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Variation in diazotrophic community structure in forest soils reflects land use history

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ABSTRACT

Microbial nitrogen fixation is an important source of nitrogen in soils of both old and secondary-growth forests. Considering that many soils, which today support secondary-growth forests were once under cultivation, no studies have examined how this kind of disturbance history affects contemporary populations of nitrogen-fixing microbes in forest soils. In the work presented here, we compare secondary-growth forest sites, which were under cultivation more than 150 years ago, to old-growth forest sites in Cadwell Forest, Massachusetts. For each site, nitrogenase activity was measured and the diversity of the *nifH* gene pools was examined. Three sites with prior agricultural history exhibited higher nitrogenase activity and were dominated by diazotrophs closely related to the α- and γ-Proteobacteria. In contrast, lower nitrogenase activity and the dominance of the anaerobic Cluster III diazotrophs characterized the three old forest sites. Further analyses of species overlap among all six sites revealed that the diazotrophic composition was closely related to previous management history, with agricultural sites clustering together and separate from old forest sites, independent of the proximity between sites. By specifically targeting one of the main functions of microbial communities in soils, the activity and diversity of nitrogen-fixing microorganisms, this work points to a long-lasting effect of former agricultural activities on secondary-growth forest soils, more than one hundred years after succession.

1. Introduction

Nitrogen in most forest soils is provided by the decomposition of organic material, inputs from atmospheric deposition, and by microbial nitrogen fixation (Widmer et al., 1999; Nadelhoffer, 2001). In most cases, nitrogen fixation represents a significant contribution to the net nitrogen input in forest soils (Johnson and Curtis, 2001). Nitrogen-fixing, or diazotrophic, microorganisms represent a physiological group of highly specialized *Bacteria* and *Archaea* that have a significant functional role in the input of nitrogen to both terrestrial and aquatic environments. The known diversity of diazotrophic microorganisms continues to grow as DNA and mRNA inventories of *nifH*, the gene that encodes nitrogenase reductase, are expanded and catalogued in databases such as Genbank (Zehr et al., 2003b; Martensson et al., 2009). Although *nifH* expression is ubiquitous in forest soils (Mergel et al., 2001), the reported diversity of diazotrophs varies dramatically between soils, and such differences in diversity can be used as an indicator of community response to a disturbance (Yeager et al., 2005).

Human activities, particularly those associated with forest management, alter the supply of nutrients in forest ecosystems (Foster et al., 1998; Compton and Boone, 2000; Johnson and Curtis, 2001; Gulledge et al., 2004; Johnson et al., 2005). In the case of the nitrogen cycle, particularly nitrogen fixation, widespread disturbances such as land-use change in the form of clear-cutting can significantly alter diazotrophic populations (Widmer et al., 1999; Shaffer et al., 2000; Johnson et al., 2005; Yeager et al., 2005). Other disturbances, such as chronic N fertilization, have been noted to have an impact on nitrogen-fixing populations in soils of Harvard Forest in Massachusetts (Compton et al., 2004; Gulledge et al., 2004). However, little work has been performed to assess the long-term permanence of such effects, particularly in relation to the long-lasting effect of previous agricultural activity on forest soil diazotrophs.

Old-field succession following abandonment after extended periods of agricultural activity, or secondary growth, is commonly found in the northeastern United States (Compton and Boone, 2000). Overall, very few long-term studies that allow an effective test of the resilience of forest soil diazotroph communities exist.

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Thus, long-term community responses to disturbance must be inferred from current manipulations or historical reconstruction (Compton and Boone, 2000). Cadwell Memorial Forest in Pelham, Massachusetts, is an excellent research site to study the long-term effect of agricultural activity on secondary-growth forest soils and to examine the resilience of the soil diazotroph community to such a disturbance. After being cleared for agricultural activities between 1783 and the 1850s, these sites were abandoned and allowed to return to forest ecosystems (D'Amato et al., 2005). In 1953, the University of Massachusetts, Amherst acquired the land. The boundaries between old forest sites and secondary-growth sites cleared for agricultural use remain delineated, making it possible to obtain comparative samples to assess microbial community composition under both management histories. Long-term secondary growth should promote homogenous plant–microbe and soil–microbe interactions on both sides of the boundaries. We therefore hypothesized that, after more than a century after the cessation of agricultural activities, few differences in diazotrophic community composition and activity should be observed in forest soils with different land-use history.

In the work presented here we aim to address the question of whether extensive land-use changes such as forest clearing and agricultural activity that occurred more than a century ago have a legacy effect on contemporary forest soil community diversity and function. For this purpose, we have compared the structure of nitrogen-fixing populations and their respective nitrogenase activity in old forest sites with that of adjacent, historically farmed secondary-growth sites.

2. Materials and methods

2.1. Soil sampling and analyses

Cadwell Memorial Forest is located in the central hardwood region of southern New England (42°21′ 47″ N, 72°26′ 17″ W) in the towns of Belchertown and Pelham, Massachusetts. The University of Massachusetts, Amherst, has managed the 468 ha of experimental forest since 1951 (Wilson and McComb, 2005). In terms of prior pastureland utilization, during the early to mid 1800s, ten farms operated in this area along with a sawmill and a small woodworking shop (D'Amato et al., 2005). Current vegetation includes red maple (*Acer rubrum* L.), red oak (*Quercus rubra*) and scarlet oak (*Quercus coccinea*), and, to a lesser degree, eastern hemlock (*Tsuga canadensis*) and white pine (*Pinus strobus*) (D'Amato et al., 2005). Soil samples were obtained from the topsoil (0–10 cm) of six sites in the southwest corner of the property (Fig. 1) after removal of overlaying litter, and transported immediately to the laboratory for analyses. At each site, triplicate samples were obtained in cores 10 cm apart from each other using a 10-cm^2 area for each site during sample collection, given the heterogeneity of the terrain. Three sites with agricultural history identified as areas enclosed by stone walls (CA1, CA2, CA3) were chosen as secondary-growth sites, and three were chosen from adjacent old forest sites (CF1, CF2, CF3) in the same area. Sites CA1, CA2, CF1, and CF2 were close to each other in an area dominated by mixed hardwood and maple. Sites CA3 and CF3 were obtained 700 m away, in an area dominated by pine and oak species. All six sites were predominantly fine sandy loams. For each site, triplicate samples were sieved through a 2-mm mesh, removing root material by hand. After sieving, each triplicate sample from each site was stored at −20 °C. DNA was extracted 1 month after sampling. Chemical analyses were performed at the UMass Soil Testing Facility (Amherst, MA) with a 50-g portion of pooled samples, where concentrations of NO_3 and NH_4 were measured colorimetrically.

2.2. DNA extraction and PCR amplification of *nifH*

DNA was extracted using the BIO101 FastDNA kit (Qbiogene, Carlsbad, CA) with minor modifications. A total of 0.5 g of each triplicate sample for each site was subjected to DNA extractions. Ballistic exposure times in a Mini-BeadBeater (Biospec, Bartlesville, OK) were increased to 1 min, and bead-beating speed was lowered to 2500 rpm for maximum DNA yield. The DNA extracted from each set of triplicates was then pooled to represent each site. The extracted DNA was used in triplicate PCR amplifications of a 330-bp fragment of the *nifH* gene, using nested outer PCR primers (Zani et al., 2000) nifH3 (5′-ATR TTR TTN GCN GCR TA-3′) and nifH4 (5′-TTY TAY GGN AAR GGN GG-3′), and inner primers (Zehr and McReynolds, 1989) nifH1(5′-GAY CCN AAR GCN GA-3′) and nifH2 (5′-AND GCC ATC ATY TCN CC-3′) as previously described (Izquierdo and Nüsslein, 2006).

2.3. Construction of *nifH* gene clone libraries

The construction of clone libraries of the *nifH* gene has been previously described (Izquierdo and Nüsslein, 2006). Briefly, after the final round of PCR amplification, pooled amplified fragments were purified and cloned into a pGEM-T-easy vector (Promega, Madison, WI). Two vector-specific primers were used for the amplification of the DNA inserts from individual clones: pGemF (5′-GCA AGG CGA TTA AGT TGG G-3′) and pGemR (5′-ATG ACC ATG ATT ACG CCA AG-3′). Clones were screened by agarose gel electrophoresis to detect those with the correct size insert. A clone library was created for every sample site and each library was screened for a total of 100 clones. To address possible contamination found in commercial primer preparations for *nifH* PCR reactions as has been previously reported (Zehr et al., 2003a; Goto et al., 2005; Bostrom et al., 2007), an additional clone library was constructed using the negative control as a target. DNA extracted from randomly selected positive clones was sequenced with a 3730xl DNA Analyzer (Applied Biosystems, Inc., Foster City, CA). The resulting *nifH* gene sequences were aligned with representative sequences obtained from GenBank using the software package ClustalX (Thompson et al., 1997). BioEdit v. 7.0.5 was used for manual editing of the sequences and their translation into amino acid sequences (Hall, 1999). For the construction of phylogenetic trees, distances between pairs of amino acid sequences were calculated using the Gamma distance correction based on the Minimum Evolution criterion in the MEGA software package, version 3.1 (Kumar et al., 2004). Bootstrap confidence values were obtained based on 1000 replicates. Translated amino acid sequences were used in tree construction instead of the original nucleotide sequences to account for adequate cluster-based phylogenetic affiliation (Zehr et al., 2003b). The clone library constructed from the negative control contained sequences that had ≥98% sequence similarity to *Dechloromonas* sp. SIUL and *Azoarcus* sp. BH72. Therefore, sequences that were ≥97% similar to these controls were not included in the phylogenetic and comparative analyses.

2.4. Diversity measurements and statistical analyses

Clones were clustered into Operational Taxonomic Units (OTUs) or *nifH* phylotypes, which are defined as sequences with at least 97% nucleotide sequence similarity. We have used a 97% similarity cutoff based on an analysis of the families *Rhizobiaceae* and *Paenibacillaceae* where the entire available collection of cultivars could be differentiated at the species level based on a range between 80% and 97% of nucleotide sequence similarity (data not shown). Other cutoffs of unique diazotrophic taxa fall within this range (Gaby and Buckley, 2011). The distributions of OTU data were used to calculate