	**0.006**	0.251	0.117	9
Negative								
Species	*Pseudotomentella tristis*	0.168	0.493	0.0303	0.779	**0.907**	**0.013**	3
Genus	*Piloderma*	**0.52**	**0.044**	**0.628**	**0.019**	0.225	0.235	6

Only taxa present in five or more plots were assessed as potential indicators. Only taxa which display a significant relationship with N availability are shown. Values in bold are significant ($P < 0.05$).

significant correlation between Bray-Curtis community dissimilarities and differences in root N concentration between transects (Table S5).

The majority of fungal taxa were not individually tested as potential indicators because they occurred in fewer than five plots. Of the 35 included species/genera, 11 show significant responses to increases in N availability (Tables 2 and S6). *Russula ochroleuca*, *Thelephora terrestris*, Pezizales sp. 3, *Amanita*, *Thelephora/Tomentella* and *Lactarius* all responded positively to increasing N, whereas *Pseudotomentella tristis*, Cantharellaceae sp. 1, *Cenococcum* 3, *Piloderma* sp. 3 and *Piloderma* responded negatively to increasing N. *Russula ochroleuca*, *Piloderma* and *Thelephora/Tomentella* displayed statistically significant responses in both linear regression and Dufrêne-Legendre indicator species analyses.

Relative importance of N compared with other environmental variables

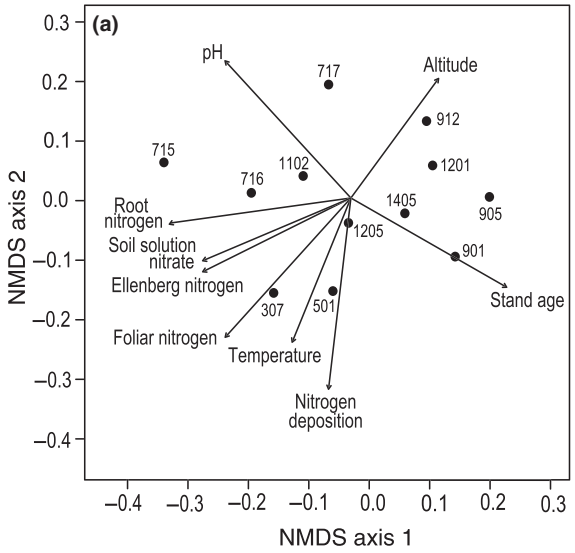
Of the 18 variables fitted as vectors to the NMDS ordination of plot community composition, nine were significantly correlated (Fig. 3). Root N concentration was the variable most closely aligned with the primary axis, which corresponds with the highest variation between plots. In addition to the five included N availability variables, soil pH, stand age, mean annual temperature and altitude were also significantly related to ECM community composition (Fig. 3).

Partial Mantel tests, in which the independent effects of matrices of related variables were sequentially tested when effects of all other matrices were removed, indicated that N availability, stand age and geography were significantly related to ECM community composition (Table 3). Of these, N availability achieved the highest Mantel r score, and stand age the lowest. Climate and soil characteristics were not significantly related to ECM composition once the effects of other variables were removed. Effects of stand age appeared largely confounded with root N concentration; partial Mantel tests, where effects of age were removed, indicated a significant correlation between ECM fungal community dissimilarity and root N concentration (Mantel $r = 0.42$, $P = 0.003$), whereas the reverse (effects of stand age when root N concentration was removed) was not significant.

DISCUSSION

This study has confronted the challenge of assessing impacts of N availability on mycorrhizas at large scales. The negative effects of increased N on fungal diversity (Fig. 2), and its impacts on community composition across plots (Fig. 3), are in agreement with other studies of below-ground ECM fungal communities (Taylor *et al.* 2000; Lilleskov *et al.* 2002a; Avis *et al.* 2003), but this study provides molecular-based evidence that N is a major factor influencing ECM communities across complex environmental gradients. These effects could have significant functional consequences at the ecosystem level; different species of ECM fungi can play important roles in acquiring N and P from distinct sources (Wallander *et al.* 1997; Lilleskov *et al.* 2002b). In addition, these transitions can have implications for below-ground food webs and processes, and they could alter carbon cycling and sequestration in forest soils – processes in which ECM fungi play a dominant role (Högberg *et al.* 2001; Godbold *et al.* 2006). Conversely, functional redundancy among ECM fungal species may buffer against significant functional changes; thus, testing for functional consequences of N-induced mycorrhizal shifts should be a priority for future research.

Only plot 717 showed a significant within-plot effect of root N concentration, but with an opposite trend to that



(b)

Variable	r^2	*P*	
Latitude	0.4405	0.0799	.
Longitude	0.4732	0.0619	.
Temperature	0.5480	0.0220	*
Precipitation	0.2692	0.2498	
Nitrogen deposition	0.8273	0.0010	***
Stand age	0.7724	0.0050	**
Foliar nitrogen	0.8196	0.0010	***
Foliar calcium	0.3305	0.1568	
Foliar magnesium	0.4118	0.0659	.
Foliar potassium	0.1490	0.5724	
Foliar phosphorus	0.5005	0.0539	.
pH	0.8080	0.0020	**
Altitude	0.5075	0.0460	*
Ellenberg nitrogen	0.6596	0.0060	**
Root nitrogen	0.8213	0.0010	***
Soil solution nitrate	0.6263	0.0030	**
Organic carbon concentration	0.2128	0.3586	
Soil type	0.6697	0.0609	.

Figure 3 (a) Fungal community compositions of the 12 plots are displayed using non-metric multidimensional scaling (NMDS). Multiple random starts were performed to avoid local minima and the percentage stress of the ordination is 14.9. The ordination is rotated so that the greatest variation between plots lies on the *x*-axis (Oksanen *et al.* 2009). Eighteen environmental variables were tested for significant correlation with community dissimilarities (b), and significantly correlated variables are shown in the plot as arrows. The length of the arrow is proportional to the strength of the correlation with the ordination, and vectors indicate the direction of the greatest change. (•: .0.1 > *P* > 0.05; *0.05 > *P* > 0.01; **0.01 > *P* > 0.001; ***0.001 > *P*).

Table 3 Partial Mantel test results, where each test relates to the independent effect of individual matrices of environmental variables on ECM community composition, when the effects of other included matrices are removed

Matrix	Component variables	Mantel *r*	*P*	Matrices partialled out
N availability	Foliar N/root N/soil solution nitrate	**0.408**	**0.006**	Soil, stand age, climate, geography
Soil	Soil type/pH/organic C content	0.116	0.172	N availability, stand age, climate, geography
Stand age	Stand age	**0.275**	**0.033**	N availability, soil, climate, geography
Climate	Temperature/precipitation/altitude	−0.017	0.478	N availability, soil, stand age, geography
Geography	Latitude/longitude	**0.330**	**0.015**	N availability, soil, stand age, climate

Values in bold are significant ($P < 0.05$).

observed across plots. This Scottish plot is noteworthy for having the lowest N deposition, greatest precipitation, lowest mean temperature and highest altitude (Table 1); this suggests that prior to a threshold level of background N availability being reached, small increases in N could increase the suite of fungi that can persist in a local area. Within the other 11 plots, we find no evidence that differences in N availability affect ECM communities, a result in agreement with previous N fertilisation studies in oak forests (Avis *et al.* 2003, 2008) which found that increased N only affected ECM communities at scales > 500 m^2. Within-plot N gradients were roughly twofold smaller than between-plot gradients, and they may not have been strong enough to influence ECM communities at within-plot scales where processes such as competition may be more significant (Koide *et al.* 2005). However, it should be noted that the inability to detect a correlation does not discount the possibility that within-plot N gradients affect ECM communities; only that we did not find evidence for a relation at the within-plot scale examined in this study. As well as the scale of sampling, reduced sample size and plant-based measurement of N within plots could influence our ability to detect fine-scale effects of N, and the presence of a relationship at one plot out of 12 could be due to chance alone. Replicated experimental studies should now be used to confirm whether N availability is a less important determinant of mycorrhizal diversity at within-plot scales.

No single fungus was detected in all 12 plots, indicative of the high spatial variability of ECM fungal communities. Over a broad geographic area, this makes the task of identifying indicator species difficult, as absence from a plot could be a result of unsuitable habitat, or dispersal limitation. Nevertheless, a number of taxa show significant responses to differences in N availability. *Thelephora/*

Tomentella spp., *Lactarius* spp. and *Piloderma* spp. display analogous responses to increasing N at both local (Lilleskov *et al.* 2002a, 2008) and intracontinental scales (Tables 2 and S6), highlighting them as strong candidates for use as indicator species. In contrast, although *Russula* and *Cortinarius* have previously been suggested as nitrophobic genera (Brandrud 1995; Peter *et al.* 2001; Lilleskov *et al.* 2002a), our results do not support this assertion. Whereas some *Russula* species, such as *R. paludosa*, appeared to decline in abundance with increasing N levels (Table S3), *R. ochroleuca* increased in abundance, consistent with previous reports of N tolerance of its sporocarp production (Arnolds 1991) and in a similar manner to *R. amoenolens* in a fertilization experiment (Avis *et al.* 2003). *Cortinarius* showed no significant relationship with N availability metrics, but had low abundances across all plots, possibly reflecting the generally high levels of N availability. *Cortinarius olivaceofuscus* was most abundant at high N plot 715, but this plot is base rich, and this species is known to be calciphilous (Hansen & Knutsen 1992); *C. olivaceofuscus* may therefore be less sensitive to elevated N, or be buffered against the acidification effects of N deposition. Responses can clearly vary between species of the same genus, highlighting the need to examine species-specific responses to elevated N. Among the more common fungi that we tested, roughly equal numbers responded positively or negatively to increased N availability (Tables 2 and S6). The overall pattern of reduced fungal diversity (Fig. 2) therefore suggests that rarer taxa may be impacted most heavily by increased N, in agreement with a fertilization study of oak forest mycorrhizas (Avis *et al.* 2008).

Unusually for surveys of below-ground fungal diversity, accumulation curves of ECM fungi were beginning to plateau at the majority of our study plots (Figure S1), suggesting that these pine plots are relatively species-poor, especially when compared to deciduous woodlands where upwards of 100 ECM fungi have been recorded (e.g. Richard *et al.* 2005; Avis *et al.* 2008). Different forest types may respond differently to increased N availability, but without additional large-scale surveys of deciduous forests, the effect of forest type on fungal responses remains unknown. The low richness of our study plots could reflect suppression of the fungal community because of cumulative N deposition effects. Alternatively, employing direct sequencing without pooling or morphotyping roots, may have avoided some of the analytical uncertainties currently inherent within other molecular methods, which could lead to over-estimation of species richness (Avis *et al.* 2008; Buée *et al.* 2009). High-throughput sequencing is likely to play an important part in future surveys of ECM fungi, but validation studies are needed (Avis *et al.* 2010); the presence of abundant sclerotia or dominant unknown ECM fungi, as was the case in this study, can strongly bias community descriptions from pooled samples. Although we collected roots from the entire soil core and did not stratify sampling by soil horizon, future studies should also consider sampling roots within individual horizons because different ECM fungi can be distributed in different horizons (Lindahl *et al.* 2007), and depths of horizons can vary across plots.

Ellenberg N values indicate that predictable shifts in understory plant species are occurring as a result of increased N, and that this is mirrored by below-ground changes in ECM fungal communities (Fig. 3); this could represent a useful warning tool for site managers. It is unlikely that shifts in understory plant communities directly cause the observed differences in ECM fungal communities, as the sampled host plant was uniform across study plots, and the majority of understory plants associate with distinct arbuscular or ericoid mycorrhizal fungi. However, it is not possible to rule out potential indirect effects of changes in competitive interactions between ECM and arbuscular or ericoid mycorrhizal fungi, which could hypothetically be altered as N availability or plant communities shift.

We utilized three measures of N availability and they all showed the same relationship with fungal communities at intracontinental scales. Soil solution nitrate data were available for only 11 of the 12 plots, and were measured at inconsistent depths across plots (Table S2), which could influence results. Foliar and root N concentrations were collected consistently; however, plant-based measures could hypothetically be influenced by differential N uptake of particular ECM fungi associated with each root system, possibly giving rise to a circular relationship between plant N concentrations and ECM communities. Nevertheless, the consistent pattern displayed by both plant and soil measures of N availability supports the finding that changes in N impact ECM fungal communities at intracontinental scales.

Variables other than N availability are also undoubtedly important determinants of fungal communities (Fig. 3). We found evidence that stand age was influencing ECM communities across plots (Table 3), but its effects appeared largely confounded with root N concentration – previous studies have shown that N availability can decrease as stands age (Prescott 1997; Bradley *et al.* 2002) and this appeared to be the case here. It is often difficult to disentangle the relative importance of direct eutrophication effects of N deposition, and indirect effects such as acidification and leaching of base cations (Lilleskov *et al.* 2002a). Soil pH was identified as a potentially important variable (Fig. 3), but neither pH nor foliar Ca^{2+} levels have the expected negative correlation with N availability (Table S2). This is probably due to the inclusion of a variety of soil types, which can vary in buffering capacity (Aber *et al.* 1998), leading us to conclude that acidification effects of N were

unlikely to be responsible for the observed relationship between N and ECM fungal communities. Nitrogen could also indirectly affect fungal communities *via* an influence on stand productivity, which is also likely to be influenced by stand age and geographic variables. Thus, the potential effects of stand productivity warrant further study.

Climate has been hypothesized as a potentially important variable structuring ECM communities across broad spatial scales (Lilleskov & Parrent 2007), and could alter edaphic factors or carbon supply from the host (Clemmensen *et al.* 2006; Craine *et al.* 2009), or differentially affect respiration rates of different ECM fungi (Malcolm *et al.* 2008). To date, limited research has been conducted on the effects of climate on ECM fungal communities (but see Gange *et al.* 2007). We found some evidence that temperature and altitude were related to ECM community composition (Fig. 3), although the independent effect of climate was not significant (Table 3). This could mean that the correlation with other removed variables masked real climatic effects, or that combining groups of related variables into a single predictive climate matrix masks the effects of individual variables.

Our findings indicate that increased N is altering ECM fungal communities at intracontinental scales, and suggest that changes in N could be altering large-scale distribution patterns of individual species. Studies to uncover baseline distributions of ECM fungi before patterns are radically altered are urgently required, and the vast network of ICP Forests plots would provide an excellent platform for this (Cox *et al.* 2010). Such work would allow future changes in ECM fungal communities to be measured and predicted, and inform ecologically relevant manipulative experiments designed to gain a mechanistic understanding of the relationship between N and changes in ECM fungal communities, and the consequences of these changes to forest ecosystem vitality.

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SUPPORTING INFORMATION

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

Figure S1 Fungal accumulation curves.

Table S1 Previous below-ground studies on ECM responses to N.

Table S2 Environmental variables for the 12 study plots.
Table S3 List of fungi and abundances.
Table S4 Within-plot regressions.
Table S5 Within-plot mantel tests.
Table S6 Dufrêne-Legendre indicator analysis.

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