Biological Conservation 217 (2018) 113–120

Contents lists available at ScienceDirect

Biological Conservation

journal homepage: www.elsevier.com/locate/biocon

High-throughput eDNA monitoring of fungi to track functional recovery in ecological restoration

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ARTICLE INFO

Keywords:
Ecosystem function
eDNA
Genomics
Land degradation
Microbiome
Restoration genomics

ABSTRACT

Fungi are key functional components of ecosystems (e.g. decomposers, symbionts), but are rarely included in restoration monitoring programs. Many fungi occur belowground, making them difficult to observe directly, but are observable with environmental DNA (eDNA) methods. Although eDNA approaches have been proposed as ecological monitoring tools for microbial diversity, their application to restoration projects is very limited. We used eDNA metabarcoding of fungal ITS barcodes on soil collected across a 10-year restoration chronosequence to explore fungal responses to restoration. We observed a dramatic shift in the fungal community towards that of the natural fungal community after just 10 years of active native plant revegetation. Agaricomycetes and other Basidiomycota – involved in wood decay and ectomycorrhizal symbiosis – increased in rarefied sequence abundance in older restored sites. Ascomycota dominated the fungal community, but decreased in rarefied sequence abundance across the restoration chronosequence. Our results highlight eDNA metabarcoding as a useful restoration monitoring tool that allows quantification of changes in important fungal indicator groups linked with functional recovery and, being underground, are normally omitted in restoration monitoring.

1. Introduction

Land clearing and unsustainable land use are driving a global land degradation crisis (Gibbs and Salmon, 2015; Nkonya et al., 2016). Ecological restoration is employed as the primary intervention to repair degraded land, largely to re-instate functional ecosystems and native biodiversity (Suding et al., 2015). Effective and targeted restoration is required, in combination with accurate biological monitoring, to achieve these restoration goals (Collen and Nicholson, 2014).

Terrestrial ecosystems consist of aboveground and belowground components that interact to shape ecological communities (Wardle et al., 2004). Plants influence the composition of belowground biota, and in turn, belowground biota feedback to influence plants. For example, fungi contribute major ecological functions such as decomposition and nutrient cycling, especially carbon and soil aggregation (Avis et al., 2017; Morriën et al., 2017). Mycorrhizal fungi can influence the status of soil nutrients, and also the establishment, diversity and succession of plants (Cavagnaro et al., 2005; Kulmatiski et al., 2008). Therefore, understanding the dynamics of fungal communities is important to influencing ecosystem functions (Gehring et al., 2014), and as such should be a key focus point of ecological restoration.

Fungi are large components of the biodiversity in many soil ecosystems, even in species-poor plant communities (Taylor et al., 2014). The diversity and community dynamics of soil fungi are often linked to soil physical, chemical, and biological properties (e.g. age, pH, nutrient levels) (Guo et al., 2016; Moon et al., 2016; Trivedi et al., 2016; Zechmeister-Boltenstern et al., 2011). These soil properties are often influenced by vegetation cover, land-use, and revegetation practices. As such, assessing changes in the fungal community during ecological restoration is an important part of determining the return of functional ecosystems and native biodiversity to restoration sites – key indicators of restoration success (Harris, 2009). Despite the potential for revegetation to influence fungal diversity, few restoration projects have monitored changes in the fungal community, and used the fungal data as part of the assessment of restoration progress and success (Harris, 2003).

A primary reason why fungi often go unmonitored in restoration is that many are belowground and microscopic, making them difficult to observe *in situ*. However, with next generation sequencing approaches, researchers can now efficiently and accurately assess such highly

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http://dx.doi.org/10.1016/j.biocon.2017.10.035
Received 19 July 2017; Received in revised form 17 October 2017; Accepted 29 October 2017

diverse and cryptic biological communities (Lindahl et al., 2013). High-throughput amplicon sequencing of environmental DNA (eDNA) – metabarcoding – can identify and quantify the biological sources of genetic material (Barnes and Turner, 2015; Corlett, 2017; Ji et al., 2013). As such, metabarcoding has been put forward as a cost-effective, efficient and easy-to-standardise approach that can be used to survey and monitor even the most cryptic biodiversity. Metabarcoding has already proven to be an effective and efficient method to survey soil bacterial and fungal microbiomes (Rime et al., 2015; Taberlet et al., 2012). However, there are few examples of using metabarcoding to explore changes in biodiversity in a restoration context (Gellie et al., 2017b; Mills et al., 2017).

In this study, we tested the hypothesis that replanting the native plant community into an ex-pasture will lead to restoration of the fungal community. To test this hypothesis, we used metabarcoding to explore the soil fungal community across a 10-year revegetation chronosequence, including samples from remnant sites (the revegetation reference sites), and cleared sites. We analysed these samples to address the following questions: (i) Does native overstory revegetation alter the soil fungal community? (ii) Which functional groups of fungi are indicators of the different stages of revegetation? (iii) How do soil physicochemical parameters respond to revegetation, and do these changes associate with the fungal community?

2. Material and methods

2.1. Site description and sampling

Our study system was an active restoration site at Mt Bold, a water catchment reserve of the Mt Lofty Ranges in South Australia (35.07°S, 138.42°E), described in detail in Gellie et al. (2017b). This catchment was dominated by open eucalypt woodland, but has been cleared and grazed from early in the 20th century. Grazing ceased in 2003, restoration began in 2005, and the restoration goal was to recreate the local *Eucalyptus leucoxylon* grassy woodland community, as found in the remnant, reference sites (Remnant A and B). Prior to 2005, Remnant A was minimally cleared and had low-density grazing, and remnant B was protected from clearing and had minimal human impact. Each reference site is in close proximity to the restoration site (< 1 km).

Revegetation methods were consistent across the study system. This included the use of the same site preparation method (i.e. shallow surface rip), plant species mix (i.e. replanting the same subset of overstory and mid-story plant species present in the local woodland community), timing (i.e. late winter planting), and maintenance (i.e. fencing to exclude livestock, annual grass slashing, woody weed removal). The sites restored between 6 and 10 years ago were revegetated with the same local, native plant species, including the overstory South Australian blue gum (*E. leucoxylon*) and manna gum (*E. viminalis*), and a shrub layer that included golden wattle (*Acacia pycnantha*), sticky hop bush (*Dodonaea viscosa*) and sweet bursaria (*Bursaria spinosa* ssp. *spinosa*). Remnant A had weed control, and remnant B was managed for conservation.

In January 2015, we sampled soil from three randomly selected 25 × 25 m quadrats at each of seven sites, including sites restored 6, 7, 8 and 10 years before sampling, a cleared site, and the two remnant, reference sites (the restoration reference sites; remnant A and B in Gellie et al., 2017b), giving a total of 21 quadrats. Soil was sampled from the 0–10 and 20–30 cm soil horizons at each quadrat. The data used for this work was generated from the Biomes of Australian Soil Environments (BASE) database workflow, and is downloadable as OTU abundance tables from the BASE download portal (samples 102.100.100/19281–19322). Below we briefly describe the BASE methods, which are described in detail in Bissett et al. (2016). Sampling was conducted as part of the Biomes of Australian Soil Environments (BASE) project according to the protocol described in Bissett et al. (2016). Briefly, nine soil samples per quadrat were pooled into a sterile plastic bag, homogenised using a sterilised trowel, and frozen on site in sterile 50 mL falcon tubes – hereafter the replicates (n = 42). 300 g of homogenised soil was also sampled for soil physicochemical analysis, quantifying soil moisture, ammonium, nitrate, available phosphorus, sulphur, organic carbon, and soil pH (H_20).

2.2. Genomic analyses

DNA extraction and sequence analysis were conducted according to the methods described in Bissett et al. (2016). Briefly, soil DNA was extracted in triplicate using MoBio PowerSoil extraction kits according to manufacturer's instructions, together with extraction blank controls. We PCR-amplified the fungal internal transcribed spacer (ITS) region for each replicate with negative controls using primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990). PCR products were screened for negative control contamination with gel electrophoresis, purified using the Agencourt AMPure XP bead PCR product purification kit as per manufacturer's instructions, concentration normalised to 10 nM, and sized on an Agilent Bioanalyze. Equal volumes of products were pooled, diluted to 4 nM and sequenced on the Illumina MiSEQ platform with MiSeq Reagent Kit v3 600 cycle chemistry, to produce 300 bp paired end reads.

Read analysis was also done as per Bissett et al. (2016) as part of the BASE dataset analysis. Briefly, the ITS1 region was extracted from Illumina R1 reads using ITSx (Bengtsson-Palme et al., 2013) and Operational Taxonomic Units (OTUs) clustered at 97% sequence similarity between ITS1 reads using USEARCH v8.0.1517 (Edgar, 2010). OTUs were classified against the UNITE v7.0 fungal database (Koljalg et al., 2013), using the Wang classifier (Wang et al., 2007) in MOTHUR. We discarded OTUs not identified as belonging to fungi, unidentified at the phylum level, or having < 100 reads across the full BASE dataset (> 900 samples) as in Gellie et al. (2017b).

2.3. Statistics

We used R v 3.3.2 (R Core Team) for all statistical analyses. OTU abundance was rarefied to the replicate with the lowest number of reads (49,724 reads for 0–10 and 51,138 reads for 20–30 cm soil samples, respectively) with the *rarefy* function in *vegan* v 2.4-3 (Oksanen et al., 2017). OTU richness was measured using the Chao 1 nonparametric richness estimator. Diversity was estimated as the effective number of species (Jost, 2006) using the Shannon-Wiener index (H) and the Gini-Simpson index (D), where the Shannon-Wiener index and Gini-Simpson index were transformed by using the formula exp(H) and 1/(1-D), respectively, to evaluate the true diversity of the fungal community.

Differences in rarefied abundances of the sequence reads, OTU richness, diversity indices, phyla, classes and soil characteristics across the restoration sites (i.e. the restoration chronosequence), soil depths, and the interaction between restoration site and soil depth were analysed using a multifactor permuted analysis of variance (PERMANOVA) with the *aovp* function implemented in *lmPerm* 2.1.0 package with 5000 permutations.

The effect of the restoration sites on fungal composition was visualised using non-metric multidimensional scaling (NMDS) ordinations using Bray-Curtis (rarefied abundance) and Jaccard (presence-absence) dissimilarity matrices, which were generated with *vegan's vegdist, metaMDS, stressplot and ordiplot* functions (Oksanen et al., 2017). Differences in fungal community composition across the restoration chronosequence and soil depths were tested using ANOSIM analysis (999 permutations) on Bray-Curtis dissimilarity matrices with the *anosim* function in vegan, estimating *R* values, where *R* close to 1 indicates high separation between groups (e.g. between restoration sites) and *R* close to 0 indicates little separation between groups.

Distance-based redundancy analyses (db-RDA) were run to visualize the relationships between soil physical and chemical variables and