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Deep Ion Torrent sequencing identifies soil fungal community shifts after frequent prescribed fires in a southeastern US forest ecosystem

Shawn P. Brown[1]*, Mac A. Callaham, Jr.[2], Alena K. Oliver[1], Ari Jumpponen[1]

1 Division of Biology, 421 Ackert Hall, Kansas State University, Manhattan, Kansas 66506

2 Center for Forest Disturbance Science, USDA Forest Service, Southern Research Station, 320 Green Street, Athens, Georgia 30602

* corresponding author

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* Corresponding Author:
Shawn P Brown
Division of Biology
421 Ackert Hall
Kansas State University
Manhattan, KS 66506
Tel. 785 532 3934
FAX 785 532 6653
Email spbrown1@k-state.edu

Abstract

Prescribed burning is a common management tool to control fuel loads, ground vegetation, and facilitate desirable game species. We evaluated soil fungal community responses to long-term prescribed fire treatments in a loblolly pine forest on the Piedmont of Georgia and utilized deep Internal Transcribed Spacer Region 1 (ITS1) amplicon sequencing afforded by the recent Ion Torrent Personal Genome Machine (PGM). These deep sequence data (19,000+ reads per sample after subsampling) indicate that frequent fires (3 year fire interval) shift soil fungus communities whereas infrequent fires (6 year fire interval) permit system resetting to a state similar to that without prescribed fire. Furthermore, in nonmetric multidimensional scaling analyses, primarily ectomycorrhizal taxa were correlated with axes associated with long fire intervals whereas soil saprobes tended to be correlated with the frequent fire recurrence. We conclude that 1) multiplexed Ion Torrent PGM analyses allow deep cost effective sequencing of fungal communities, but may suffer from short read lengths and inconsistent sequence quality adjacent to the sequencing adaptor; 2) frequent prescribed fires elicit a shift in soil fungal communities; and, 3) such shifts do not occur when fire intervals are longer. Our results emphasize the general responsiveness of these forests to management, and the importance of fire return intervals in meeting management objectives.

Introduction

Prescribed fire is a tool in widespread use by forest managers particularly across the southeastern United States. Prescribed fire is an economical way to manage the accumulation of fuel, to control woody competing vegetation, and to restore structural and functional attributes of the understory communities of natural and plantation forests (Waldrop *et al.*, 1992). Fires also promote a diverse understory of grasses and forbs, which can support populations of desirable game species (Phillips & Waldrop, 2008). Previous studies have also suggested shifts in soil (Bastias *et al.*, 2006; Anderson *et al.*, 2007) and root-associated (Baar *et al.*, 1999; Chen *et al.*, 2002; Bastias *et al.*, 2006; Buscardo *et al.*, 2010) fungal communities in response to fire particularly if the fire intervals are short. Frequent fire regimes have also been reported to suppress ectomycorrhizal species (Smith *et al*., 2005), and to decrease root colonization by ectomycorrhizal species (Dahlberg *et al.* 2001; Schoenberger & Perry, 1982). Similarly, studies focusing on wildfire events indicate that microfungal communities respond to the fires but recover over time (Wicklow & Whittingham, 1978; Lucarotti *et al.* 1978). In addition to the community composition, prescribed fires may also alter the biomass (Smith *et al*., 2005; Dooley *et al*., 2011) or functional redundancy and richness of soil fungal communities (Artz *et al.*, 2009).

In the past decade, next generation sequencing (NGS) has improved our understanding of bacterial (Lozupone & Knight, 2007; Sogin *et al*., 2006) and fungal (Buee *et al.*, 2009; Jumpponen & Jones, 2009; Öpik *et al.*, 2009) diversity in complex environments as a result of a

more comprehensive interrogation of microbial communities (Gilbert *et al*., 2010). However, the high costs of NGS tools have limited small laboratories' access but lowering these costs might enable 'democratization' of sequencing in microbial ecology (Caporaso *et al.*, 2012; Glenn, 2011). Here, we adopted Ion Torrent Personal Genome Machine (PGM) (Life Technologies, Gulford, Connecticut) for investigating fungal communities in a long-term prescribed fire experiment. Ion Torrent PGM does not depend on a costly light detection system (Glenn, 2011) but measures hydrogen ion release during nucleotide incorporation resulting in changes in pH (Rothberg *et al*., 2011). We used this young technology to generate deep sequencing data of Internal Transcribed Spacer (ITS) amplicons (more than 19,000 reads per sample after quality control and subsampling). The resulting data are congruent with studies in other forested systems focusing on either ectomycorrhizal or soil-borne fungal community responses to fire and suggest that different guilds of soil-inhabiting fungi respond differently to recurring fires.

Materials and Methods

Study site

The study sites are located on the Hitchiti Experimental Forest (HEF) in Jones County, Georgia, U.S.A. (32º58'N, 90º44'W), within the Ocmulgee River drainage, part of the Altamaha River Basin. Historically, much of the area was used for cotton agriculture prior to conversion to forestry. The HEF is a sub-unit of the Oconee National Forest and is situated approximately 18 km east of the town of Gray, Georgia, between the North and South Units of the Piedmont National Wildlife Refuge.

Hydrologic and edaphic conditions at the study site are typical of the Georgia Piedmont with highly eroded Alfisols and Ultisols planted with loblolly pine (*Pinus taeda* L.). Soils are mapped as Wilkes and Davidson Series according to the United States Department of Agriculture Soil Conservation Service mapping. Wilkes soils consist of shallow, well-drained soils with moderately slow to slow permeability. These soils formed in residuum weathered from intermediate and mafic crystalline rocks on uplands in the Piedmont, and are classified as loamy, mixed, active, thermic, shallow Typic Hapludalfs. Davidson soils consist of very deep, well-drained, moderately permeable soils that formed in materials weathered from dark colored rocks high in ferromagnesian minerals. These soils reside mainly on gently sloping to moderately steep uplands in the Piedmont, and are classified as fine, kaolinitic, thermic Rhodic Kandiudults.

Experimental design

The long-term prescribed fire experiment was established at the HEF in the winter of 1988/1989 when all plots (except the unburned controls) were burned with low intensity backfires. Each burn treatment is replicated in a total of four ~0.8 ha plots. These plots were all located in a 70+ year old natural stand of mixed pine and hardwood that had not been burned for at least 50 years prior to the initiation of this study. The primary objective of the study was to evaluate long- and short-term soil and vegetative effects of recurring prescribed fire with different return intervals (3 yr, 6 yr, and an unburned control). The HEF has suffered from southern pine beetle outbreaks in the last 7-10 years. We sampled the plots regardless of insect damage to determine whether legacy effects of recurring fires since 1988 can be detected in the soil fungal communities. For this study, sampling included only one insect damaged plot – we retained it in our analyses after determining that including it did not change our conclusions on the fire responses.

Soil sampling

We separated the plots into four sampling blocks so one plot representing each treatment could be sampled in a single day to minimize any temporal confounding effects. As a result, all samples were collected in a total of four days between November 1 and 16, 2011. For plots with living overstory, sampling consisted of randomly selecting fifteen representative dominant canopy trees (loblolly pine; *Pinus taeda* L.) distributed across the whole plot (but at least 10m away from the plot edge). At each of these fifteen trees, 3m directly south of the bole, we collected one 10 cm deep 5 cm diameter soil core; all fifteen samples were composited into one sample per plot. For one plot, where outbreaks of southern pine beetle had recently occurred (resulting in mortality of most overstory trees), we sampled in a systematic grid of fifteen points (3 x 5 points) spaced at approximately 5 m apart.

The soil samples were stored on ice until frozen at –20°C and shipped frozen and packed in dry ice to Kansas State University (KSU). Upon receipt at KSU, the samples were stored at -80ºC until further processing. The samples were thawed at room temperature and passed through a 1mm mesh (No. 18) sieve to remove rocks, large particles, and roots. The sieved soil was homogenized by hand for approximately 5min and ~10g (9.07-10.7g fresh weight) soil placed into a 50mL Bead Solution Tube (UltraClean Mega Soil DNA Kit, MoBio, Carlsbad, California) and stored at -80°C until DNA extraction according to manufacturer's protocol. Extracted DNA was eluted in 100 µl of S5 buffer.

PCR reactions were performed in triplicate for each experimental unit to minimize PCR biases.

The technical replicates were combined into one per experimental unit after amplicon clean up. In order to produce amplicons sufficiently short to accommodate the Ion Torrent emPCR chemistry (~300 bp maximum) whilst specifically targeting fungal community, we opted to use a nested PCR protocol utilizing common fungus specific primers (ITS1f-ITS4) in the primary reactions. To produce the desired short amplicons, we chose more generic primers (ITS1-ITS2) nested within the primary PCR products in the secondary reactions. The conditions for the initial 25 µL PCR reactions were: 10ng template DNA per sample, 200 µM exACTGene dNTPs (Fischer BioReagents, Fair Lawn, New Jersey), 10 µM forward (ITS1f, Gardes & Bruns, 1993) and reverse (ITS4; White *et al.*, 1990) primers, 25mM $MgCl_2$, 5 µL 5x Green GoTaq® Flexi Buffer (Promega, Madison, Wisconsin), 7.8 µL molecular biology grade water, and 1 U GoTaq® Hot Start Polymerase (Promega, Madison, Wisconsin). All reactions were performed using Eppendorf Mastercyclers (Hamburg, Germany) with initial 94°C denaturation for 4 min followed by 25-cycles of denaturing in 94°C for 1 min, 55°C annealing for 1 min, and 72°C extension for 2 min with a final extension at 72°C for 10 min. Short nested amplicons were produced using primers ITS1 and ITS2 (White *et al.*, 1990) in a step-down PCR with 2µL of initial PCR product as template and PCR reagents as above in a reaction with 10 cycles that consisted of initial 94°C denaturing for 4 min, and 1°C step-down from 58°C to 55°C for 1 min in four cycles of 94°C denaturing for 1 min and 72°C extension for 2 min followed by six additional cycles with annealing at 55°C. As above, the reaction was completed with a final extension at 72° for 10 min. To minimize the DNA-tagged-primer bias, the Ion Torrent specific adapters and the sample specific DNA tags (Multiplex Identification DNA-tags; MIDs [see Supplemental Table S1 for complete MID list]) were incorporated into the PCR amplicons in an additional PCR reaction to reduce 3' end primer biases (Berry *et al.*, 2011). These reactions consisted of 4µL of the nested

PCR product as template, 200 μM dNTPs, 5 μM Ion Torrent PGM sequencing primer (ITS2–MID–P1) and emPCR primer (ITS1-A), 25mM $MgCl_2$, 10 μL 5x Green GoTaq® Flexi Buffer, 14.6μL molecular grade water and 2 U GoTaq® Hot Start Polymerase in following PCR conditions: 94°C for 2 min, 5 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 10 min. Negative controls consisting of molecular grade water in place of extracted DNA underwent the three PCR reactions under the same experimental conditions and were amplicon free indicating library production was contamination free. The three replicate PCR amplicons per each experimental unit were cleaned with Agencourt® Ampure® using a SPRIplate 96-ring magnet (Beckman Coulter, Beverly, Massachusetts) per manufacturers protocol with the modification of a 1:1 bead to product ratio to further increase discrimination against small fragments. After cleaning, the three technical replicates were combined into one per experimental unit. The amplicons were pooled equimolarly such that each experimental unit was equally represented in the final pool. To remove lingering short (~50bp) non-target DNA, the final pool was cleaned once more as described above. Prior to sequencing, the cleaned amplicons were assessed for size distribution and DNA concentration using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California). The amplicon library was sequenced using one Ion 318™ Chip at Cofactor Genomics (St. Louis, Missouri) on August 4, 2012. The raw sequence data (.fastq file) are available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under accession number SRR630872.

The sequence data were analyzed using the MOTHUR pipeline (v. 1.25.0; Schloss *et al.*, 2009) following a modified standard operating procedure (Schloss *et al.*, 2011). Briefly, the data were

subjected to quality control whereby each sequence was screened for a match to the sequencing primer (ITS2-P1), a valid DNA tag (MID), and thresholds for average Phred quality score ($Q \geq 25$), ambiguous bases (count = 0), and homopolymers (length ≤ 8). Each sequence that passed quality filtering was truncated to a 150bp length after removal of primer and MIDs; shorter sequences were omitted. To control for PCR- or sequencer-generated errors, we pre-clustered the remaining sequences to remove sequences within one base pair's distance from a more abundant sequence using a pseudo-single linkage algorithm implemented in mothur (pre.cluster, diff=1). All potentially chimeric sequences were identified using mothur-embedded uchime (chimera.uchime; Edgar *et al.*, 2011) and removed (439 unique sequences were determined to be chimeric representing 773 total sequences). The sequence data were sub-sampled to 19,292 sequences per sample to ensure comparable estimators across experimental units (see Gihring *et al.*, 2011). Unique sequences were pairwise aligned (Needleman-Wunch; Needleman & Wunch, 1970) and the resultant distance matrix clustered using the nearest-neighbor algorithm at 97% similarity. We used the nearest-neighbor algorithm because the short reads in our study (150 bp) do not allow the sensitivity necessary to cluster using the more common average-neighbor algorithm at 97% sequence similarity without severely underestimating cluster numbers. Because of their uncertain origin, all global singletons (clusters that were only found once across all experimental units) were omitted (Tedersoo *et al.*, 2010). Rarefaction (Magurran, 1988), observed OTU richness (S_{obs}), diversity (Simpson's complement; $1\text{-}D = 1 - \sum p_i^2$), evenness (Simpson's equitability; $E_D = (1/\sum p_i^2)/S$), and Good's coverage (complement of the ratio between local singleton OTUs and the total sequence count) were estimated. Where appropriate, analysis of variance was performed in JMP (v.7.1; SAS Institute, Cary, North Carolina) to identify treatment level differences. Soil fungal community compositions were visualized using

Nonmetric Multidimensional Scaling (NMDS; Borg, 1997) based on Yue and Clayton's dissimilarity matrix (Yue & Clayton, 2005). Treatment differences based on the Yue and Clayton's dissimilarity matrix were tested using an Analysis of MOlecular VAriance (AMOVA; PERMANOVA in Anderson, 2001). In addition, we also tested for treatment differences utilizing a Bray-Curtis dissimilarity matrix, which provides dissimilarity values that are inherently more constrained than Yue and Clayton's dissimilarities to test for congruence of these two metrics.

Taxonomic affinities of a randomly selected representative sequence for each OTU were obtained using BLASTn (nr/nt) after exclusion of uncultured/environmental data. The best BLAST match that reported taxonomic levels below phylum was recorded for the 200 most abundant OTUs by frequency (Supplemental Table S2). Representative sequences of all called OTUs (including singletons) as well as representative sequences of the 200 most abundant OTUs are provided (see supplemental information). To confirm BLAST-based affinities, we MUSCLE-aligned representative OTU sequences of four common BLAST-assigned genera (*Cenococcum, Hygrocybe, Leohumicola, and Mortierella*) with sequences of isolated or vouchered specimens, including closely related genera as an outgroup, from GenBank (http://www.ncbi.nlm.nih.gov/genbank) and generated Neighbor Joining trees (Supplemental Figures S1a-d) in Geneious Pro (v. 5.3.4; Biomatters Ltd., Christchurch, New Zealand). The OTU sequences usually nested with references and these affinities were supported in bootstrap analyses (1,000 bootstrap replicates). The 200 most abundant OTUs were analyzed for responses to treatments (ANOVA of arcsine transformed OTU frequencies). We used False Discovery Rate (FDR) to correct for multiple comparisons. Despite the liberal 5% false discovery rate, none of

the OTUs differed in frequency across treatments. It is of note that the inclusion or omission of global singletons did not affect our overall conclusions, indicating minimal singleton effect on compositional, diversity or evenness in our system, but likely exaggerate observed and extrapolative richness estimators.

Results

We interrogated fungal responses to long-term prescribed fire manipulations using an Ion Torrent PGM dataset comprised of >3,000,000 sequences with an average read length of 129.4 ± 69.9 (mean ± SD). After omitting ~80% of sequence data because of homopolymers exceeding eight nucleotides, low average quality scores (Q < 25), or insufficient read length (< 150bp), we retained a total of 333,919 high-quality sequences that were burdened with high variability in quality scores in the first 30bp and subsequent decline as read length increased (Fig. S2). These attributes necessitated our choice to use the average Q values ≥25, instead of a sliding window analysis recommended in the mothur standard operating protocol.

The obtained sequence data represented a diverse soil fungal community comprised of 1987 OTUs (6,499 OTUs with singletons included) across the three burning treatments. Many OTUs (747, ~38%) were unique to a treatment (Fig. 1) suggesting high beta-diversity. Observed OTU richness (Fig. 2a) estimators did not differ among the burn treatments ($F_{2,9} = 0.46$, $P = 0.646$). Rarefaction analyses indicated a plateau (Fig. 2b) and our coverage estimators (Fig. 2c) were remarkably high (0.992 ± 0.001, mean ± SD across all treatments) indicating overall an excellent OTU coverage afforded by the deep sequencing. The coverage estimators did not differ among

the treatments ($F_{2,9} = 0.09$, $P = 0.916$) indicating similarly deep recovery of the community constituents across the treatments. Diversity (Fig. 2d) and evenness (Fig. 2e) differed among the treatments ($F_{2,9} = 4.77$, $P = 0.038$; $F_{2,9} = 9.10$, $P = 0.007$, respectively) and post-hoc tests (Tukey's HSD at $\alpha = 0.05$) indicated that 3 year burn treatments were less diverse and even than 6 year or un-burned control treatments.

AMOVA on the community distance matrix (Yue and Clayton's dissimilarity matrix based on shared and non-shared OTUs) and NMDS analyses (Fig. 3) indicated that soil-inhabiting fungal communities differed among the treatments ($F_{2,9} = 2.07$, $P = 0.007$). The 3 year burn treatment was distinct from the 6 year ($F_{2,9} = 2.63$, $P = 0.003$) and un-burned control treatments ($F_{2,9} = 2.46$, $P = 0.025$), but the 6 year and control treatments were not observed to differ significantly from each other ($F_{1,6} = 1.25$, $P = 0.243$). It is important to bear in mind that our data matrix contained a large number of OTUs unique to a given treatment (~38%; Fig. 1) that may exaggerate compositional differences among the treatments, despite the considerable number of OTUs that were shared among the treatments. For this reason, AMOVA based on the more constrained Bray-Curtis dissimilarity matrix was performed and results were largely congruent. However the overall community differences were marginally significant ($F_{2,9}=1.24$, $P=0.085$) and the 3 year burns differed from the unburned control ($F_{1,6}=1.68$, $P=0.025$).

Analyses of the 200 most frequent OTUs (representing 196,399 or 60.0% of all sequences, Table S2) indicated that the soil fungal communities were dominated by ascomycetes by OTU count and sequence count (113 of 200 OTUs, 96,913 sequences representing 49.34% of the 200 most

frequent OTU sequences) followed by basidiomycetes (78 of 200 OTUs, 92,161 sequences (46.92% of sequences)). OTUs representing Glomeromycota (1 of 200, 172 sequences) or basal fungal lineages (in total 8 of 200 OTUs, 7153 sequences (3.64% of sequences)) distributed across Mucorales, 3 OTUs; Mortierellales, 3 OTUs; Entomophthorales, 2 OTUs) were rare. The observed fungal communities were diverse and dominated by Herpotrichiellaceae (17 OTUs, 9,130 sequences) and Pyronemataceae (16 OTUs, 13,839 sequences) within Ascomycota; Russulaceae (14 OTUs, 7,407 sequences) and Pucciniaceae (6 OTUs, 2,360 sequences) within Basidiomycota. None of the most common 200 OTUs differed in frequency across the treatments after correction for multiple comparisons. However, when the OTU frequencies and NMDS axes were analyzed for correlation, 12 of the 200 most frequent OTUs were strongly and significantly correlated with Axis 2 (Table 1) suggesting OTU associations with fire treatments (negatively correlated OTUs are implicitly associated with 3 Year burning regimes whereas positively correlated OTUs are associated with 6 Year and unburned control burring treatments). Of the top 200 most frequent OTUs, six were significantly negatively correlated with Axis 2 (53.2% of community variation). These six potentially phoenicoid OTUs (Carpenter & Trappe, 1985) are all putative or potential soil-borne saprobes including OTU 1329 (*Trichophaea abundans*) – a known pyrophilous species and a genus common in recently burned ground (Turnau, 1983; Fujimura *et al.,* 2005). In contrast, six OTUs were positively and significantly correlated with NMDS Axis 2 indicating their correlation with unburned or long fire return interval plots. These OTUs were assigned to putatively mycorrhizal (OTU 4 – *Cenococcum*, OTU 53 – Atheliaceae sp., and OTU61 – *Ramariopsis*), lichenized (OTU 3353 – *Lecanora*), and plant pathogenic (OTU 3347 – *Puccinia*) taxa (Table 1).

Discussion

We analyzed fungal communities in a prescribed fire experiment and tested the utility of the Ion Torrent PGM. Prescribed fire can be used to control fuel accumulation and can alter understory plant communities structurally and functionally (Waldrop *et al.*, 1992) favoring diverse grasses and forbs that support game species (Phillips & Waldrop, 2008). In addition to these shifts in aboveground communities, previous studies have suggested that both soil fungal communities and biomass may be sensitive to prescribed burning regimes (see Smith *et al.*, 2005; Dooley *et al.*, 2011). Our analyses indicated that soil-inhabiting fungal communities differed compositionally between the 3 year burn treatments and the 6 year burn or unburned control treatments. Similar responses in soil fungal communities to short-term fire intervals have also been observed in Australian sclerophyll forest using RFLP and DGGE analysis (Bastias *et al.*, 2006; Anderson *et al.*, 2007). Our observations are congruent with studies focusing on the fire effects on ectomycorrhizal fungi, whose communities also shift in response to prescribed burning (Baar *et al.*, 1999; Chen *et al.*, 2002; Bastias *et al.,* 2006; Buscardo *et al.*, 2010) suggesting differing fire tolerances among mycorrhizal fungi. However, our results contrast those of Artz and colleagues (2009), who cloned and sequenced functional laccase genes to evaluate responses of soil Basidiomycete functional diversity and recorded a greater diversity and evenness in short-term burn treatments than in unburned controls. We observed no responses in fungal community richness, but recorded decrease in diversity and evenness as a result of frequent recurring fires. This likely indicates that frequent fires favor few members of the communities and increase their dominance. This conclusion is also supported by the observed compositional shifts in our soil fungal communities.

The observed community shifts were likely attributable to small changes in relative frequencies of common OTUs and – to a greater extent – changes in frequencies in the less frequent OTUs. We base these conclusions to two primary lines of evidence. First, our analyses suggested that although fungal communities shift in response to fire treatment, they rarely responded strongly to prescribed fire and, when they did, the differences among the fire treatments did not remain significant after correction for multiple comparisons in ANOVA. Second, in contrast to ANOVA, correlation analyses identified twelve among the most frequent 200 OTUs that correlated with fire treatments. This suggests that the community shifts were driven largely by a limited number of commonly observed OTUs that responded to the treatments. Our results support earlier observations that community shifts are often driven – not by the loss of dominants – but shifts in the less frequent subordinate taxa (see e.g. Jumpponen & Jones, 2009), which were included in our NMDS analyses but too infrequent to analyze by ANOVA.

The common OTUs that correlated with the ordination axes are interesting. While the list was dominated by soil fungi with uncertain ecologies, three putatively ectomycorrhizal OTUs (*Cenococcum*, *Ramariopsis,* Atheliaceae sp.) were negatively correlated with the frequent, 3 year interval fire treatment. Although these data are sparse, it is tempting to speculate whether or not the frequent fires favor non-symbiotic fungi in forested ecosystems. The response by *Cenococcum* supports such speculation as it appears to be strongly suppressed by short-term burn regimes (Smith *et al*., 2005). More generally, our results are seemingly congruent with previous studies suggesting that frequent burn regimes favor non-mycorrhizal over ectomycorrhizal fungal communities (Chen *et al*., 2002). This was demonstrated here by the

abundance and negative correlation of six putative saprobic soil-borne fungi along Axis 2. It is important to note that due to the relatively short reads (150 base pairs) the assignment of OTUs into known taxa becomes potentially problematic and confidence in assigned OTU ecologies should be treated with caution.

Ion Torrent PGM performance

NGS technologies have opened the microbial ecologists' toolbox for a deeper interrogation of microbial communities. The Ion Torrent PGM provides a new platform that relies on measuring shifts in hydrogen ion concentrations resulting from nucleotide incorporation rather than utilizing light emitting reporter molecules that are quantitated with high-resolution cameras. As a result, Ion Torrent PGM machines are small, relatively inexpensive and sequencing can be performed in just few hours continuing the democratization of NGS (Caporaso *et al.*, 2012). The first bacterial community analyses using amplicon sequencing on the PGM platform are starting to emerge (*e.g.* Jünemann *et al.*, 2012; Whiteley *et al.*, 2012; Yergeau *et al.*, 2012). Our goal was to test PGM utility for fungal community analysis. In doing so, we also highlight challenges in adopting emerging technologies (primer selection, careful lab procedures, bioinformatic support, interpretation of quality control standards).

The sequences we obtained with Ion Torrent PGM chemistry aiming for 200bp read length provided an average read length of 129 bp. Although PGM read lengths are expected to increase, they remain shorter than those from GS-FLX Titanium unidirectional amplicon sequencing. Our data were similar to Whiteley *et al.* (2012), where the quality scores for bacterial amplicons

sequenced on chips preceding our 318™ also fell remarkably with sequence length (see Fig. 2d in Whiteley *et al.*, 2012). The current emPCR chemistry for Ion Torrent PGM is limited to fragments ~300bp in length. While this limitation may not present an obstacle in bacterial 16S community analysis, finding fungal primers that do not introduce lineage specific biases may prove difficult (see Ihrmark *et al.*, 2012). In addition to the steady decline of sequence quality after 100bp, we observed highly variable sequence quality adjacent to the Ion Torrent PGM adapter (Fig. S2). While some NGS platforms suffer from platform inherent errors, including homopolymer errors (Margulies *et al.*, 2005) and base calling biases (Erlich *et al.*, 2008), the low sequence quality on PGM did not previously map to homopolymers (Whiteley *et al.*, 2012). Further systematic comparisons between Ion Torrent PGM and other NGS platforms are necessary to better understand these variations in sequence quality as has been done for other platforms (Luo *et al.*, 2012).

One difficulty in adopting new NGS technologies is how to filter, sort, and analyze the resultant sequence data. Established data tools and pipelines for 454 sequencing have two main methods for ensuring high-quality sequence yields. The first method uses platform-generated data to correct flowgrams for near-neighbor light seepage using an expectation-maximization algorithm such as PyroNoise (Quince *et al.*, 2011). The second, more common method uses Phred scores to cull questionable sequences by implementing a minimum average quality threshold for entire sequences (Ribosomal Database Project, PyroTagger) or utilizing a sliding window average analysis (mothur standard operating protocol) or culling sequences that have one nucleotide below Phred score threshold. The first option (flowgram correction) is currently unavailable for Ion Torrent PGM due to lack of established ionogram correction data. Accordingly, to analyze

our data, we filtered sequences by quality scores. The very low quality scores adjacent to the sequencing primers made the use of this option problematic. Recommendations for processing NGS data from emerging technologies will likely be forthcoming if the platform gains popularity for analyses such as ours.

Conclusions

Our results show that for fungal community analyses, the PGM platform provides a low cost, scalable and high throughput solution for multiplexed DNA tag sequencing and should therefore lower the threshold for conducting such experiments. Our conclusions on the fungal community responses to prescribed fire are congruent with others and indicate that frequent, recurring prescribed burning leads to shifts in soil fungal communities, whereas greater fire intervals permit system resetting back to a state that resembles unburned conditions. In sum, we conclude that the forest systems – above and below ground – are generally responsive to management by prescribed fire. If prescribed fire management aims to shift understory plant community, maintaining short fire intervals may be necessary as no such changes occur with longer fire intervals. This, however, may have functional consequences in the belowground compartment as high fire frequency may increase the non-symbiotic fungi at the cost of mycorrhizal associates.

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Table 1. OTUs with significant Spearman rank correlations with NMDS Axis 2 (53.2% community variation). OTUs represent those that belong to the 200 most common OTUs and 60.0% of all analyzed sequences. OTUs with negative coefficients are associated with 3 year burn treatments; positive coefficients are associated with 6 year and control burn treatments. Table shows OTU BLASTn affinity to genus where available

OTU	BLASTn affinity	ρ	*P*-value
2	Wallemiomycetes sp.	-0.9091	0.0004
36	*Umbelopsis*	-0.6923	0.0125
40	*Sorocybe*	-0.6014	0.0386
115	*Udeniomyces*	-0.6900	0.0013
1166	Dothideomycetes	-0.6609	0.0193
1329	*Trichophaea*	-0.6818	0.0146
4	*Cenococcum*	0.6084	0.0406
53	Atheliaceae sp.	0.6223	0.0307
61	*Ramariopsis*	0.6972	0.0117
414	*Hyphoderma*	0.6983	0.0115
3347	*Puccinia*	0.6149	0.0333
3353	*Lecanora*	0.5966	0.0405

Fig. 1 Venn diagram displaying shared and unique OTU counts among the three long-term prescribed fire treatments.

Fig. 2 Community richness, diversity, coverage, and evenness estimators across the three long-term prescribed fire treatments: a) observed OTU richness (S_{Obs}), b) rarefaction curves, c) Good's coverage, d) complement of Simpson's diversity (1-D), e) evenness based on Simpson's diversity index. Bars represent average values for a given treatment ± 1SD. Letters represent results of Tukey's HSD at $\alpha = 0.05$.

Fig. 3 Nonmetric MultiDimensional Scaling (NMDS) of the soil-borne fungal communities in the three long-term prescribed fire treatments. Symbols represent loading scores for experimental units. Analysis of MOlecular VAriance (AMOVA) indicates that 3 year burn interval treatment differs from 6 year burn interval treatment and unburned control treatment at $\alpha = 0.05$.

Fig. S1 Neighbor Joining trees representing four common genera of the 200 most abundant OTUs (a – *Cenococcum*; b – *Hygrocybe*; c – *Leohumicola*; d – *Mortierella*). Numbers at the nodes indicate bootstrap support (1,000 replicates) and the scale bar values represent the number of substitutions per site. The best BLASTn affinities of prevalent OTUs are consistent with the NJ analyses placement of these OTUs.

Fig. S2 Average quality scores across all sequences show dramatic decrease over read length. Early base calls adjacent to the sequencing adaptors (PGM P1 primer) show great stochasiticy while base calls toward 3' of obtained sequences exhibit similar quality scores. Mean Q-Values (black boxes) and Standard Deviation (grey bars). The horizontal and vertical lines represent the threshold of average sequence quality to be retained and the 150bp truncation point, respectively.