



Chronic nitrogen additions fundamentally restructure the soil fungal community in a temperate forest



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ABSTRACT

Fungi dominate the microbial biomass of temperate forest soils and are a key driver of ecosystem nutrient cycling. Chronic nitrogen (N) amendments frequently cause the accumulation of soil organic matter within soils, suggesting that elevated N disrupts decomposition by altering fungal communities. To link previously observed increases in soil organic matter with potential changes in the fungal community, we assessed the effects of soil N amendment on fungal community structure at a long-term N addition experiment at Harvard Forest (Petersham, MA, USA). A decline in the relative abundance of ectomycorrhizal fungi following long-term N addition was offset by an increase in the relative abundance of saprotrophs. Species richness and diversity of ectomycorrhizal fungi declined, while ascomycetes and saprotrophs responded positively to N enrichment. However, nitrophilic species included ectomycorrhizal as well as saprotrophic fungi, especially the ectomycorrhizal *Russula vinacea*, whose relative abundance increased from 10 to 37% of the entire community across N treatments. Two decades of soil N enrichment appears to have fundamentally altered the soil fungal community of this temperate forest.

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1. Introduction

Soil fungi play key roles in ecosystem C and N cycles, as decomposers of organic matter and symbionts of plants. Fungi are the primary producers of the extracellular enzymes that break down lignin and cellulose (Schneider et al., 2012), two of the most abundant compounds in plant biomass (Dix and Webster, 1995; de Boer et al., 2005). However, fungi vary considerably in their decomposition capacities. For example, some species can fully decompose lignin, while others can only partially decompose this polymer (Fernandez-Fueyo et al., 2012). Production of the multiple enzymes involved in cellulose decay also varies among fungal species. An environmental disturbance that selectively impacts particular groups of fungi may influence the concentrations and chemical structure of lignin and other key plant constituents, and

ultimately shape rates of soil C cycling (Filley et al., 2002; Grandy et al., 2008). This is especially the case in the organic horizon of temperate forest soils where lignin is a dominant constituent whose presence limits plant litter decomposition.

Our research focuses on fungal responses to atmospheric nitrogen (N) deposition, a significant global change stressor in many parts of the world. Nitrogen deposition causes the inadvertent fertilization of historically N-limited ecosystems and has the potential to saturate biotic demand for N, causing a cascade of effects, including nitrate leaching to groundwater, enhanced trace gas emissions to the atmosphere, shifts in plant community structure, altered ecosystem carbon (C) storage, nutrient imbalances, and overall ecosystem decline. On a global basis, atmospheric N deposition has more than doubled over the last 150 years, from approximately 34 Tg N yr⁻¹ in 1860 to >100 Tg N yr⁻¹ currently (Galloway et al., 2008). Nitrogen deposition is predicted to reach global levels of 200 Tg N yr⁻¹ by 2050, with some areas of the world expected to receive N deposition rates exceeding 30–50 kg N ha⁻¹ yr⁻¹ (Galloway et al., 2008). Even now, parts of Western Europe experience rates of N deposition as high as

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25–50 kg N ha⁻¹ yr⁻¹ (total wet and dry deposition; Holland et al., 2005); these rates are likely to characterize parts of Asia as well.

In temperate forest ecosystems, long-term N deposition often results in significant accumulation of soil organic matter (Pregitzer et al., 2008; Zak et al., 2008, 2011; Janssens et al., 2010; Lovett et al., 2013; Frey et al., 2014). While the exact mechanisms causing soil C accumulation remain unclear, increasing evidence suggests this accumulation is tied to the suppression of organic matter decomposition, rather than to an enhancement of plant C inputs (Zak et al., 2008, 2011; Frey et al., 2014). Observed soil C accumulation is often associated with reduced total soil fungal biomass (e.g., Frey et al., 2004, 2014; Wallenstein et al., 2006), decreased ligninolytic enzyme activity (e.g., Deforest et al., 2004; Frey et al., 2004; Hofmockel et al., 2007; Zak et al., 2008), and, in some cases, the down-regulation of fungal ligninolytic gene expression (Edwards et al., 2011; Hesse et al., 2015).

Research on soil N enrichment and fungal community structure typically focuses on specific subsets of fungi, for example, mycorrhizal sporocarps or root tips (Arnolds, 1991; Dighton and Jansen, 1991; Egerton-Warburton and Allen, 2000; Lilleskov et al., 2001, 2002; Treseder, 2004; Parrent and Vilgalys, 2007; van Diepen et al., 2011), saprotrophic taxa (Allison et al., 2007), or lignin-decomposing basidiomycetes (Blackwood et al., 2007; Hofmockel et al., 2007; Hassett et al., 2009; Lauber et al., 2009; Entwistle et al., 2013). More holistic research has targeted short-term fertilization regimes (≤ 5 years; Weber et al., 2013; Mueller et al., 2014), or favors descriptions of entire communities and phyla over discussions of species (Hesse et al., 2015). Moreover, research often involves low sequencing depths, unlikely to comprehensively describe changes in entire soil fungal communities. Because responses measured in the early years of global change experiments can change significantly or reverse over time (e.g., Melillo et al., 2002; Janssens et al., 2010), and because species within a phylum may have different autecologies (Wolfe et al., 2012), it is critical to describe the long-term effects of environmental change manipulations at multiple levels of the taxonomic hierarchy.

We sought to comprehensively describe the fungal community in the organic soil horizon of a temperate deciduous forest exposed to long-term simulated N deposition. We conducted our work at the Harvard Forest Long Term Ecological Research (LTER) site in Petersham, MA, USA where the Chronic Nitrogen Amendment Study was established more than 25 years ago to explore effects of N deposition on local habitats (Aber et al., 1989). By performing a deep sequencing analysis of the entire fungal community, we were able to examine the effect of long-term N additions on the fungal community at different taxonomic levels (phylum, genus, OTU), and on multiple, key functional groups (saprotrophs, ectomycorrhizal fungi).

2. Materials and methods

2.1. Experimental design and sampling

The Chronic Nitrogen Amendment Study was established in 1988 in a mixed hardwood stand composed primarily of black and red oak (*Quercus velutina* and *Quercus rubra*). Soils are Typic Dystrudepts of the Gloucester series (Peterjohn et al., 1994) with no significant variation amongst the N treatments in pH or concentrations of the base cations Ca and Mg (Turlapati et al., 2012). Three 30 × 30 m plots receive one of three N treatments: no N addition (N0); 50 kg N ha⁻¹ yr⁻¹ (N50), a level of N deposition found in parts of Europe and Asia (Vet et al., 2014) and predicted for many parts of the world by 2050 (Galloway et al., 2008); or 150 kg N ha⁻¹ yr⁻¹ (N150), a treatment designed to push the system towards N saturation and serve as a space-for-time substitution, enabling

explorations of a high N ecosystem (J. Aber, personal communication). Although the experiment is sometimes criticized as pseudoreplicated, previous work at the site has confirmed clear treatment differences among plots not caused by random variation (Aber and Magill, 2004; Frey et al., 2014). Moreover, the choice to create fewer, very large plots encompassing multiple entire trees, including root systems, may be an asset to explorations of the fungal community, as larger plots may be more likely to encompass entire communities of multiple fungal individuals (Douhan et al., 2011). The rate of ambient N deposition at Harvard Forest was estimated as 8–10 kg N ha⁻¹ yr⁻¹ in 2010, down somewhat from the 10–15 kg N ha⁻¹ yr⁻¹ estimated in the early 2000s (Schwede and Lear, 2014). The N-fertilized plots are treated with an aqueous solution of ammonium nitrate applied monthly during the growing season (Aber et al., 1989). Water added from fertilizer additions is equal to approximately 0.5% of mean annual precipitation at the site. Each treatment plot is divided into 36, 5 × 5 m subplots.

We collected four soil samples (2.5 cm diameter) from the organic soil horizon (O_e and O_a layers) in each of three randomly chosen subplots within each of the three N treatment plots. Edge subplots were avoided. Samples were combined into one composite sample per subplot, resulting in three replicates per treatment. Samples were taken to the University of New Hampshire on ice and then homogenized by passing through a 2-mm sieve sterilized with ethanol. Fine roots (<2 mm) were removed from sieved soils using forceps. Soils were flash frozen in liquid N and stored at –80 °C within 6 h of sampling.

2.2. DNA extraction, amplification and 454-pyrosequencing

Our aim was to generate robust descriptions of fungal diversity and community composition across plots and so we deeply sequenced three loci. First we sequenced the internal transcribed spacer (ITS) region, designated as a barcode for fungi (Schoch and Seifert, 2012). Because sequencing of either ITS1 or ITS2 alone can give different estimates of community composition (Mello et al., 2011; Bazzicalupo et al., 2012), we amplified both loci, each independently, using primers anchored in either the 18s and 5.8s (ITS1) or 5.8s and 28s (ITS2). We next sequenced the large subunit (LSU) of basidiomycetes, because basidiomycetes are the primary decomposers of lignin, and lignin is accumulating in the N-enriched soils at our site (Frey et al., 2014). We targeted the LSU because this locus is amenable to analyses involving phylogenetic approaches (e.g. UniFrac; Lozupone and Knight, 2005); whereas, the ITS regions are not (Lindahl et al., 2013), and so the LSU provides information complementary to ITS-based analyses.

DNA was extracted from 0.75 g of soil with MoBio Powersoil RNA/DNA co-isolation kits (MoBio 12866–25, 12867–25) and purified (MoBio 12877–50). PCR amplification of the ITS1 and ITS2 regions of fungal rDNA and the LSU D2–D3 region of basidiomycete rDNA was performed with primer pairs ITS1f–5.8s (Vilgalys and Hester, 1990; Gardes and Bruns, 1993), 5.8sr–ITS4 (Vilgalys and Hester, 1990; White et al., 1990), and LR21r–LR5f (Hopple and Vilgalys, 1994; Tedersoo et al., 2008), which were combined with MID tags and 454 sequencing primers (see Table S1, Supplementary Information for full primer sequences).

High-fidelity Phusion Polymerase (New England Biolabs M0530) and 27 PCR cycles were used to minimize errors and primer bias (see Appendix S1, Supplementary Information for full PCR protocol). An equimolar amplicon library was submitted for Roche 454 sequencing in two 1/8th plate reactions at the Roy J. Carver Biotechnology Center at University of Illinois with all samples sequenced in each sequencing reaction.

2.3. Sequence processing and taxonomic and functional identification

Sequences were filtered for quality and demultiplexed using AmpliconNoise (Quince et al., 2011). AmpliconNoise was chosen after comparison with alternative denoising methods (Appendix S2, Supplementary Information; also see Gaspar and Thomas, 2013). Remaining ITS1 and ITS2 sequences were clustered into operational taxonomic units (OTUs) at 95% sequence similarity, while LSU sequences were clustered at 97% sequence similarity, using UCLUST (Edgar, 2010). We chose a 95% sequence similarity cutoff for the ITS regions to avoid over-splitting data due to sequencing errors (Jumpponen et al., 2010; Tedersoo et al., 2010) and a 97% cutoff for the LSU because this region is more conserved than either ITS region, requiring a more stringent clustering threshold (Köljalg et al., 2005). The most abundant sequence in each cluster was chosen as a representative sequence. Chimeras were removed using *de novo* UCHIME (Edgar, 2010) and Perl v5.12.4. Singleton OTUs were removed, because singleton elimination is accepted as the more conservative approach to describing fungal communities (Tedersoo et al., 2010). However, trends in diversity and community composition were the same or similar whether singletons were retained or removed (Table S2, Supplementary Information). Samples were rarefied to 820, 345, and 280 sequences, representing the minimum number of reads across samples for the ITS1, ITS2, and LSU loci, respectively. All sequence processing and subsequent analyses were performed in QIIME (Caporaso et al., 2010) unless otherwise noted.

Taxonomy was assigned by comparing representative sequences to the NCBI nt database using the BLASTn algorithm, and consensus taxonomies were generated at the genus level using MEGAN (Huson et al., 2011) with minimum support of 3, top 5% of matches, and all other parameters set to default (see MEGAN user manual for a description of these settings). Non-fungal sequences were manually removed. In order to identify the most abundant OTUs in the ITS1 and ITS2 datasets with more certainty, we compared sequences to voucher specimens in the NCBI nt database. The most abundant OTU in the ITS1 dataset represented 29.5% of sequences overall, while the most abundant OTU in the ITS2 dataset represented 20.3% of sequences. Genus level taxonomic assignments from MEGAN were checked by manual inspection of BLASTn search results for those OTUs with greater than 1% relative abundance. Moreover, whenever possible, we confirmed identification of OTUs by comparing the representative sequences to sequences derived from ectomycorrhizal root tips or cultures collected from our site (for details see Appendix S3, Supplementary Information). Genus level taxonomic assignments from MEGAN were accurate in greater than 95% of the 43 total OTUs checked in this way, including the most abundant OTU in the dataset.

Generic identifications enabled us to classify OTUs into broad functional groups, for example as saprotrophs, ectomycorrhizal fungi, or pathogens (*sensu* Tedersoo et al., 2014; see Table S3, Supplementary Information for functional classifications). While the functional roles of many fungi are poorly described, especially the functions of uncultured fungi, the approach we adopt is demonstrated as useful for the interpretation of taxonomic data (for example see Parrent and Vilgalys, 2007; Talbot et al., 2013; Voříšková et al., 2013; Štursová et al., 2014; Talbot et al., 2014; Tedersoo et al., 2014). Assignments were checked manually for accuracy. In cases where a genus can take more than one functional role, we assigned the primary function given by Tedersoo et al. (2014), but discuss alternative roles for those genera found to respond significantly to N enrichment.

2.4. Relative abundances of genera and functional groups

Relative abundances estimated from the sequencing of environmental DNA may not match relative abundances estimated by counting individuals, or measuring biomass, because of interspecific variation in ITS copy numbers and differences in PCR amplification efficiencies. However, comparisons of relative abundance generated from molecular data are informative when comparisons are made for a particular species across samples (e.g. Amend et al., 2010; Ihrmark et al., 2012) and provide ecological data not found in analyses of presence-absence data. Moreover, we have evidence that the relative abundances of ectomycorrhizal genera generated by our sequencing experiment are highly correlated with relative abundances as measured by counting the colonized root tips at our site (Fig. 1). To confirm our relative abundance estimates as robust, we compared the molecular data to root tip data. We calculated an average relative abundance for each ectomycorrhizal genus by treatment. We then used linear regression to compare these data to the average relative abundance of ectomycorrhizal genera colonizing root tips. Root tip colonization data were generated for a separate PhD thesis (by JJS) by sequencing ectomycorrhizal fungi on individual root tips (methods for root tip collection and sequencing are given in Appendix S4).

Next, we more fully explored the changes in relative abundance as measured by sequencing. In these analyses we included genera detected by at least one ITS locus at $\geq 1\%$ relative abundance, on average, across the three samples of a treatment. Genera below this threshold were excluded because quantitative comparisons of taxa with low read abundances are likely to be unreliable (Lundberg et al., 2012; Smith and Peay, 2014). Relative abundance data for various genera were generated by summing the relative abundance of each OTU within a specific genus in each sample. We also compared the relative abundance of the most abundant OTUs in the ITS1 and ITS2 datasets, and functional groups representing $\geq 2\%$ relative abundance, on average, across loci and treatments.

Tests of the relative abundances of genera and functional groups

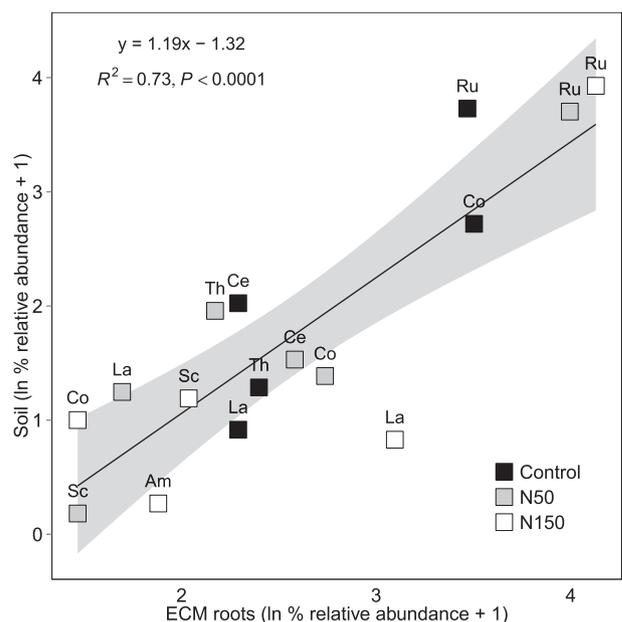


Fig. 1. Relative abundance of ectomycorrhizal (ECM) genera as measured by sequencing individual ECM root tips compared to relative abundance of ECM genera as measured by 454 sequencing of whole soil. Abbreviations: control (ambient N deposition); N50 (50 kg N ha⁻¹ yr⁻¹); N150 (150 kg N ha⁻¹ yr⁻¹); Am (*Amanita*); Ce (*Cenococcum*); Co (*Cortinarius*); La (*Lactarius*); Ru (*Russula*); Sc (*Scleroderma*); Th (*Thelephora* and *Tomentella*). The gray band is the 95% confidence interval.

were based on linear mixed-effects models in R 2.15.2 lme4 (Bates et al., 2012; R Core Team, 2012) with locus, subplot, or both as random effects, depending on the goodness of fit of full versus reduced models, and with N treatment as a fixed effect. Residuals were visually examined for normality and homoscedasticity. When these assumptions were violated, data were log or square-root transformed. Type I error was controlled using the Benjamini and Hochberg FDR procedure (Verhoeven et al., 2005). We used a similar linear mixed-effects model procedure to test for effects of N on the relative abundance of functional groups, but allowed for heterogeneous variance using the function lme in R 2.15.4 nlme (Pinheiro et al., 2014). Significant differences between means were determined using the Satterthwaite approximation and Tukey tests in R lmerTest and multcomp (Hothorn et al., 2013; Kuznetsova et al., 2013).

2.5. Community diversity and composition

To compare alpha diversity across plots, we calculated the Shannon diversity, Simpson's diversity, and OTU richness of each ITS locus for each of the three samples from each N treatment. Next, to document N effects on the species richness and diversity of ascomycetes, basidiomycetes, ectomycorrhizal fungi, and saprotrophs, respectively, the same alpha diversity metrics were calculated for each phylum and functional group individually. Differences in alpha diversity were compared using linear models fit by generalized least squares (Pinheiro et al., 2014), and significant differences were assessed using Tukey tests in R 2.15.4 nlme, because these tests allow for a heteroscedastic variance structure.

To compare community composition we calculated Bray-Curtis distances using presence-absence of ITS OTUs. This approach ignores the phylogenetic distances between ITS OTUs because the ITS regions are difficult to align across deeply divergent lineages (Lindahl et al., 2013). However, including a measure of phylogenetic distance may be useful to the extent that functional differences between fungi correlate with phylogenetic distance. Towards that end we calculated a phylogenetically based community composition distance metric, the unweighted UniFrac (Lozupone and Knight, 2005), using the basidiomycete LSU data. This metric gives an indication of the branch length that is unique to a sample relative to other samples, and provides pairwise sample distances as a proportion, similar to classical distance metrics. Unweighted UniFrac (Lozupone and Knight, 2005) was calculated for the LSU region using a neighbor-joining tree constructed in ClustalW 2.1 (Larkin et al., 2007), with *Saccharomyces cerevisiae* sequence RDN25 (www.yeastgenome.org) as the outgroup.

Statistically significant differences in community composition were determined by adonis in the R vegan package (Oksanen et al., 2013) and by Monte-Carlo simulations (Caporaso et al., 2010). Principal coordinates and Procrustes analyses were used to visualize differences across plots. To determine whether ITS1 and ITS2 provided similar estimates of community composition we tested the correlation between ITS1 and ITS2 Bray-Curtis distance estimates with Mantel tests (Caporaso et al., 2010). To aid in interpretation of the LSU unweighted UniFrac, we regressed family level relative abundance from the LSU data against the principal coordinate axes using the envfit function in the R vegan package, and plotted those families that had significant correlations.

3. Results

3.1. Sequence processing and taxonomic and functional identifications

Following denoising, quality control, and the removal of chimeras and non-fungal sequences, we obtained 16,150 ITS1

sequences, 8,164 ITS2 sequences, and 11,681 LSU sequences (see Appendix S5, Supplementary Information for details on the filtered, low-quality sequences). We generated an average of 1794, 907, and 1298 sequences per sample (9 samples = 3 N treatments × 3 plots per treatment) for the ITS1, ITS2, and LSU, respectively. Clustering at a 95% (ITS) or 97% (LSU) similarity threshold resulted in a total of 575, 485, and 155 OTUs for the ITS1, ITS2, and LSU, respectively, with 0.4–12% of sequences forming singleton OTUs. The number of non-singleton OTUs used in final analyses totaled 351, 313, and 100 for the ITS1, ITS2, and LSU, respectively.

High-throughput bioinformatics, particularly denoising (Gaspar and Thomas, 2013), sequence clustering (Nilsson et al., 2008, 2011), and the subsequent choice of a representative sequence used as a comparison with public databases, may cause one of several minor variants to be chosen as the true sequence. By comparing our sequences to sequences obtained from ectomycorrhizal root tips or fungal cultures isolated from the same treatment plots, we confirmed the identification of 58 OTUs, including OTUs from three genera subsequently identified as increasing in relative abundance with added N.

The ITS1 and ITS2 markers provided similar results, as regards to the broad effects of N fertilization on fungal community composition (Table S4, S5), but the two markers gave different estimates of relative abundance of different taxa (Table S3), confirming the utility of using multiple loci to describe fungal communities (Mello et al., 2011; Monard et al., 2012). The ITS1 locus was biased towards detection of basidiomycetes, and the ITS2 was biased towards ascomycetes—81% of sequences detected by ITS1 were identified as basidiomycetes versus 55% for ITS2 (ANOVA $P < 0.001$), while 38% of ITS2 sequences were identified as ascomycetes versus 15% for ITS1 (ANOVA $P < 0.001$).

The total number of 664 OTUs we identified using ITS1 and ITS2 sorted into 158 fungal genera or groups (clusters of OTUs that could not be assigned to a genus with confidence are named as groups). Thirty-one genera or groups were unique to ITS1, 77 to ITS2, and 50 genera or groups were found using both loci. A complete list of genera and their functional classifications is provided in the Supplementary Information (Table S3). In terms of sequence abundance, 19 genera or groups, each representing >1% relative sequence abundance when averaged across N treatments, comprised 80% of the total sequences (highlighted in Table S3, Supplementary Information). Of the 158 genera/groups identified, 18 were classified as ectomycorrhizal (21% of all identified OTUs), 74 as saprotrophic (49% of all OTUs), and 29 as plant pathogenic (12% of OTUs). We were able to assign 87% of genera to a specific ecological role, which translates to 89% of OTUs and 68% of sequences (Table S3, Supplementary Information).

3.2. Comparing relative abundances of 454 sequences and root tips

The relative abundances of ectomycorrhizal fungi in the organic horizon detected by 454 sequencing were highly correlated with relative abundances detected by Sanger sequencing of ectomycorrhizal root tips collected in the field from the same plots (Fig. 1; $R^2 = 0.73$, $P < 0.0001$), suggesting our sequencing of environmental samples provides an accurate description of species' abundances in soil.

3.3. Changes in the relative abundances of different phyla and functional groups

Basidiomycetes dominated the fungal community in all treatment plots, ranging from 63 to 71% of relative sequence abundance across the N treatment gradient (Fig. 2A). Ascomycetes averaged 26% across treatments. Other phyla (Chytridiomycota,

Mucoromycotina, Neocallimastigomycota) each made up <5% of identified sequences. Sequences that had no match in the NCBI database represented <2% of the total. Members of the orders Russulales (45%) and Agaricales (14%) were the most abundant across the treatment plots.

There were no significant N treatment differences in relative abundance of ascomycetes or basidiomycetes (Fig. 2A). However, the relative abundances of broad functional groups (*sensu Tedersoo et al., 2014*) did change significantly across the N addition treatments (Fig. 2B). Ectomycorrhizal fungi were dominant in all N treatments, representing 59–72% of all sequences, a finding typical for the organic horizon of forest soils (Baldrian et al., 2012; Weber et al., 2013; Talbot et al., 2014). However, their relative abundance declined from 72% in the control treatment to 62% and 59%, in the N50 and N150 treatments, respectively. In contrast, the relative abundance of saprotrophic fungi increased significantly, changing from only 18% of total sequences in the control treatment to 28% in the highest N addition treatment (Fig. 2B). Saprotrophic basidiomycetes, a subset of the total saprotrophic functional group, also increased in relative abundance under N enrichment, resulting in no apparent change in basidiomycete relative abundances (Fig. 2A). Plant pathogens made up 1–3% of sequences in the control and N50 treatments and increased to 5% of sequences in the N150 treatment (Table S3, Supplementary Information).

3.4. The discovery of nitrophilic organisms

The ectomycorrhizal genus *Russula* made up 40–50% of sequences across N treatments (Fig. 3). The ectomycorrhizal genus *Cortinari* was the second most abundant genus (6% abundance on average across N treatments), with remaining genera comprising <5% of total sequence abundance. Eight genera, representing 16% of all OTUs, underwent significant changes in relative abundance in response to N addition (Table 1). The ectomycorrhizal genus *Cenococcum* declined significantly with N fertilization, while

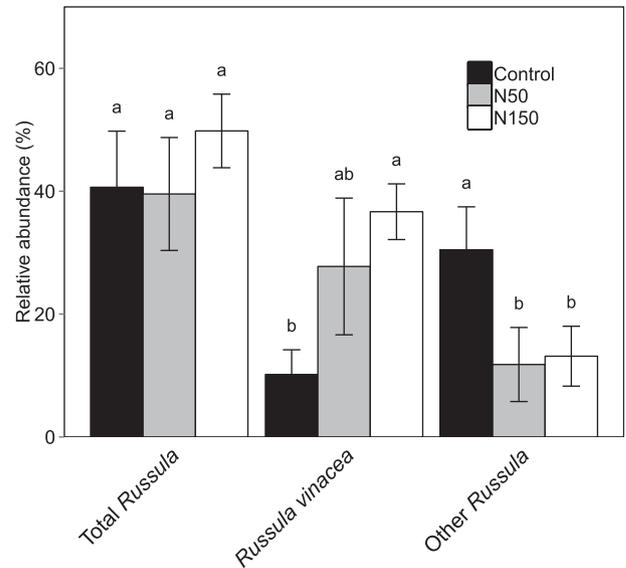


Fig. 3. Relative sequence abundance of *Russula* calculated for the entire genus (total *Russula*), for *R. vinacea* on its own, and for all OTUs of *Russula* but without *R. vinacea* (other *Russula*). Relative abundances are average estimates from ITS1 and ITS2. Error bars are standard errors. Different letters denote significant differences within a group across N addition treatments.

Scleroderma and *Rhizoscyphus* increased. Three saprotrophic taxa, all ascomycetes, also increased under the highest N addition: *Hypocrea*, *Phialophora*, *Trichophyton*.

While there were no significant treatment differences in sequence abundance for the genus *Russula* as a whole (Fig. 3), the most abundant OTU in our dataset, identified as *Russula vinacea*, increased from 10% relative abundance in the control treatment to 28–37% in the N-fertilized plots. This was the largest change

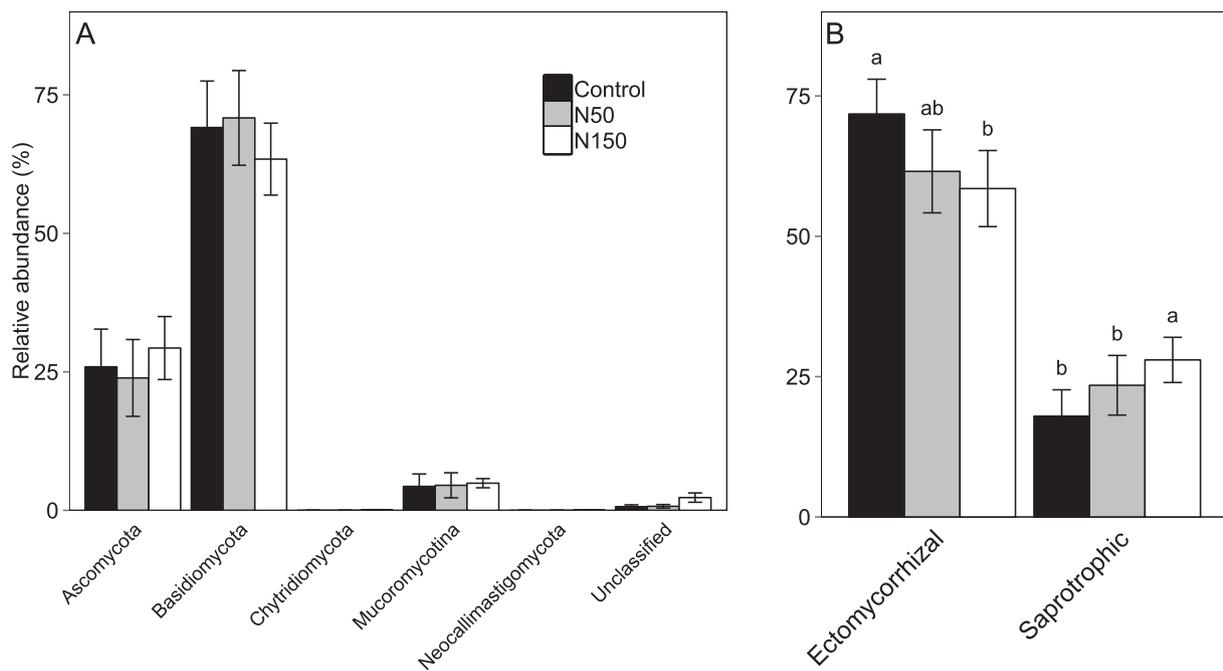


Fig. 2. Relative sequence abundance of (A) fungal phyla and (B) broad functional groups (ectomycorrhizal or saprotrophic fungi). Relative abundances are an average of estimates from ITS1 and ITS2 to account for differences in detection of fungal taxa between the loci. Error bars are standard errors. Different letters denote significant differences within a functional group across N addition treatments. The phyla Chytridiomycota and Neocallimastigomycota were detected in the N150 treatment at 0.06% and 0.04% of relative sequence abundance, respectively, and were not detected in the control or N50 treatments.

Table 1

Fungal genera showing significant differences in relative abundance in response to chronic N addition. 'Control', 'N50', and 'N150' represent the N addition level (0, 50, or 150 kg N ha⁻¹ yr⁻¹). Values followed by different letters within a genus are significantly different. Standard errors are presented in parentheses. Ecological information is derived from Alexopoulos et al. (2002), Cairney and Chambers (1999), Cannon and Kirk (2007), and listed references.

Phylum	Genus	Relative abundance (%)			Ecological summary
		Control	N50	N150	
Ascomycota	<i>Cenococcum</i>	6.6 (3.5) ^a	3.6 (1.9) ^a	0.3 (0.3) ^b	Ectomycorrhizal; broad host range; cosmopolitan distribution (Jany et al., 2002)
Ascomycota	<i>Hypocrea</i>	0.6 (0.2) ^b	0.3 (0.1) ^c	2.3 (0.4) ^a	Saprotrophic, cellulolytic; cosmopolitan distribution (Liu et al., 2012)
Ascomycota	<i>Phialophora</i>	0.2 (0.1) ^b	0.2 (0.1) ^b	1.5 (0.7) ^a	Saprotrophic or biotrophic, cellulolytic, xylanolytic, proteolytic; commonly associated with roots, cosmopolitan distribution (Caldwell et al., 2000)
Ascomycota	<i>Rhizoscyphus</i>	0.8 (0.6) ^b	1.0 (0.4) ^b	2.7 (1.4) ^a	Ericoid mycorrhizal; root endophyte or ectomycorrhizal, proteolytic, cellulolytic, and capable of partial lignin degradation; cosmopolitan distribution (Read et al., 2004; Grelet et al., 2010)
Ascomycota	<i>Trichophyton</i>	0.2 (0.1) ^b	0.3 (0.2) ^b	0.9 (0.5) ^a	Saprotrophic in soil, decomposing keratin; dermatophytic
Ascomycota	<i>Verticillium</i>	0.2 (0.1) ^b	0.7 (0.4) ^{ab}	1.0 (0.5) ^a	Plant pathogen; parasite of insects or nematodes
Basidiomycota	<i>Agaricus</i>	0.5 (0.3) ^{ab}	4.1 (2.5) ^a	0.1 (0.1) ^b	Saprotrophic, ligninolytic (Bonnen et al., 1994)
Basidiomycota	<i>Scleroderma</i>	0.0 (0.0) ^b	0.2 (0.1) ^b	2.3 (1.3) ^a	Broad host range, ectomycorrhizal, may be important in organic phosphorous acquisition (Antibus et al., 1991)

recorded for any single OTU. This OTU represented 29.5% and 20.3% of sequences in the ITS1 and ITS2 datasets, respectively, whereas the second most abundant OTU in these datasets represented 6.3% and 7.0% of sequences, respectively. Identification of the OTU as *R. vinacea* was based on a match to sequences from ectomycorrhizal root tips, also isolated from our study site (>99% similarity) and a match to GenBank accession EU598181 (99% sequence similarity) which was confirmed as *R. vinacea* by B. Buyck (pers. comm.; ITS1 OTU 691, ITS2 OTU 281 in Table S6, Supplementary Information). The increased abundance of *R. vinacea* in N-amended soils appears to have occurred at the expense of other *Russula* species, which as a group declined by more than 50% in response to both levels of N addition (Fig. 3).

3.5. Community diversity among treatments

Simpson's diversity, a measure of the probability that two randomly chosen individuals (in our analyses, sequences) represent different species, averaged 0.84 across treatments, suggesting a relatively even distribution of species within samples across the three plots despite the dominance of a single OTU (*R. vinacea*). The levels of fungal diversity (Fig. 4) and evenness across plots are consistent with recent high-throughput sequencing analyses of the soil fungal community (e.g., Baldrian et al., 2012). There was a significantly higher taxonomic richness in the highest N treatment (N150), compared to control and N50 treatments (Fig. 4A). This overall increase in richness was driven largely by an increased number of ascomycete OTUs; the number of basidiomycete OTUs was not different across N treatments. OTU richness of the ectomycorrhizal taxa declined in the highest N treatment (Fig. 4A), concomitant with a decline in Shannon diversity (Fig. 4B) and relative sequence abundance (Fig. 2B). As with relative abundances, the lack of apparent change in basidiomycete diversity and decline in ectomycorrhizal diversity corresponds to an increase in the diversity of saprotrophic basidiomycetes with N addition. The number of saprotrophic taxa, many of which are ascomycetes (Table S3, Supplementary Information), was significantly higher in the N150 compared to the control and N50 treatments; estimates of the Shannon diversity of saprotrophs and ascomycetes were also significantly higher in the N150 treatment (Fig. 4B).

3.6. Community composition among treatments

The fungal community as a whole, as measured by ITS, was significantly different among N addition treatments (Fig. 5A). The ITS1 and ITS2 markers gave somewhat different results with ITS1 suggesting that total fungal community composition was significantly different among all three treatments and ITS2 suggesting a significant result only for the highest N level (N150). Loadings of log-transformed OTU sequence abundance indicated that differences between the treatments were driven in part by ectomycorrhizal basidiomycetes, including *Russula* and *Cortinarius*, and saprotrophic ascomycetes (Fig. S1, Supplementary Information). When ascomycetes and basidiomycetes were plotted separately, the basidiomycetes showed stronger separation across treatments (Fig. 5B and C), with both N addition treatments separating from the control, particularly for the ITS1 region. This result is likely caused by a shift in composition of ectomycorrhizal basidiomycetes (Fig. 5D). The saprotrophic component of the community exhibited less clear separation with only the ITS2 locus detecting a separation of the highest N treatment (N150) from the control and the N50 treatments (Fig. 5E).

An unweighted UniFrac analysis of the basidiomycete component of the community, based on LSU data, suggests a significant effect of N addition on basidiomycete community composition (Fig. 5F), with different communities in all three levels of N fertilization. The relative abundance of the ectomycorrhizal family Cortinariaceae was significantly associated with the control treatment (N0), while the ectomycorrhizal Russulaceae was associated with the highest N treatment (N150).

4. Discussion

Here we demonstrate that more than twenty years of N additions have caused fundamental changes in the relative abundances, diversity, and community composition of soil fungi in a temperate hardwood forest. Long-term N enrichment caused a significant decline in the relative abundance and diversity of ectomycorrhizal taxa, which dominate the forest floor under ambient N deposition, with a concomitant increase in the diversity of ascomycetes and the relative abundance of several saprotrophic ascomycete taxa. We discovered a nitrophilic species, *R. vinacea*, whose relative abundance more than tripled under N fertilization. These results build on previous work at our site showing that chronic N additions

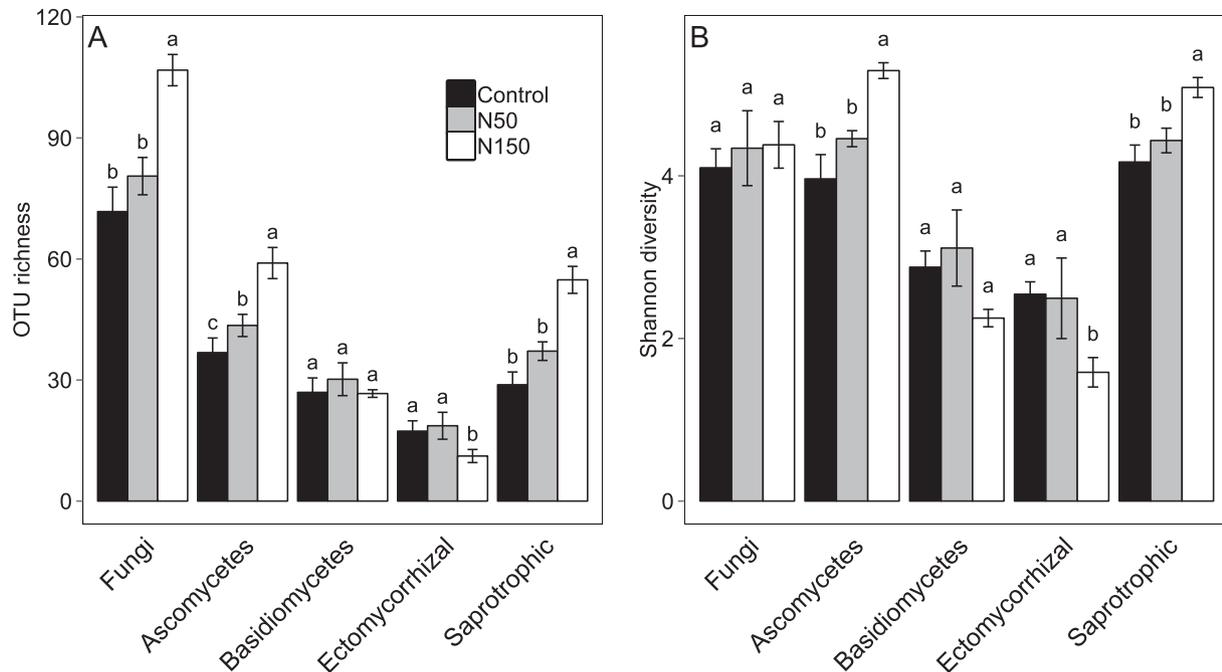


Fig. 4. Differences in (A) OTU richness and (B) Shannon diversity of fungi, ascomycetes, basidiomycetes, ectomycorrhizal fungi, and saprotrophic fungi across N treatments. Error bars are standard errors. Richness estimates are averages of estimates calculated from ITS1 and ITS2. Letters indicate significant differences between treatments.

significantly reduce total fungal biomass (Frey et al., 2004, 2014) and ligninolytic enzyme activity (Frey et al., 2004). Although there have been numerous studies examining the effects of N deposition on soil fungi, ours is the first to comprehensively describe the long-term (>20 yr) effects of soil N enrichment on multiple levels of fungal biodiversity (phylum, genus, OTU, functional groups, community-level).

Ectomycorrhizal responses were taxon specific, with N-enrichment disfavoring *Cortinarius* species and most *Russula* species, while significantly enhancing the relative abundance of *R. vinacea*. The general decline in ectomycorrhizal taxa was unlikely associated with reduced root biomass, as that was unaffected by N-enrichment at our site (Frey et al., 2014). The observed increase in *R. vinacea* abundance is consistent with work at the same experimental plots showing that the proportion of ectomycorrhizal tree roots colonized by *R. vinacea* doubles in response to N addition, with this species colonizing about 70% of all root tips analyzed in the highest N treatment (J. Sadowsky, unpubl. data). We have thus identified *R. vinacea* as a nitrophilic species which positively responds to soil N enrichment and dominates the ectomycorrhizal community following long-term N fertilization. This confirms previous observations of *Russula* species as having the potential to grow well in high N soils, although *R. vinacea* is not in the *Russula* clade currently defined as nitrophilic (Avis, 2012). *Russula* species are known to have variable responses to N, although it is unclear why some species have nitrophilic tendencies while others decline or are even unable to persist in N enriched environments (Avis et al., 2003; Lilleskov et al., 2011; Avis, 2012). One of the primary roles of ectomycorrhizal fungi is to transport N to hosts in exchange for plant C, and ectomycorrhizal fungi may be excluded from symbiosis under conditions of high N availability as plants allocate less photosynthate C to N acquisition structures (Wallenda and Kottke, 1998). The increase in the relative abundance of *R. vinacea* in response to N fertilization suggests that this species is either able to maintain symbiosis by conferring benefits other than N acquisition to its host (e.g., P acquisition), is a parasite, or is able to forage for its own soil C resources. The responses of both the Russulaceae

and Cortinariaceae (Fig. 5F), combined with previous observations that fungi in these families access different kinds of N resources (Hobbie et al., 2013), identify these fungi as important targets for future research.

Chronic N additions also led to significant changes in the structure of the less abundant (non-mycorrhizal) component of the community, including an increase in ascomycete diversity, the abundance of several ascomycete genera (e.g., *Trichophyton* spp., *Phialophora* spp., *Hypocrea* spp., *Rhizoscyphus* spp.), and the number and diversity of saprotrophic taxa, many (but not all) of which are ascomycetes. Previous studies have shown that N enrichment increased ascomycete richness in a temperate pine stand (Weber et al., 2013) and increased ascomycete abundance in a spruce boreal forest (Allison et al., 2007), suggesting that these trends are consistent across forest types and ecosystems. The genera that increased in abundance in our study have differing capacities for decomposition of soil organic matter (Table 1), and shifts in their abundance may represent shifts in dominant fungal species among different groups of decomposers (Moorhead and Sinsabaugh, 2006). The increase in relative abundance of saprotrophic ascomycetes with soil N enrichment lends support to the hypothesis that N additions may change the outcome of competition between functional groups by favoring growth and competitive ability of fast-growing, N-tolerant copiotrophs (Ågren et al., 2001), taxa that may have a lower capacity for efficient lignin degradation. This type of mechanism has been proposed to operate in the structuring of bacterial communities in N-enriched soils, with N enrichment favoring copiotrophs and disfavoring oligotrophs that degrade relatively recalcitrant organic matter (Fierer et al., 2012). However, it is also possible that N-tolerant fungi come to dominate the community simply because fungal taxa less able to withstand high N levels decline.

Although our study was based at a particular site and in the specific context of Harvard Forest's long-running Chronic Nitrogen Amendment Study, commonalities with previously published work are abundant, and point to generalities. These are: (1) most ectomycorrhizal fungi decline in response to N enrichment, but there

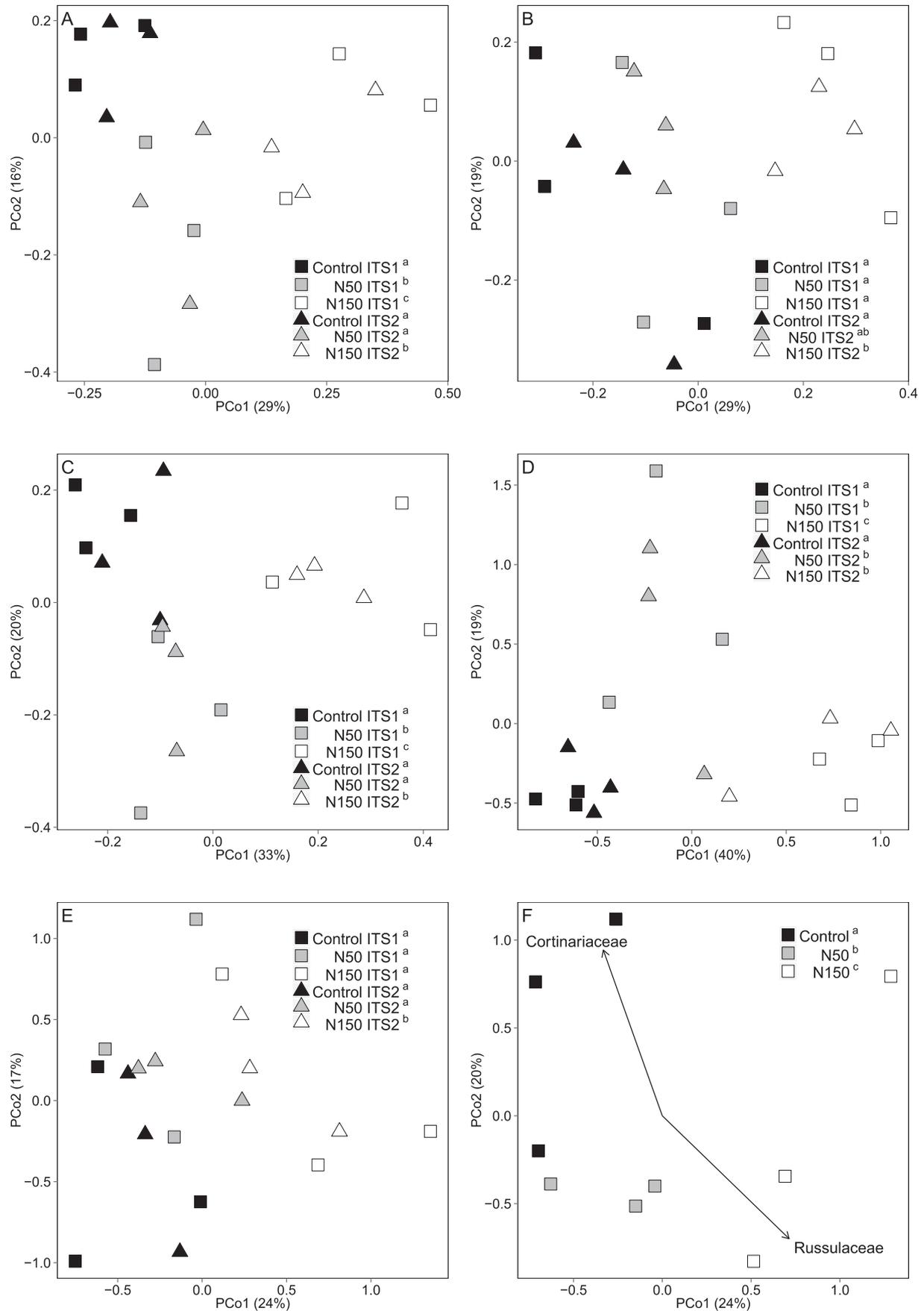


Fig. 5. Species composition across N treatments for (A) fungi, (B) ascomycetes, (C) basidiomycetes, (D) ectomycorrhizal fungi, and (E) saprotrophs using ITS1 and ITS2 data, or unweighted UniFrac for (F) basidiomycetes using LSU data. Treatment designations in the legend followed by different letters in superscript indicate significant differences between treatments. Vectors in panel F represent the strength and direction of the correlation between family level relative abundance from the LSU data and the first two principal coordinate axes from unweighted UniFrac. Only significant correlations are plotted as vectors.

are nitrophilic taxa that come to dominate the ectomycorrhizal community following long-term N additions; and (2) N additions increase the diversity and abundance of those saprotrophic ascomycetes whose primary niche appears to be cellulose decomposition, over saprotrophs capable of degrading more recalcitrant C compounds. These fungal responses to soil N fertilization are associated with a suppression of organic matter decomposition overall and a significant accumulation of soil C (Pregitzer et al., 2008; Zak et al., 2008, 2011; Frey et al., 2014) that has a higher relative abundance of lignin (Frey et al., 2014). In the aggregate, fungal responses to N enrichment are likely to play a critical role in altering soil nutrient cycling.

Data accessibility

- DNA sequences from 454 sequencing: NCBI Sequence Read Archive accession number SRP022908.
- DNA sequences from Sanger sequencing of ectomycorrhizal root tips: GenBank accession numbers KP348013–KP348282.

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Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.funeco.2016.05.011>.

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