ECOSYSTEM ECOLOGY - ORIGINAL RESEARCH

Ectomycorrhizal fungal succession coincides with shifts in organic nitrogen availability and canopy closure in post-wildfire jack pine forests

Stephen D. LeDuc · Erik A. Lilleskov ·
Thomas R. Horton · David E. Rothstein

Abstract Successional changes in belowground ectomycorrhizal fungal (EMF) communities have been observed with increasing forest stand age; however, mechanisms behind this change remain unclear. It has been hypothesized that declines of inorganic nitrogen (N) and increases of organic N influence changes in EMF taxa over forest development. In a post-wildfire chronosequence of six jack pine (*Pinus banksiana*) stands ranging in age from 5 to 56 years, we investigated EMF community composition and compared shifts in taxa with detailed soluble inorganic and organic N data. Taxa were identified by internal transcribed spacer rDNA sequencing, and changes in community composition evaluated with non-metric multi-dimensional scaling (NMDS). Dissimilarities in the community data were tested for correlations with N variables. We observed a successional shift along NMDS axis 1 from such taxa as *Suillus brevipes* and *Thelephora terrestris* in sites age 5 and 11 to species of *Cortinarius* and *Russula*, among others, in the four older sites. This change was positively correlated with soluble organic N (SON) ($r^2 = 0.902$, $P = 0.033$) and free amino-acid N ($r^2 = 0.945$, $P = 0.021$), but not inorganic N. Overall, our results show a successional shift of EMF communities occurring between stand initiation and canopy closure without a change in species of the dominant plant–host, and associated with SON and free amino-acid N in soil. It is uncertain whether EMF taxa are responding to these organic N forms directly, affecting their availability, or are ultimately responding to changes in other site variables, such as belowground productivity.

Keywords Ectomycorrhiza · Fungal diversity · Pinus banksiana · Organic nitrogen · Disturbance

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S. D. LeDuc · D. E. Rothstein
Department of Forestry and the Ecology, Evolutionary Biology and Behavior Program, Michigan State University, 126 Natural Resources, East Lansing, MI 48824, USA

Present Address:
S. D. LeDuc (✉)
US Environmental Protection Agency, Ariel Rios Bldg, 1200 Pennsylvania Ave., NW, 8623P, Washington, DC 20460, USA
e-mail: leduc.stephen@epa.gov

E. A. Lilleskov
U.S. Department of Agriculture Forest Service, Forestry Sciences Laboratory, 410 MacInnes Dr., Houghton, MI 49931, USA

T. R. Horton
Department of Environmental and Forest Biology, State University of New York–College of Environmental Science and Forestry, 1 Forestry Drive, Syracuse, NY 13210, USA

Introduction

Studies of changes in community composition over forest development have primarily focused on aboveground biota, yet ecologists increasingly realize that above and belowground communities are intimately linked (Wardle et al. 2004). Despite this realization, we have a poor understanding of how soil microbial communities may change after disturbance and subsequently with forest stand age, and the driving factors behind any shifts in composition. Given their intimate symbiosis with plant roots, changes in

mycorrhizal fungi can affect ecosystem structure and functioning (van der Heijden et al. 1998), and studies have shown shifts in the composition of ectomycorrhizal fungi (EMF) with forest age following a disturbance event (Visser 1995; Twieg et al. 2007). Variation in the ability to disperse or persist in the soil and quickly colonize root tips via spores or other propagules likely determines the initial composition of the EMF community immediately following disturbance (Deacon and Fleming 1992; Taylor and Bruns 1999; Lilleskov and Bruns 2003). As forests age, however, these fungi are either completely replaced by other EMF taxa or decline in abundance (Visser 1995; Twieg et al. 2007), and the underlying mechanisms behind this successional shift remain unclear.

One characteristic that changes profoundly with stand age, soil nitrogen (N) availability, has been shown to be a potent modifier of the EMF community (Taylor et al. 2000; Lilleskov et al. 2002a; Cox et al. 2010), and it has been hypothesized that declines of inorganic N and concomitant increases of organic N may drive changes in EMF taxa during stand development (Abuzinadah and Read 1986; Finlay et al. 1992). Inorganic forms of N, i.e., ammonium (NH_4^+) and nitrate (NO_3^-), are typically elevated in young, recently disturbed stands (reviewed in Wan et al. 2001). By contrast, organic forms of N, such as amino acids, proteins and chitin, are thought to predominate in older stands with the accrual of organic horizons (Read 1991). EMF taxa have been shown to increase free amino-acid N uptake for their plant–host (Plassard et al. 2000; Boukcim and Plassard 2003), and similar to inorganic N, differences may exist between EMF taxa in uptake rates (Finlay et al. 1992; Jones et al. 2009). Some EMF taxa can also directly access more complex forms of organic N, such as protein N, but others cannot—or have a limited ability to do so (Abuzinadah and Read 1986; Finlay et al. 1992; Smith and Read 1997; Lilleskov et al. 2002b). Lilleskov et al. (2002b), for example, found that *Laccaria bicolor* and *Lactarius theiogalus*, common at high inorganic-N sites, grew poorly on protein in pure culture, while other species, such as *Cenococcum geophilum* and *Tricholoma inamoenum*, found at low inorganic-N sites, grew well. Regulatory mechanisms within host plants, such as higher mortality rates of root tips colonized by poor versus good mutualists or the reduced supply of carbohydrates to relatively poor mutualists, could result in a shift from fungal taxa with a high affinity for inorganic N early in stand development to those better able to acquire organic forms of N in older stands (Hoeksema and Kummel 2003; Kiers et al. 2011).

A few previous studies have evaluated in situ relationships between EMF succession and inorganic N and total soil N, but not forms of available organic N. Wallander et al. (2010) found a negative relationship between NH_4^+ concentrations and fungal biomass in younger, but not

older, sites, and did not report finding any relationship between these concentrations and shifts in EMF diversity. Twieg et al. (2009) found no association between successional changes in EMF taxa and total soil C to N ratios, extractable inorganic N and potentially mineralizable N. The lack of relationship between EMF taxa and total N may not be surprising, however, since total N includes both organic and inorganic forms and varies widely in its availability to soil microorganisms. In contrast, EMF taxa may respond to shifts in more available pools of organic N. A laboratory test of protein use yielded mixed results for EMF species associated with differing stages of stand development (Finlay et al. 1992). Despite this finding, however, changes in EMF taxa and plant–mycorrhizal available organic N have never been followed over a single, successional sequence even with the recognition that these fungi play a large role in N cycling (Schimel and Bennett 2004).

Alternatively to N, other edaphic or host changes over the course of stand ontogeny could favor a successional shift in EMF taxa. Deacon and Fleming (1992) suggested that differences in life history strategies and photosynthate requirements between fungi could explain changes in EMF community composition with stand age. Colonization by hyphae from living roots might become more important with stand age, for example, conferring a competitive advantage to fungi that invest in mycorrhizal networks (Deacon and Fleming 1992). Peay et al. (2011) recently suggested that ectomycorrhizal exploration types (Agerer 2001) may explain successional dynamics, arguing that long-distance types should be favored in young stands with low root densities, while short-distance types should be favored in older stands with higher root densities. In such a case, N availability may not be the primary influence on the EMF community shift between young and old stands.

The jack pine (*Pinus banksiana*) forests of northern Lower Michigan, USA provide an ideal setting to examine potential changes in EMF community composition with stand age and N availability. These forests exhibit frequent disturbance with minimal confounding variation in climate, topography, soil or vegetation-type. In particular, this allows the dominant plant–host species to be held constant through time. Previous work in these stands has characterized temporal changes in not only inorganic N dynamics (Yermakov and Rothstein 2006; LeDuc and Rothstein 2007; LeDuc and Rothstein 2010) but also easily soluble organic N, including free amino acids (LeDuc and Rothstein 2010). Moreover, unlike many successional or stand-age sequences (Vitousek et al. 1989), this jack pine ecosystem exhibits a "U-shaped" pattern of inorganic N availability with stand development, driven by the decrease and subsequent re-accrual of the organic horizon and N mineralization therein (Yermakov and Rothstein 2006;

LeDuc and Rothstein 2010). Thus, in this system, inorganic N availability is not simply moving uniformly in concert with stand development, potentially allowing its effects to be better separated from that of other variables.

In this present study, we examined changes in EMF community composition using molecular methods across a six-site chronosequence of Michigan jack pine stands varying in age since severe wildfire disturbance. We asked the following questions: (1) do EMF community diversity and composition change with forest stand age following disturbance? And, (2) are compositional changes related to either inorganic or organic N availability, specifically soluble organic N and free amino-acid N?

Materials and methods

Study sites and sampling

Our chronosequence study sites were located within the highplains district of northern Lower Michigan, USA (44°30′N, 84°30′W), a region characterized by a harsh, continental climate with a short growing season (82 days) (Albert et al. 1986) and cold temperatures (annual, January, and July mean temperatures = 6.8, −7.2, and 20.3 °C, respectively) (Michigan State Climatologist's Office 2012). The landscape of this area is dominated by broad outwash plains generally consisting of acidic, excessively drained, poorly developed sands of the Grayling series (mixed, frigid Typic Udipsamments) (Albert et al. 1986; Werlein 1998). The outwash plains are dominated by jack pine, and the combination of dry conditions, flat topography, and highly flammable vegetation has resulted in a frequent fire-return interval of approximately 60 years (Cleland et al. 2004). Due to frequent disturbance (a short fire-return interval and extensive harvesting), the vast majority of jack pine forests in this region are composed of stands 70 years of age or less (Yermakov and Rothstein 2006).

Despite the general uniformity of this region, variation in topography, relative landscape position and soil texture can influence ecosystem productivity (Kashian et al. 2003; Walker et al. 2003); therefore, we used field-scouting to further restrict chronosequence sites to jack pine dominated, uniformly flat, sandy outwash sites, lacking any clay or gravel banding to a depth of 2 m. In each site, the overstory of the preceding stand was killed by a stand-replacing wildfire of at least 80 ha in size, and, as a result, all sites were even-aged stands, with a single, mono-dominant jack pine overstory and a shrub-and-sedge (*Carex* spp.) understory. As part of developing our chronosequence, we rejected other fires, and locations within these fire perimeters, where fire severity did not result in overstory mortality.

Using these criteria, we developed a larger chronosequence (LeDuc and Rothstein 2010), and, from this, selected six sites aged 5, 11, 19, 23, 47, and 56 years post-wildfire. As part of the previous study, these sites had been characterized a year prior for a suite of forest floor and soil characteristics, including inorganic N, net N mineralization, amino-acid N and soluble organic N (SON). Extractions of N had been collected three times during the growing season, and averaged at the site-level (see Table 1 for values and methods; for complete description of sites and methods, see LeDuc and Rothstein 2010). Inorganic N dynamics of these six sites followed the "U-shaped" pattern evidenced by the chronosequence as a whole (Fig. 1a) (LeDuc and Rothstein 2010). Free amino-acid N and SON were the mirror image: lowest in the youngest stands, increasing rapidly to peak values in the 23- to 47-year-old sites, and then declining to an intermediate level in the 56-year-old stand (Fig. 1b, c) (LeDuc and Rothstein 2010).

At each site, we identified a 80 × 100 m sampling area of uniform, flat terrain, surrounded by at least a 2-m buffer from any stand boundary. In September 2006, we collected 80 soil cores (4 cm diameter × 10 cm below the O horizon; starting at the Oe layer) per site, along four transects (80–100 m in length), spaced 20 m apart. The cores were collected at the drip-line of jack pine seedlings/trees, spaced at least 4 m apart. We chose this distance to reduce autocorrelation between samples and thus maximize the number of species captured for a given sampling effort. Previous work has found high autocorrelation in dominant taxa in samples occurring within 3 m (Lilleskov et al. 2004). Sampling near any EMF host-species other than jack pine was specifically avoided. All samples were transported to the laboratory on ice, storing them for less than 7 days at 4 °C prior to processing. Using tap water, the roots in each core were washed over a 1-mm sieve and frozen at −20 °C for later analysis.

Molecular identification of EMF taxa

To identify EMF taxa, each frozen root sample was slowly thawed by first placing the sample in 50 % ethanol and water for 1 h, transferring to 75 % ethanol for 1 h, and then finally transferring to 100 % ethanol prior to sampling (Lilleskov et al. 2002a). Similar to the sampling method of Peter et al. (2001), a root subsample was spread out on a 1-cm grided Petri dish, and randomly selected grids were viewed under a stereomicroscope, with the ectomycorrhiza closest to each grid center selected, avoiding clearly desiccated root tips. In this manner, a primary root tip was chosen. No more than one root tip per core was amplified, yielding 80 root tips or less per site. This approach minimizes spatial autocorrelation of sampled tips, and provides a good estimate of frequency of operational taxonomic

Table 1 Age, plant community, soil characteristics and nitrogen pools for each chronosequence site

Stand age (year)[a]	5	11	19	23	47	56
Plant community (%)[b]						
Jack pine (*P. banksiana*)	93	100	100	98	94	97
Red pine (*P. resinosa*)	–	–	–	–	6	–
Northern pin oak (*Q. ellipsoidalis*)	7	–	–	2	–	3
Median jack pine DBH[c]	ND	2.5	5.7	7.5	11.5	15.4
Jack pine site index[d]	51	45	44	48	43	48
Organic horizon C and N (Oe/Oa)[e]						
C (g m^{-2})	919.8	1,402.7	335.7	1,119.0	1,465.5	1,092.6
N (g m^{-2})	37.8	55.4	13.3	40.3	52.2	38.8
Soil characteristics						
pH[f,g]	3.83	3.74	3.70	3.81	3.47	3.46
Silt + clay (%)[h]	10	16	10	12	7	12
Phosphorus (g m^{-2})[f,i]	3.19 (0.61)	1.41 (0.13)	2.59 (0.32)	2.04 (0.51)	0.99 (0.22)	2.35 (0.64)
Soil nitrogen						
Total N (g m^{-2})[f,e]	285 (20)	298 (10)	221 (09)	209 (20)	258 (49)	268 (22)
Total soluble N (mg m^{-2})[f,j,k]	432.5 (31.0)	327.5 (22.6)	531.0 (37.7)	584.4 (54.2)	763.1 (86.1)	686.1 (76.4)
Soluble organic N[f,j,k]	301.8 (34.3)	287.1 (24.3)	475.8 (32.8)	524.8 (49.3)	655.5 (83.8)	414.8 (52.7)
Amino-acid N (mg m^{-2})[f,j,l]	24.5 (3.8)	18.5 (4.3)	75.1 (13.7)	116.7 (9.9)	127.2 (18.1)	67.0 (6.0)
Inorganic N (mg m^{-2})[f,j]	130.7 (34.1)	40.5 (7.1)	55.2 (9.3)	59.6 (12.2)	107.6 (15.1)	271.3 (27.5)
NH_4^+-N	109.2 (28.4)	25.6 (5.7)	40.5 (10.7)	51.2 (11.3)	92.4 (9.5)	255.4 (28.0)
NO_3^--N	21.6 (8.3)	14.9 (5.8)	14.7 (3.7)	8.3 (2.4)	15.2 (6.5)	15.9 (1.7)
Soluble inorganic N/organic N ratio	0.43	0.14	0.12	0.11	0.16	0.65
Total net N mineralization (g N m^{-2})[m]	1.76 (0.46)	0.84 (0.17)	0.94 (0.15)	1.25 (0.40)	1.49 (0.47)	3.04 (0.30)
Total net nitrification (g N m^{-2})[m]	0.27 (0.25)	0.03 (0.02)	0.01 (0.01)	0.00 (0.00)	0.00 (0.00)	0.12 (0.12)

Values in parentheses are SEs ($n = 4$) unless otherwise noted; soil characteristics and N data are from LeDuc and Rothstein (2010)

ND No data

[a] Years since stand-destroying wildfire

[b] Values expressed in percent number of stems for the 5-year-old site and in percent basal area (≥ 2 cm in diameter at 1.3 m) for sites age 11–56

[c] Diameter at breast height (1.3 m)

[d] Height, expressed in US feet, attainable by the average dominant or co-dominant jack pine at 50 years

[e] Measured via combustion–gas chromatography (NA1500 elemental analyzer; Carlo-Erba, Milan, Italy)

[f] For combined upper 10 cm of Oe/Oa and mineral soil (soil cores taken at surface of Oe horizon to a depth of 10 cm). For complete description of methods used to measure phosphorus and N pools see LeDuc and Rothstein (2010)

[g] Measured in a 1:2 soil–0.01 M $CaCl_2$ slurry

[h] Fine earth fraction (≤ 2 mm) in upper B horizon

[i] Orthophosphate-P measured by the Murphy and Riley (1962) method; 6 g air-dried soil extracted in 60 ml Melich-3 extractant

[j] 18 g fresh soil extracted in 27 ml 4 mM $CaCl_2$

[k] Measured using a total organic carbon/nitrogen analyzer (TOC-VCPN/TNM-1; Shimadzu, Columbia, MD, USA); soluble organic N calculated by subtracting inorganic N from total soluble N values

[l] Free-amino acids measured by fluorometric procedure modified from Jones et al. (2002); values multiplied by a factor of 1.4 to estimate N content (LeDuc and Rothstein 2010)

[m] Measured using buried-core method; extracted 7 g fresh soil in 50 ml of 2 M KCl

units (OTUs). Backup tips were stored in 100 % ethanol at −20 °C, and in case of failure to amplify and sequence deoxyribonucleic acid (DNA) from the primary root tip, at least three backup tips were tried before discarding the sample.

DNA was extracted from each primary tip using a method modified from Avis et al. (2003). We used a plant DNA extraction kit (REDExtract-N-Amp Plant PCR Kit; Sigma-Aldrich, St. Louis, MI, USA), following the manufacturer's instructions, except for the following: the root
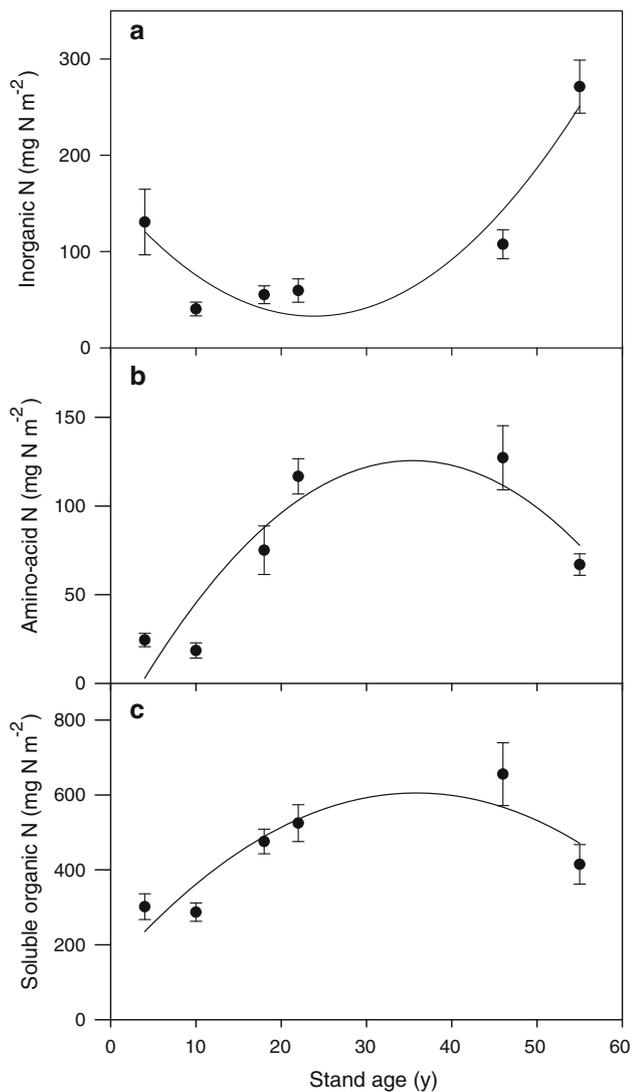
Fig. 1 **a** Inorganic N standing pools, **b** amino-acid N, and **c** total soluble organic N as functions of stand age; data are stand means ± 1 SE. *Lines* represent the following best fit equations: **a** inorganic N = 159.90 − 10.68 × age + 0.22 × age^2, $r^2 = 0.722$, $P < 0.0001$; **b** amino-acid N = −30.25 − 8.80 × age − 0.12 × age^2, $r^2 = 0.648$, $P < 0.0001$; **c** soluble organic N = 136.29 + 26.18 × age − 0.37 × age^2, $r^2 = 0.492$, $P < 0.001$. Data are from LeDuc and Rothstein (2010)

tip was removed from the 100 % ethanol and blotted dry prior to placing in extraction solution, and 20 μl each of extraction and dilution solutions were used. The internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) were amplified by polymerase chain reaction (PCR) using the PCR mix supplied with the kit, and the primer pair, ITS-1F (Gardes and Bruns 1993) and ITS-4 (White et al. 1990). Amplified DNA was purified using a Qiagen QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA), and submitted to Michigan State University's Research Technology Support Facility (E. Lansing, MI,

USA) with the primer ITS-5 for a forward sequencing reaction.

Amplified sequences were edited in SeqMan 7.0, and OTUs were preliminarily identified according to the closest Blast search matches in Genbank or Unite. Only matches to sequences deemed reliable were included, i.e., Genbank or Unite sequences from samples identified morphologically (sporocarps, cultures), preferably from a taxonomic or phylogenetic study if available. Sequences were aligned with close matches in Bioedit using ClustalW. Alignments were manually edited, and submitted to similarity analysis in Bioedit. Sequences with ≥97 % similarity to only one species over >90 % of their length were considered the same species or species group. At <97 % match, genus or higher level assignments were made by examining the BLAST matches or by phylogenetic analysis. If the matches were not consistently to one species or genus, we aligned unknowns to close matches, and ran a neighbor-joining analysis with bootstrap (heuristic search, 100 replicates, optimality criterion = parsimony) in PAUP (v.4.0b10), and determined bootstrap support for placement at a taxonomic level. Typically genus assignments were >89 % matches and family assignments were >80 % matches. One notable exception was *Ceratobasidium*, which has a high level of infrageneric ITS variability (Sharon et al. 2008). Matches at 86–87 %, with consistent BLAST matches to only *Ceratobasidium/Rhizoctonia*, were considered to be the same genus. One representative sequence per OTU was submitted to Genbank (Table S1).

EMF community diversity and composition

All successfully amplified samples were grouped to characterize the EMF community at a given site. We used the software EstimateS 7.52 to compute taxa accumulation curves and first-order Jackknife richness estimators (100 randomizations without sample replacement). Numbers of taxa observed were divided by Jackknife richness estimators to yield a percent estimate of the community captured. We also calculated the Shannon diversity index, and the Simpson's diversity (1/D) and evenness indices to estimate stand-level diversity (Magurran 2004). Changes in diversity indices with stand age were evaluated using linear and non-linear regressions. The function that best fit each relationship was based on the combination of minimum sum of squares error, lowest *P*-values and conceptual strength.

In addition to diversity, employing the metaMDS function in R 2.10.1, we compared EMF community composition across the chronosequence using non-metric multi-dimensional scaling (NMDS) with random starts to avoid local minima (Oksanen et al. 2010). Bray–Curtis

distances of the original data were used. Ordinations were conducted at both the genus and species level.

EMF taxonomic shifts and soil N availability

We used the envfit function in R 2.10.1 (Cox et al. 2010; Oksanen et al. 2010) to explore potential associations between changes in the EMF community with soil N variables (inorganic N, amino-acid N and SON). Since the EMF community was characterized at the site level, comparisons were made to site-level N means. We also included soil phosphorus (P) and organic horizon (Oe/Oa) carbon (C), as index of O horizon mass, in this analysis. The variables were fitted as vectors to both the genus and species-level NMDS, and significance was determined by the r^2 fit generated via 10,000 permutations. To further explore relationships between N variables and the community data, we regressed the NMDS axis 1 and 2 scores against inorganic N, N mineralization, amino-acid N, SON and the ratio of inorganic N to SON. We also carried out partial Mantel analyses to assess the potential effect of these N pools after accounting for stand-age.

When necessary, data were log-transformed to meet assumptions of normality, and curve fitting was conducted in SigmaPlot 11.0. All other statistical analyses were conducted in R 2.10.1, and significance was accepted at $\alpha \leq 0.05$.

Results

Chronosequence characteristics

Overall, all of the chronosequence sites exhibited similar soil and vegetation characteristics (Table 1). Soils in all sites were acidic, extremely well-drained, loamy sand to sand. There was no systematic variation in soil texture, and all sites had a low jack pine site index (≤ 51). Jack pine was the dominant overstory vegetation across all stands, ranging from 94 to 100 % of basal area. Similarly, jack pine accounted for 93 % of the total number of stems in the 5-year-old stand. Though overwhelmingly jack pine, there was a small percentage of red pine (*Pinus resinosa*) and northern pin oak (*Quercus ellipsoidalis*) in the overstory in select sites.

EMF community diversity and composition

We successfully identified EMF genotypes in approximately 70 % of the samples across all sites, ranging from 69 % in the 23, 47 and 56 year-old sites to 74 % in the 5-year-old site. Total number of OTUs identified was 106. Taxa accumulation curves (Fig. 2) in all sites deviated

from a linear slope. According to observed taxa expressed as a percentage of jackknife values (data not shown), sampling effort on average captured 63 % of the site-level richness, with a range from 57 to 72 % in the 47- and 11-year-old sites, respectively. Changes in the Shannon diversity and Simpson's evenness indices were best described by an asymptotic curve, increasing sharply between the 5- and 19-year-old sites, before remaining relatively constant (Fig. 3a, c). We were unable to fit a statistically significant curve to changes in the Simpson's diversity index due, in part, to a high value in site age 23 (Fig. 3b).

In the NMDS at the genus level, the older sites diverged along axis 1 from the two youngest sites (Fig. 4a). Along axis 2, the mid-age sites (19- and 23-years-old) separated from the two older sites (47- and 56-years-old) forming discrete pairs. In the NMDS of the species-level data (Fig. 4b), sites age 5 and 11 were tightly clustered as were sites age 19, 23 and 47; however, the 56-year-old site fell approximately in between the two groups along axis 1 and separated from all other sites along axis 2. The change in community observed along axis 1 was primarily driven by a shift away from such taxa as *Thelephora terrestris*, *Suillus brevipes* and *Rhizopogon* cf. *rubescens* and towards taxa of *Cortinarius* and *Russula*, among others (Table S2). The most abundant taxa in the youngest sites, *Thelephora terrestris* and *Suillus brevipes*, were not observed in the three oldest stands (Table S2).

EMF taxonomic shifts and soil N availability

We found a significant relationship, particularly at the species level, between EMF taxonomic changes along NMDS axis 1 and organic N availability, but no association
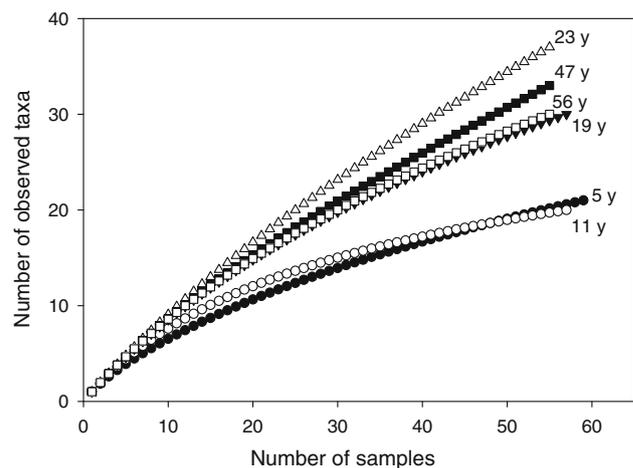

Fig. 2 Taxa accumulation curves for each site, calculated in EstimateS 7.52 by averaging 100 randomizations without replacement. Sites noted by year since fire
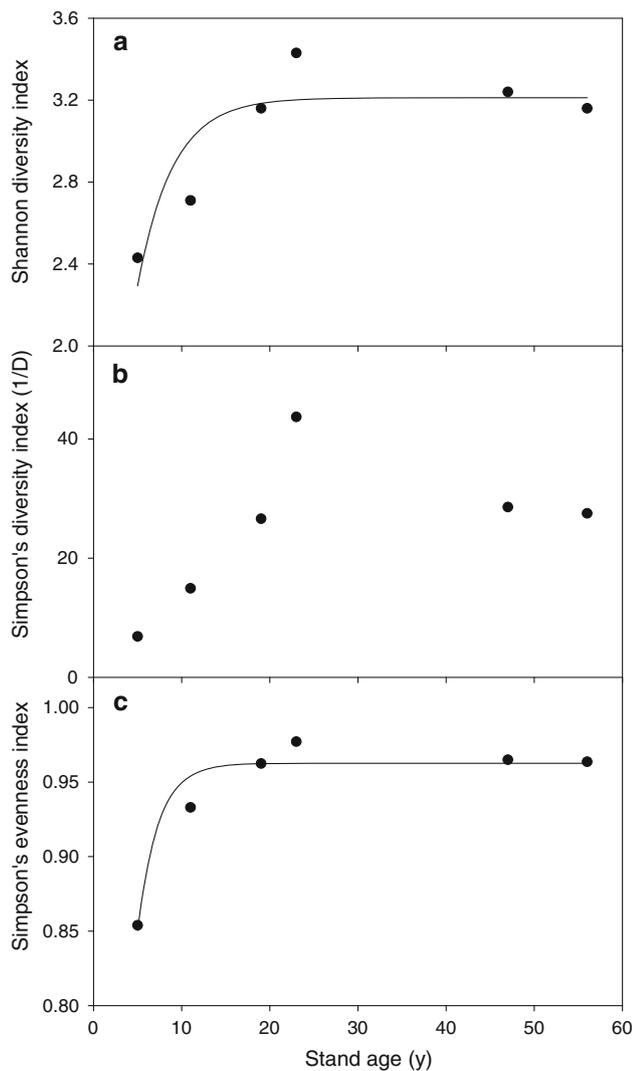
Fig. 3 a Shannon diversity index, b Simpson's diversity index and c Simpson's evenness index of the EMF community as a function of stand age. *Lines* represent the following best fit equations: a Shannon diversity index $= 3.212 \times [1 - \exp(-0.250 \times \text{age})]$, $r^2 = 0.708$, $P = 0.022$; c Simpson's evenness index $= 0.963 \times [1 - \exp(-0.430 \times \text{age})]$, $r^2 = 0.919$, $P = 0.002$

with inorganic N. According to the envfit function, the genus-level NMDS ordination was not significantly related to any environmental variable, although direction of maximum change in amino-acid N and SON did parallel axis 1 (Fig. 4a). At the species level, amino-acid N and SON were significantly correlated to the NMDS ordination, with higher values associated with sites 19, 23, and 47 along axis 1 (Fig. 4b). There were no significant correlations between either the genus or species ordinations and P, organic horizon C or inorganic N (Fig. 4a, b).

In the linear regression analysis, amino-acid N and SON correlated with NMDS axis 1 scores in both the genus and species ordinations ($r^2 = 0.577$, $P = 0.049$ and $r^2 = 0.640$, $P = 0.034$, respectively) (Fig. 5a, b). Similarly,

we also found a significant relationship for changes in the EMF community and organic N after accounting for stand age using the partial Mantel tests (genus-level Mantel $r = 0.730$, $P = 0.029$; species-level Mantel $r = 0.559$, $P = 0.019$). In contrast, there was no relationship between genus or species NMDS axis 1 scores and either inorganic N alone, N mineralization or the ratio of inorganic to SON. Species-level axis 2 scores did correlate with inorganic N (Fig. 5c) and N mineralization ($r^2 = 0.764$, $P = 0.014$), but this was primarily influenced by site age 56. The correlations between genus-level axis 2 scores and inorganic N and N mineralization were not significant ($r^2 = 0.388$, $P = 0.111$ and $r^2 = 0.291$, $P = 0.156$, respectively). Likewise, we found no significant relationship between inorganic N and shifts in the EMF community across the chronosequence according to the partial Mantel tests (genus-level Mantel $r = -0.082$, $P = 0.536$; species-level Mantel $r = -0.010$, $P = 0.462$).
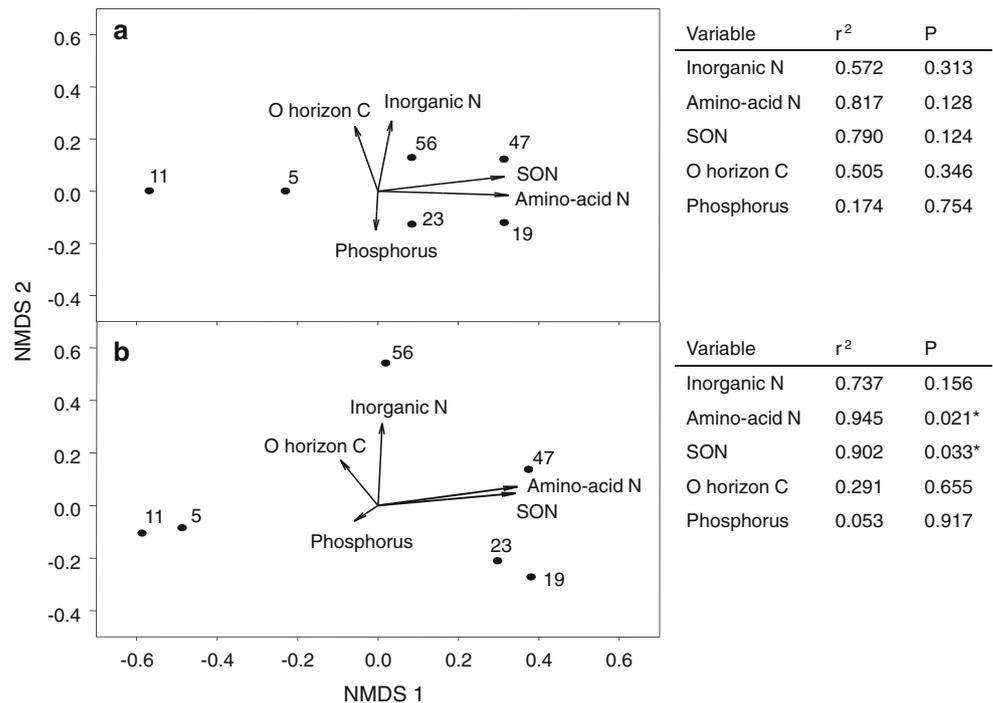
Discussion

EMF community diversity and composition

Variation in the soil mycorrhizal community can have profound effects on ecosystem structure and functioning (van der Heijden et al. 1998), yet we still have a limited understanding of how mycorrhizal communities change over time and potential drivers of that change. Here, we observed that EMF community diversity and composition clearly shifted with stand age. Due to the dominance of basal area by jack pine at all sites (Table 1), the root type and therefore the plant–host was likely overwhelmingly jack pine throughout stand ontogeny. Some of the younger stands had stump sprouts from fire-killed overstory oaks, and there were scattered oak seedlings in the understory of the older sites. For example, in the 56-year-old site, oak seedlings, though the most common tree seedling (88 % of stems), were established at a low density of 0.17 stems per m², or one seedling for almost every 6 m² (Rothstein, unpublished data). The open understory of these stands, and dominance by jack pine, allowed us to easily avoid sampling near oak seedlings or mature oak trees.

Our finding that EMF diversity was lowest following disturbance is not surprising as multiple studies have shown that severe fires, especially those that result in overstory mortality, alter EMF community composition (Visser 1995; Baar et al. 1999; Dahlberg et al. 2001; Smith et al. 2005; Kipfer et al. 2011). Fire may affect the EMF community directly through heat-induced mortality, indirectly through altering the environment, including plant–host composition or characteristics, or through a combination of direct and indirect effects (Peay et al. 2009). As a result, the post-fire EMF community is

Fig. 4 NMDS of EMF community composition at the **a** genus and **b** species level across the chronosequence sites (stress for both ordinations $= 2.08 \times 10^{-14}$). Sites noted by year since fire. Variables tested for significant correlation with community dissimilarities shown as *arrows*, with orientation and length indicating direction of greatest change and strength of correlation, respectively. Variables significantly correlated to changes in the community indicated by *asterisk* (*)

Variable	r^2	P
Inorganic N	0.572	0.313
Amino-acid N	0.817	0.128
SON	0.790	0.124
O horizon C	0.505	0.346
Phosphorus	0.174	0.754

Variable	r^2	P
Inorganic N	0.737	0.156
Amino-acid N	0.945	0.021*
SON	0.902	0.033*
O horizon C	0.291	0.655
Phosphorus	0.053	0.917

typically dominated by a few taxa of fungi, either absent or rare prior to disturbance. Fire severity is measured by its effect on vegetation or soil properties (Barnes et al. 1998), and the wildfires here were considered severe since they killed the preceding overstory. In contrast, fires that do not remove the overstory or consume substantial portions of the forest floor generally have a reduced impact on the EMF community (Jonsson et al. 1999; Dahlberg et al. 2001).

In the present study, *Thelephora terrestris* and *Suillus brevipes* were dominant in the youngest stand. *Thelephora terrestris* is often found in greenhouses, nurseries or reforested sites, due to its ability to disperse and quickly colonize a plant host from spores (Colpaert 1999). Other taxa found in the young sites (e.g., *Rhizopogon* and *Tomentella* species) are often part of the resistant propagule bank, persisting for years in the soil as dormant spores or other propagules (Taylor and Bruns 1999; Ashkannejhad and Horton 2006; Bruns et al. 2009). Similar to our results, Buscardo et al. (2011) found that *Rhizopogon* (a sister genus to *Suillus*) taxa dominated the EMF community after wildfire in a Mediterranean pine forest. Likewise, Baar et al. (1999) found that bishop pine (*Pinus muricata*) seedlings were dominated by *Rhizopogon* taxa, *Wilcoxina mikolae* and *Tomentella sublilacina* (a close relative of *T. terrestris*) 18 months after a severe fire. Horton et al. (1998) did observe that some EMF initially colonizing bishop pine seedlings in the first few months after fire were also found in the pre-fire forest community. Their results suggest that the EMF community may initially survive the fire, but are quickly out-competed in the first year by

disturbance-adapted fungi such as *Rhizopogon* spp. (Baar et al. 1999).

Though the dominant fungi in the 5-year-old site, *Thelephora terrestris* and *Suillus brevipes*, were largely absent from the three oldest sites (Table S2), other taxa persisted in low abundances over the entire chronosequence. Over 50 % of the taxa in the 5-year-old site, for example, were observed in at least one of the three oldest stands. Diversity increased with stand age due to both the persistence of taxa found in the young sites and the gradual accumulation of new taxa. Thus, the succession observed here did not involve complete replacement (relay floristics), but resulted at least in part from a shift in dominance (initial floristics sensu Egler 1954), with the persistence of a group of fungi throughout the stand age sequence. This finding is consistent with that of Visser (1995), who also observed that most EMF species found on young jack pine persisted through succession and were not completely replaced.

Notably, the greatest change in EMF community composition and diversity occurred prior to canopy closure. The first two decades of stand development in these xeric jack pine ecosystems coincide with a movement from stand initiation to a stem-exclusion phase, either at the stand or patch-level (Oliver and Larson 1996; Spaulding and Rothstein 2009). In this study, canopy closure coincided with sites age 19 and 23. This finding is similar to those of Visser (1995) for jack pine and Twieg et al. (2007) for Douglas-fir (*Pseudotsuga menziesii*), suggesting this successional shift in EMF taxa coincides with canopy closure
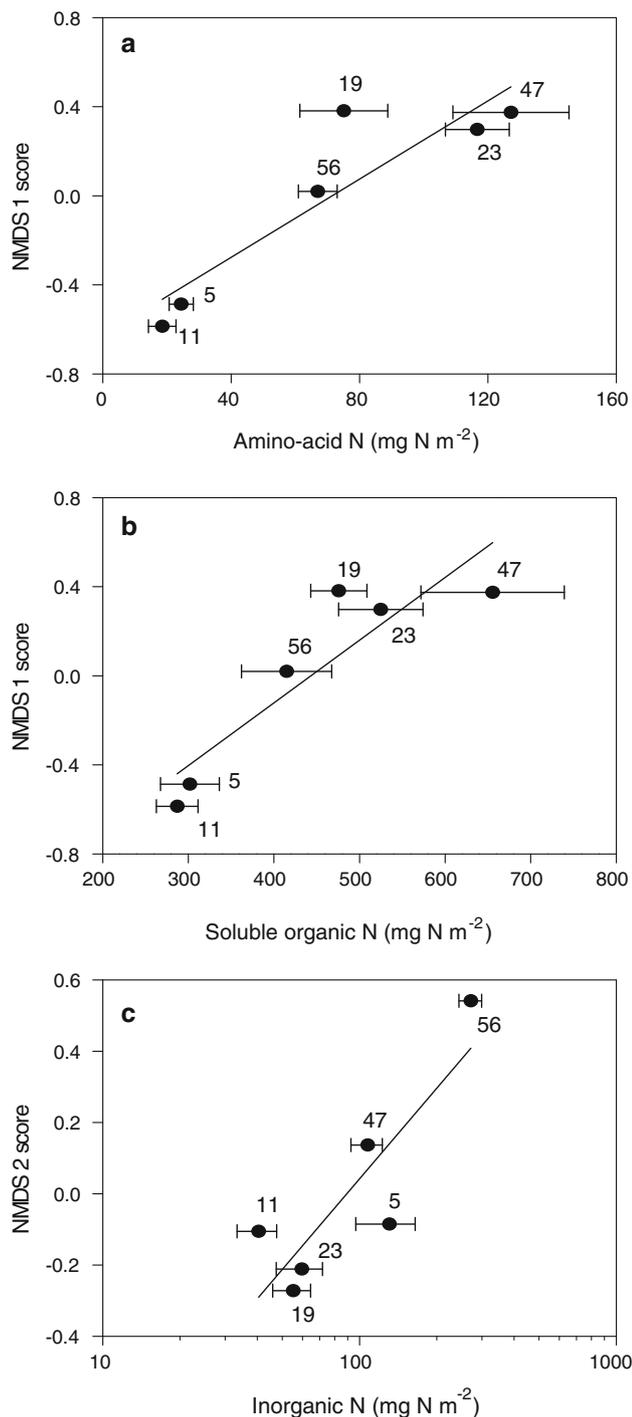
Fig. 5 NMDS axis 1 scores regressed against site average **a** soluble amino-acid N and **b** soluble organic N; NMDS axis 2 scores regressed against site average **c** inorganic N. All scores are from the species-level ordination and data are stand means ± 1 SE. *Lines* represents the following linear equations: **a** NMDS 1 score = −0.63 + 0.009 × amino-acid N, $r^2 = 0.779$, $P = 0.013$; **b** NMDS 1 score = −1.24 + 0.001 × soluble organic N, $r^2 = 0.768$, $P = 0.014$; **c** NMDS 2 score = 1.93 + 0.891 × log10 inorganic N, $r^2 = 0.692$, $P = 0.025$. Sites are noted by year since fire

across multiple ecosystems. This finding would also be consistent with Last et al.'s (1987) hypothesized importance of canopy closure for structuring EMF succession, except contrary to their hypothesis, we failed to observe a decrease in EMF diversity in older stands.

EMF taxonomic shifts and soil N availability

The finding of a successional change in EMF taxa with stand age raises the question of underlying mechanisms. The shift did not correlate with inorganic N availability, either alone, in a ratio with SON, or after accounting for stand-age. Indeed, sites age 5 and 11 grouped closely in the species-level NMDS ordination (Fig. 4b) despite site age 5 having the second highest inorganic N availability and site age 11 having the lowest (Table 1; Fig. 1). This finding is consistent with Twieg et al. (2009), who found no correlation between changes in EMF taxa and extractable inorganic N and potentially mineralizable N. Although we cannot rule out community inertia leading to lags between a variable and shifts in taxa, the results of this study, combined with those of Twieg et al. (2009), suggest that inorganic N cannot by itself account for successional shifts in EMF community composition. We caution that this should not be interpreted as inorganic N having little effect on the structure of the EMF community. Inorganic N could still favor nitrophilic EMF fungi outside the dynamics of succession. The fact that NMDS axis 2 scores correlated with inorganic N, albeit primarily driven by site age 56, raises this as a possibility.

By contrast, we observed a close relationship between the successional shift in taxa and SON and amino-acid N (Figs. 4b, 5a, b). This association could be potentially explained by selection of EMF taxa better able to access organic forms of N for their plant hosts. While we did not measure the specific compounds of the SON pool (aside from amino-acid N), it likely contained easily soluble peptides and proteins. There were ten genera across the chronosequence present in at least four stands with a minimum relative abundance of 5 % in at least one stand. Of these ten, five have been shown to utilize protein N (Lilleskov et al. 2011 and references therein): *Amanita*, *Cortinarius*, *Russula*, *Suillus* and *Tricholoma*. Amongst this group, all except *Suillus* were positively related in relative abundance with SON. Despite this finding, however, species differences within these genera may exist that limit this comparison. Moreover, the dominant fungi found in the 5-year-old site, *Thelephora terrestris*, has been shown to use protein N in pure culture (Finlay et al. 1992). Nygren et al. (2007) recently observed proteolytic activity across a range of EMF taxa, suggesting that the ability to access protein N may be more widespread than previously considered. They found, for example, that *Laccaria* cf.

bicolor and *Lactarius chrysorrheus*, two taxa observed in low abundances in our youngest sites, could access protein N. In our study, *Lactarius chrysorrheus* was found across the entire chronosequence, but *Laccaria* cf. *bicolor* was only found in the youngest sites with low SON and amino-acid availability—although, because of its low abundance, we could have missed sampling this fungus in the oldest sites. Overall, our results appear mixed regarding the relationship between SON and the abundance of fungi able to access protein N.

Rather than responding to organic N levels, it is alternatively possible that the relationship between EMF taxa and organic N may be caused by the fungi themselves. In a girdling experiment, Högberg and Högberg (2002) estimated that EMF produced approximately a third of the microbial biomass C and, with their associated roots, half of the extractable dissolved organic C in a forest soil in northern Sweden—although they did not observe a concomitant effect on dissolved organic N. In a chronosequence of Norway spruce (*Picea abies*) stands, Wallander et al. (2010) found that EMF biomass peaked around canopy closure. Here, both SON and amino-acid N increased sharply between stand initiation and canopy closure (Fig. 1). High EMF biomass could increase extracellular enzyme activity, fungal biomass turnover or rhizosphere fluxes of N to the bulk soil, potentially leading to the peak in amino-acid N and SON. LeDuc and Rothstein (2010) found no difference in gross production of amino acids via proteolysis across these same sites, suggesting that the peak in amino-acid N is not due to EMF extracellular enzyme activity. These measurements, however, were taken in the bulk soil and not also in the rhizosphere, and so we cannot definitely rule out this particular mechanism.

EMF taxonomic shifts and other site characteristics

While the association observed between EMF community composition and organic N pools is compelling, it is also possible that this relationship is not causative. Other potential drivers of the successional shift in taxa include: changes in belowground allocation, and the interaction of root density and fungal exploration type. Root exudates, turnover and microbial turnover are all sources of soil amino acids (Jones and Darrah 1994; Lipson and Nasholm 2001), and LeDuc and Rothstein (2010) found that amino-acid N pools closely mirrored stand net primary productivity in these jack pine forests. Thus, it may be that amino-acid N and/or SON are correlated with stand-level belowground plant productivity, including root biomass. This could also be influencing EMF successional changes. Peay et al. (2011) recently proposed a two-step model for EMF successional dynamics based on root density and EMF exploration type. First, they suggested

that following disturbance, EMF community composition is largely determined by spore abundance and reactivity. Second, as root density increases, the optimal colonization strategy switches from colonization via spores and propagules to colonization via mycelium from living roots (Newton 1992; Peay et al. 2011). Colonization from mycelium is generally thought to be more efficient than from spores, since fungi with mycelia networks have a larger C resource base for colonizing new root tips (Deacon and Fleming 1992). As root density continues to increase with forest stand age, fungi with shorter hyphal exploration types should be more efficient than longer distance exploration types (Peay et al. 2011).

To examine this possibility, we characterized the exploration type by genera across our chronosequence (Table S3). Numbers of fungi with mid-range exploratory types did not vary across sites, but consistent with Peay et al. (2011), long-distance exploration types were the most common in site age 5, presumably with the longest distance between roots. Also across all sites, short-distance types were the least common at site age 5. In contrast, site age 11 appeared to have comparable exploration-type abundances with the remaining older sites. Given that sites age 5 and 11 grouped closely in the species ordination, distinct from the older sites, we would have expected both sites to be similar in exploration types if root density and exploration type completely explained the successional shift. Nevertheless, given that the change in taxa coincided with canopy closure, it is possible that the successional shift occurred as stand-level root density and/or belowground C allocation reached high enough levels to favor colonization via mycelium. Thus, it may be that both organic forms of N and EMF successional shifts are related to changes in belowground productivity. Of course, all these potential drivers are not mutually exclusive and could be working in concert to influence EMF community succession over the course of stand development.

Finally, the ordination of site age 56 in the NMDS species biplot is potentially noteworthy. Its placement along the mid-point of axis 1 corresponded to a decline in organic N (Figs. 4b, 5a, b), and was driven by an increase in the numbers of taxa and their relative abundance in common with sites age 5 and 11. The separation along axis 2 corresponded to an increase in inorganic N (Fig. 5b). Caution should be used in evaluating these results, however, since these dynamics were found in only one site; the same ordination was not observed at the genus-level; and the increase in common taxa with sites age 5 and 11 was marginal compared to the other older sites (Table S2). Annual stand-level measurements in this site from 2007 to 2010 have shown a decline in live aboveground biomass with high rates of tree mortality (Rothstein, unpublished data), and thus declining productivity could be contributing

to both the decrease of organic N and changes in the EMF community. Alternatively, *Lactarius chrysorrheus* exhibited the greatest overlap in abundance between the 56- and 11-year-old stands, but is a fungus generally associated with oaks (Richard et al. 2004). Thus, even though jack pine was the dominant EMF host species, the presence of this particular fungus might reflect a small number of oak roots in our samples. Further research is needed to evaluate whether EMF community composition truly shifts again in older forests—specifically those declining in productivity—and mechanisms behind any potential shift.

Because our study is observational not experimental, it is important to evaluate potential caveats that may affect our interpretation. The number of root tips we sampled was relatively low because we chose to sequence a single root tip per soil core without morphotyping. Our EMF sampling approach is a method that minimizes autocorrelation among sequenced root tips. This approach is fundamentally different from the morphotype and sequencing methods used in other studies that typically involve full characterization of samples in each core. At the DNA level, we sequenced 480 root tips and found 106 OTUs. Studies using the morphotype and sequence approach have shown similar results. For example, by sequencing 541 root tips, Twieg et al. (2007) found 105 OTUs, remarkably similar results to this study. Whereas our method better estimates frequency and minimizes autocorrelation, the morphotyping and sequencing method provides better estimates of abundance. Overall, our sampling method likely adequately captured shifts in dominant or common taxa, particularly given that our findings were consistent with previous studies of EMF succession (Visser 1995; Twieg et al. 2007). Nevertheless, comparisons of relatively rare taxa across stands should be made carefully. This is a limitation typical of studies of EMF assemblages, which are generally dominated by a few taxa with a "long tail" of relatively rare taxa (Taylor 2002).

Additionally, N availability and EMF community composition ideally would have been measured in the same growing season. However, differences in inorganic N availability across different sites and stand ages have been shown to be robust across different sampling years in this study area (Yermakov and Rothstein 2006; LeDuc and Rothstein 2010; Rothstein and Spaulding 2010). Only the very youngest stands (≤2 years) have shown large yearly differences due to the initial spike in net mineralization and nitrification immediately following wildfire (Yermakov and Rothstein 2006). All of our stands were 4 years or older when measured for soil N, suggesting that year-to-year differences are likely to be small. The stand measures of inorganic and organic N used were also growing season averages, integrating across fluctuations in temperature and precipitation—suggesting our measures were also robust to potential differences in short-term environmental variation.

Conclusion

Overall, the results of this study clearly show a successional shift in EMF community composition with stand age. We observed higher relative abundances of *Suillus brevipes* and *Thelephora terrestris* in the youngest sites, while *Clavulina*, *Cortinarius*, and *Russula*, among other taxa, were more abundant in the mid-aged and older sites. Notably, this successional shift occurred without corresponding changes in the composition of the dominant plant–host over stand ontogeny, and coincided with canopy closure in these jack pine ecosystems. It was not associated with changes in inorganic N availability, but instead was significantly correlated to an increase in SON and free amino-acid N. However, the causal mechanisms behind this relationship are unclear. Increases in these organic N forms could be favoring certain EMF taxa directly, or could in turn be caused by shifts in EMF abundance or community change. Alternatively, these organic N forms and EMF assemblages may both be causally associated with other site variables, such as belowground productivity and root density.

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