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Effects of Disturbance and Environmental Gradients on Soil Microbial Diversity and Community Structure

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Effects of Disturbance and Environmental Gradients on Soil Microbial Diversity and Community Structure

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ABSTRACT

Microorganisms are the major component of global biodiversity, and it is found in the various habitats of terrestrial ecosystems. However, their ecological roles in nature and the patterns of microbial diversity are still poorly understood. Also the dominant factors regulating soil microbial community composition and diversity variation within these ecosystems are still unknown. In this study, the extent of soil microbial diversity was investigated in both experimental and Mountain ecosystem and the way microbial communities are affected by disturbance and environmental gradients, as well as the extent to which ecological processes or other environmental factors contribute to structuring the soil microbial communities.

Firstly, I tested fungal community responses to disturbance gradients in a laboratory environment (Microcosms). Although disturbance is thought to be important in many ecological processes, responses of fungal communities to soil disturbance have been experimentally little studied and remained unknown about the responsiveness of soil fungal community structure to disturbance although there is a long history of the effects of disturbance on community structure in larger organisms. I subjected a soil microcosm to physical disturbance, at a range of frequencies designed to simulate ecological disturbance events. A soil microcosm is subjected to physical disturbance, sterilizing 90% of the soil volume each time, at a range of frequencies. The fungal community structure was analyzed using Illumina HiSeq sequencing of the ITS1 region. It was found that fungal diversity decline with the increasing disturbance frequencies, with no sign of the ‘humpback’ pattern found in many studies of larger sedentary organisms. There is thus no evidence of an effect of release from competition resulting from moderate disturbance – which suggests that competition and niche overlap may not be important in limiting soil fungal diversity. Changing disturbance frequency also led to consistent differences in community composition. There were clear differences in

OTU-level composition, with different disturbance treatments each having distinct fungal communities. The functional profile of fungal groups (guilds) was changed by the level of disturbance frequency. These predictable differences in community composition suggest that soil fungi can possess different niches in relation to disturbance frequency, or time since last disturbance. Fungi appear to be most abundant relative to bacteria at intermediate disturbance frequencies, on the time scale we studied here.

Also, bacterial community responses to environmental gradients were tested in Mt.Norikura, Japan. Little is known about the factors affecting the relative influence of stochastic and deterministic processes on environmental gradients. The investigation on the community assembly, phylogenetic diversity and the relative role of both deterministic (niche-based) process and stochastic process may play in delimiting the bacterial phylogenetic community structure was conducted. Soil DNA from samples collected at a range of elevations was sequenced using Illumina MiSeq of the 16S rRNA gene. Mt. Norikura showed no increase in phylogenetic clustering in upper elevations, suggesting that this may not be a general pattern in elevational systems, no greater role of stochasticity towards upper elevations. However, the strength of phylogenetic clustering and the role of stochasticity was strongly related pH, with structuring and determinism being strongest at lower pH. This pattern follows that found in an earlier study of successional environments, where pH also dominates community structuring. The possibility that pH is a dominant factor in bacterial community structure, as well as in diversity, should be considered.

Keywords: Fungal diversity, soil microcosm, Intermediate disturbance hypothesis, Bacteria, soil pH, community assembly, phylogenetic diversity, deterministic process, stochastic process, phylogenetic clustering

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ABBREVIATIONS

OTU: Operational taxonomic unit

PCR: Polymerase chain reaction

qPCR: Quantitative polymerase chain reaction

NGS: Next generation sequencing

MPN: Most probable number

rRNA: Ribosomal ribonucleic acid

NMDS: Non-metric multidimentional scaling

ITS: Internal transcribed spacer

EcM: Ectomycorrhiza

DNA: Deoxyribonucleic acid

MAT: Mean annual temperature

MAP: Mean annual precipitation

ANOVA: Analyses of variance

NTI: Nearest taxon index

βNTI: Beta nearest taxon index

PD: Phylogenetic diversity

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Chapter 1. Soil Microbial Diversity and Metagenomic Approaches used for Studying Soil Microbial Community: An Introduction

1.1. The extent of Soil microbial diversity and its pattern in ecosystem

Microorganisms are by far the most abundant living beings on Earth. The number of prokaryotic cells on Earth was estimated approximately 4-6 x 10^{30} (Whitman et al., 1998), representing over half of the carbon present in living organisms. It is generally considered that the microorganisms are widespread all over the world. It is also clear that the majority of life is microbial when adding to prokaryotic cells to the constitution of the eukaryotic microorganisms. The number of species of protozoa described is nearly an order of magnitude higher than that of the prokaryotes, but still these numbers are small in comparison to those of the eukaryotic macroorganisms (Finlay, 1998).

Soil is known to be one of the habitats in which the most diverse microorganisms inhabit (Torsvik et al., 2002; Gans et al., 2005). It has been estimated that the number of bacteria species per gram of soil vary between 2,000 and 8.3 x 10^{6} (Gans et al., 2005; Schloss and Handelsman 2006), of which fewer than 1% are culturable (Amann et al., 1995). It is known that estimating the true extent of diversity in soil at present stage is impossible due to the large number of unseen bacterial cells on earth. On the other hand, there are some generally accepted patterns in soil so the patterns of diversity seems somewhat clearer. It is shown that the geographical distribution patterns of soil microbial communities vary across different spatial gradients (Martiny et al., 2006; Green et al., 2008). Also many studies have shown that environmental factors are responsible for these spatial changes at local, regional and continental scales (Rousk et al., 2011; Griffith et al., 2011; Tripathi et al., 2012).

There is a long history of description and analysis of the effects of disturbance on community structure in microbial ecology (Willig et al., 1996; Jones et al., 2008). It is generally accepted

that the role of disturbance allowed coexistence of sedentary organisms (Connell, 1978). Disturbance is also adding to community diversity through creating a range of niches for differing degrees of dispersal ability (Grime 2006; Tilman 1994). In contrast to studies on larger organisms, the response of microbial communities to disturbance has been relatively little studied. Several studies of bacteria have conducted on the habitats where newly created or drastically disturbed (Trosvik et al., 2010; Langenheder and Szekely, 2011). However, these previous bacterial ecology studies focused mainly on early colonization or successional changes after on strong disturbance event, not the response of community structure following a history of different frequencies of disturbance. One study investigated the response of the assembly pattern of soil bacterial communities to repeated disturbance on a microcosm scale in incubated pots of soil (Kim et al., 2012). A similar pattern was observed from the same soil system that investigated another important aspect of the soil biota- the fungi – in relation to disturbance (Chapter 2).

The elevational diversity gradient is another well studied diversity pattern in macro ecology. The most diverse species are found in lower parts of the mountain and the relatively fewer number of species are present in upper elevations in general (Lomolino 2001). However, elevational diversity patterns have focused mostly on larger organisms and relatively few studies was conducted on microbes. Recently, a hump-shaped relationship between prokaryotic diversity and elevation was observed on volcanic Mt. Fuji, Japan (Singh et al., 2012a; Singh et al., 2012b). It is currently not clear the diversity pattern in microbe parallels those observed in larger organisms.

Also, many studies have shown that abiotic factors are known to affect soil microbial diversity (Lozupone and Knight, 2007; Fierer and Jackson, 2006). Among them, it has been clearly shown that soil pH is the best predictor of bacterial diversity on regional (Griffiths et

al., 2011; Tripathi et al., 2012) and continental (Lauber et al., 2009) scale. On the other hands, stochastic factors also play an important role in microbial community structuring and composition (Green and Bohannan 2006; Telford et al., 2006). There has been a long debate on the role of niche-based structuring and stochastic process as the main mechanism determined biodiversity and composition of species (Wang et al., 2013; Stegen et al., 2013). In this study, there was no consistent trend in phylogenetic diversity and community structuring of bacterial with elevation. Soil pH is dominant factor in phylogenetic diversity and community structure of overall bacteria, as well as in dominant bacterial phyla or classes (Chapter 3).

1.2. What makes soil microbial communities different?

1.2.1. Disturbance effects on soil microbial communities

Disturbance is considered as a worldwide theme in current ecology (Sousa 1985; Pickett and White 1985). Regardless to geographic or climatic characteristics, almost every area in the world is affected by natural or anthropogenic disturbance. Natural (non-human) disturbance is driven by natural elements (e.g. earthquake, volcano, erosion, flood, and hurricane). On the other hands, anthropogenic disturbance is caused by human (e.g. agriculture, logging, transportation, and industry). Both type of disturbance are common phenomenon of various ecosystems that happened at all ecological organization levels (Zak 1992). Disturbance events can alter the density, biomass or spatial distribution of populations by affecting the availability or resources and substrate (Walker 1999).

Disturbances are often characterized by the availability and distribution of three aspects (Frequency, extend and magnitude). First, frequency measures the number of events happening per unit of time or probability of happening. Second, extent means the physical are affected by

disturbance in a given time period. Third, Magnitude includes the intensity which is the strength of the disturbing force of an event and the severity which is the consequence of the event (Sousa 1985). The impact of disturbance on the ecosystem depends on several factors (e.g. soil, vegetation cover, and climate).

There is a long history of description and analysis of the effects of disturbance on community structure. It is regarded as an important influence on larger organisms and described for forest trees (Molino and Sabatier 2001), herbaceous plants (Ikeda 2003), algae (Lubchenco and Menge 1978) and corals (Connell 1978). Naturally disturbed systems were observed in some studies (Paine and Levin 1981; Sousa 1984). Disturbance was applied artificially in other cases (Huston 1979). It is generally accepted that the role of disturbance in allowing coexistence of sedentary organisms (Connell 1978).

However, the response of microbial communities to disturbance has been much less studied than for larger organisms (Allison and Martiny 2008; Buckling et al., 2000). The development of culture-independent methods has transformed understanding of natural and human-influenced microbial communities and opened up potentially new perspectives on their responsiveness to disturbance. Few studies conducted on studies of disturbance to such systems using advanced culture-independent methods (Griffths et al., 2000; Shade et al., 2011). So far, most studies focused more on functional redundancy, resilience, resistance to environmental perturbations rather than community assembly pattern or variability of community composition resulting from disturbance (Allison and Martiny 2008; Bowen et al., 2011; Peter et al., 2011).

1.2.2. Environmental gradients structuring soil microbial communities

An environmental gradient is a gradual change in abiotic factors through space or time. Environmental gradients can be related to factors such as altitude, temperature, depth, soil pH and soil humidity. Species abundances usually change along environmental gradients in a more or less predictive way. Understanding the processes responsible for variation in species richness and community composition along gradients is a central challenge in ecology. Many studies on animals suggest that the environmental gradients is a key factor driving species variation (Werner et al., 2007; Dunn et al., 2009). However, plant species are not randomly distributed in the landscape along environmental gradients (Pottier et al., 2013). Among the environmental factors, elevation and soil pH are recognized to strongly constrain the bacterial species distribution (Singh et al., 2012; Wang et al., 2012; Lauber et al., 2009; Chu et al., 2010). Quantifying how functional traits relate to different environmental factors may provide insight into the mechanisms governing microbial species distributions.

From the classical point of view, it has been believed that species richness of larger organisms (e.g. plants and animals) decreases with elevation (Stevens 1992). However, recent studies on mountainside describes a more complex relationship between elevation and species richness influenced by various factors (e.g. climatic variation, taxonomy, temperature, water and energy availability, and humidity) (Rahbek 1997; Kluge et al., 2006; Beck and Chey 2008). One study reported that the microbial diversity patterns were fundamentally different from the patterns of plant (Bryant et al., 2008). In this study, plant showed that unimodal patterns with the highest species richness and phylogenetic diversity at mid-elevations. In contract, phylogenetic diversity and taxon richness of prokaryote decreased overall as the increasing elevational gradients. In recent study, Fierer et al. (2011) and Wang et al. (2012) revealed that microbial

diversity differed from plant diversity in their distribution patterns across elevational gradients. More recently, Shen et al. (2015) studied trends in phylogenetic structuring of soil bacterial communities with elevation on Changbai Mountain, China (Shen et al., 2015). They found stronger phylogenetic relatedness at higher elevations on the mountain.

It is known that the pH determines microbial ecology and biogeography from local to continental scales (Lozupone and Knight 2007). The pH does not only influences microbial community composition, functional groups and biogeography (Fierer and Jackson 2006; Lauber et al., 2008) but also their mediated ecological processes (Hussain et al. 2007). Fierer and Jackson (2006) revealed that the microbial diversity was largely explained by soil pH. The highest diversity was found around neutral soil pH and lowest diversity in extreme (acidic or alkaline) soil pH (Fierer and Jackson 2006; Lauber et al. 2008). Overall, many studies support that the most apparent predictor determining soil microbial diversity and community composition is soil pH (Fierer and Jackson 2006; Lauber et al., 2008; Lauber et al., 2009).

1.3. A general procedure of soil microbial diversity and community analysis

1.3.1. Metagenomic approaches in soil microbial study

Metagenomics is the study of the metagenome which is total genetic material (genomic DNA) from environmental samples (Handelsman et al., 1998). The development of metagenomic techniques have changed the environmental microbiological studies. The use of metagenomic approaches allows to understand the structure (gene/ species richness and distribution) and functional (metabolic) potential of environmental microbial communities. Metagenomics is also called environmental genomics, community genomics or microbial population genomics. Traditionally, microbiology has been based on growing microorganisms in pure cultures in the

laboratory. However, culture-independent molecular way of analyzing environmental samples of cohabiting microbial populations has extended a new perspective on microbiology (Hugenholtz and Tyson, 2008). This metagenomics approach is grouped into unselective (shotgun metagenomics) and targeted (sequence-driven metagenomics) metagenomics based on their sequencing strategies.

Shotgun metagenomics provides useful information about unculturable microorganisms that are difficult to analyze (Delong et al., 2006; Gill et al., 2006). However, in predicting the function of more diverse ecosystems like soil, the power of the shotgun metagenomics is rather limited because of inherent problems related with limited accessibility to the genomes of less abundant members of the community. More recently, the development of NGS (next-generation sequencing) technologies has revolutionized the field of metagenomics, allowed microbiologists to obtain much more DNA information from complex microbial communities (Mardis, 2008). These NGS platforms (e.g. 454 pyrosequencing, Illumina and Ion Torrent^{TM}) are much faster and cheaper than the traditional Sanger method in DNA sequencing.

A particular genes or genomic regions of interest is subjected to sequencing to reduce genetic complexity in targeted metagenomics approach (Suenaga 2012). There are two different ways of targeted metagenomics: (1) sequence-driven targeted metagenomics and (2) function-driven targeted metagenomics. Sequence-driven targeted metagenomics is normally conducted by PCR-based directed sequencing of metagenomic DNA. Using the ribosomal RNA genes (e.g., small subunit [SSU] and large subunit [LSU]) as phylogenetic marker gene is one of the most common cultured or that play an important role in the environment (Acinas et al ., 2004). Although taxonomic resolution of the 16S rRNA gene is not adequate for delineating taxa at the species or strain level in some cases (Fox et al., 1992), the SSU of ribosomal RNA (16S rRNA) has been used widely as a standard marker gene in Prokaryotes. Microbial community

analysis using taxonomic marker genes (e.g. 16S rRNA gene) commonly uses an operational taxonomic unit (OTUs) based approach, as the definition of microbial species is still vague and despite the massive sequencing efforts, currently available public databases are still devoid of the full extent of microbial diversity. As an alternative to sequence-driven targeted metagenomics, function-driven targeted metagenomics is a more direct route to the discovery of the major rumen enzymes approach (Ferrer et al., 2005). Function-driven targeted metagenomics can be used to obtain novel findings of targeted biological functions.

1.3.2. Microbial community analysis procedure

1.3.2.1. Initial processing and sequence quality control

Initial processing and sequence quality control analysis begins from raw sequence files (fastq formats) obtained from Illumina Hiseq or Miseq sequencing results. Raw sequence data are firstly de-multiplexed according to the unique barcode for each sample. The adaptor/primer/linker sequences were removed subsequently. The low quality sequences (i.e. maximal homopolymer at 9bp, minimum ambiguous base of 1, and minimum quality score of 25) are trimmed off. These steps normally allows for the minimum level of nucleotide degeneracy and sequencing errors when sorting out barcode and primer sequences (i.e. allowing one mismatch in barcode and two mismatches in each primer sequence). Paired-end sequences were assembled using PANDAseq software (Masella et al., 2012).

1.3.2.2. Sequence alignment, pre-clustering, chimera removal and taxonomic classification

The sequences are aligned against a reference alignment database (EzTaxon-e with 1,457 columns), using a combination of k-mer and pair-wise alignment in Mothur (Schloss et al., 2009). After complete sequence alignment, sequences are de-noised with 'pre.cluster' command in mothur, which applies a pseudo-linkages algorithm to remove the sequences that are linked due to sequencing errors (Huse et al., 2010). Next, putative chimeric sequences that generated recombinants artificially between two or more parental sequences are detected and removed using the Chimera Uchime algorithm implemented in mothur (Edgar et al., 2011). Then taxonomic classification of each sequencing read is obtained by classifying alignments against EzTaxon-extended reference taxonomy using the Naïve Bayesian algorithm (Wang et al., 2007). Eztaxon-e database provides a representative sequence information (type sequence) among closely related but unidentified 16S rRNA gene sequences (Kim et al., 2012). After that, closely related sequences are clustered together on the basis of sequence similarity. Therefore, it provides taxonomically more meaningful information than that of just similarity-based OTU clustering and the taxonomic resolution and coverage of the database will be better as more sequences are available.

Once the sequences are aligned and most of the erroneous sequences are removed, alignment-based distance matrix is calculated and clustering process generates OTU at varying cutoffs. There are generally three types of clustering methods: Complete-, average-, and single-linkage clustering. Basically, all linkage clustering methods calculated distances between clusters in a hierarchical way and types of methods vary depending on how to define cluster boundary. This OTU-based approach is now generally accepted in microbial community analysis.

1.3.2.3. OTU based and phylogenetic analysis

Once OTU table or species/ sample matrix are generated, versatile diversity analyses can be performed such as measuring diversity indices. To compare the level of diversity between samples, using the standardized number of sequences per sample is recommended. If the sequences are already accurately aligned to each other, it can be used for building phylogenic tree, which makes it possible to analyze many things such as measuring phylogenetic diversity, the extent of clumping or over-dispersion of certain lineages in phylogeny, and generating distance matrix between communities based on their evolutionary information. Additionally, distance or dissimilarity matrices generated from phylogeny and classical species/ sample table can be used for community-level comparison between samples or relating environmental variables to each community to see that factors contribute more to structuring bacterial community within a given sample. Using the OTU community matrix and the matrix generated from the phylogeny has given rise to the field of phylogenetic community ecology (Webb et al., 2002).

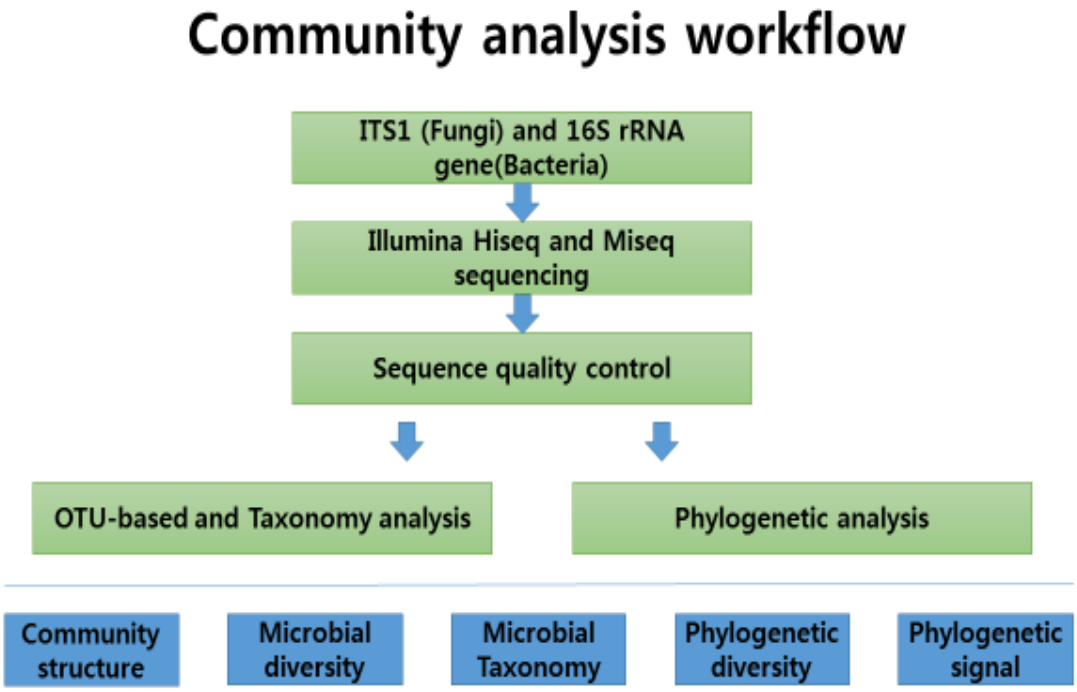

Figure 1. A community analysis procedure and protocol using Illumina Hiseq and Miseq for ITS1 region and 16S rRNA data.

1.4. Objectives of this study

There have been many theoretical and empirical studies on biodiversity and biogeography in larger organisms in terrestrial environments. However, patterns of microbial diversity and their spatial distribution are relatively less studied. Despite of the importance of microorganisms in soil ecology, few studies have focused on how microbial community assembly patterns or community composition are affected by disturbances. Also, few investigations about the effects of environmental gradients on microbial diversity and phylogenetic patterns are reported. By studying the changes in microbial diversity and community composition in response to varied environmental conditions, it would lead us to find out what particular mechanisms or factors could explain the observed patterns. Here I investigate the community structure of the two soil biota (bacteria and fungi) that play an ecologically important role in soil biochemistry and processes and the roles of niche-based and neutral processes in delimiting these communities. I tested two general patterns on soil microbial communities, the 'Disturbance – fungal diversity relationship' and the 'environmental gradients – bacterial diversity relationship', which have frequently been used to explain the patterns of biodiversity in larger organisms, such as plants and animals. These studies will provide a deep understanding of microbial ecology in these two ecosystems (e.g. experimental ecosystem and mountain ecosystem) and also shed more light on what ecological processes are important in assembling fungal and bacterial communities respectively.

Chapter 2. Changes in Soil Fungal Community Structure with Increasing Disturbance Frequency

2.1. Introduction

Disturbance is widely regarded as an important influence on community structure of larger organisms. The effect of disturbance have been described for algae (Lubchenco and Menge, 1978), corals (Connell, 1978), forest trees (Molino and Sabatier, 2001) and herbaceous plants (Ikeda 2003), amongst others. Natural or human-induced disturbance plays an essential role in shaping global vegetation (Sousa, 1984; Glenn-Lewin and van der Maarel, 1992). Disturbance also influences microbial community structure, such as soil (Willig et al., 1996) and aquatic (Jones et al., 2008) bacterial communities. It can release nutrients from breakdown of cells of organisms, provide available resources for primary productivity, and also lead to changes in species richness. To test hypotheses concerning disturbance effects on larger organisms, studies have been conducted in both naturally and artificially disturbed systems (Sousa 1984; Huston 1979; Paine and Levin 1981). An intermediate disturbance pattern has often been observed, with species diversity being lower in relatively undisturbed environments, highest in locations with moderate levels of disturbance, but with a decline in diversity beyond a certain frequency or intensity of disturbance (Connell, 1978). It is thought that with moderate levels of disturbance, 'lottery effects' provide ready access to resources of all species which have overlapping similar niches, regardless of their effectiveness in steady competition. By allowing exploitation of random opportunities, it may be possible for more species to coexist in a disturbed community (Chesson and Warner, 1981).

By creating a range of niches for differing degrees of dispersal ability, disturbance may also be important in adding to community diversity (Tilman, 1994; Grime, 2006). In contrast to studies on larger organisms, the response of microbial communities to disturbance and primary successional environments has been relatively little studied – and most of what has been done has tended to focus on observation of naturally occurring processes rather than experimental manipulation of controlled conditions (Allison and Martiny, 2008). As in the study of microbial

ecology in general, culture-independent methods of community analysis have provided new perspectives on the responses of microbial communities to disturbance, through both natural and anthropogenic processes (Griffiths et al., 2000; Shade et al., 2011; Kim et al., 2013).

Several studies of bacteria have concentrated on newly created or drastically disturbed habitats, such as the infant gut (Trosvik et al., 2010) and coastal rock pools (Langenheder and Szekely, 2011), observing and explaining the mechanisms of community assembly or reassembly during colonization. However, these previous bacterial ecology studies focused mainly on early colonization or successional changes after one strong disturbance event, not the response of community structure following a history of different frequencies of disturbance. In an earlier study using the same experimental system as we report on here (Kim et al., 2013), we investigated the response of the assembly pattern of soil bacterial communities to repeated disturbance on a microcosm scale in incubated pots of soil. That study found distinct, predictable bacterial assemblages occurring at each frequency of disturbance, highlighting the potential role of niche differentiation of bacteria in relation to disturbance events, and also its possible importance in producing the diversity of bacterial communities seen in nature.

Here, we used DNA samples from the same soil system (Kim et al., 2013) to investigate another important aspect of the soil biota – the fungi - in relation to disturbance. Fungi constitute one of the most diverse and dominant groups of organisms in soil, and they play important ecological roles in the ecosystem as decomposers, pathogens and plant mutualists (Oriazzi et al., 2012).

Fungi can have profound impacts on plant communities through increasing the fitness of certain plant species or genotypes, for instance by increasing abiotic and biotic stress tolerance, increasing plant biomass and decreasing water consumption. They can also have important effects in decreasing fitness of plants by acting as pathogens and also by altering resource

allocation patterns (Rodriguez et al., 2009). Understanding the structure and diversity of soil fungal communities is also likely to be fundamental to understanding their function in the ecosystem and their impact on plant communities.

In spite of the importance of fungi in soil ecology, few studies have focused on how fungal community assembly patterns or community composition are affected by disturbances of any sort. The responses of soil fungal communities to disturbance, whether physical, chemical or physiological (e.g. temperature fluctuations, waterlogging, salinity changes, heavy metal pollution) is potentially very important in understanding ecosystem sustainability and restoration (Kandeler et al., 2000; Classen et al., 2003; Mohamed and Martiny 2011). It is clear that in nature, aspects of fungal community structure are altered by the disturbance history of the ecosystem, to the extent that certain groups are used as bio-indicators for past disturbance, or absence of recent disturbance (Jonsson and Jonsell, 1999; Parmasto, 2001).

As a starting point for the study of soil fungal community responses to disturbance, we set out to address several basic questions:

1) How does disturbance history affect overall diversity of soil fungi? Is there any evidence of an 'intermediate disturbance effect' on diversity, as is often seen for larger organisms?

Effects of disturbance on communities of various types of organisms are well documented (Huston and Huston 1994). Here, we were interested in studying whether disturbance has strong effects on diversity of soil fungi. In particular we aimed to test whether a certain amount of disturbance would increase fungal diversity, with a decrease in diversity at higher disturbance frequencies. This intermediate disturbance pattern – with greatest diversity at intermediate frequencies of disturbance - has often been found for sedentary organisms such as coral and plants (Huston and Huston 1994).

2) Do particular taxa of soil fungi each tend to respond differently (e.g. being more disturbance-tolerant or disturbance-sensitive) in relation to differing disturbance frequency?

In the ecology of plants, animals and other large organisms, it is clear that certain taxa are associated with either disturbed or stable conditions (Grime, 2006). The ability of populations to recover after disturbance is an important niche dimension in the ecology of macroscopic organisms, with large differences based on inherited species traits (Grime, 2006; Tilman, 1988). We were interested in determining the extent to which the response to disturbance differs amongst fungal OTUs and higher level fungal taxa, providing an additional dimension to the niche of soil fungi which can explain community structure and the coexistence of soil fungal diversity.

3) Is there a predictable fungal community structure that is associated with each disturbance frequency? Does a greater role of randomness take over at higher disturbance frequencies, due to stochastic colonization of resources from small founder populations?

Stochastic events during times of population bottleneck and recovery may produce communities that differ in their dominant species for no other reason than luck (Poulin et al., 2008). There has been a long debate on the role of predictable niche-based structuring versus stochastic process as the main mechanism which determines biodiversity and composition of species in a local community. Community ecologists generally acknowledge that both mechanisms play an important part in structuring communities (Adler et al., 2007; Gravel et al., 2006; Leibold and Mcpeek 2006). Most studies testing the relative importance of stochasticity and determinism have been performed on macroscopic eukaryotes, but recently some studies have tried to explain changes in bacterial community composition through invoking a role for both mechanisms (Langenheder and Szekely, 2011; Ofiteru et al., 2010).

However, so far no study that has considered how the relative importance of stochasticity versus niche structuring of soil fungi depends on differing disturbance frequencies. By studying the replicability of community structure between different disturbance frequencies, we aimed to compare how the two mechanisms varied in relative strength.

4) Do fungi overall become less abundant in the soil at higher disturbance frequencies, due to their growth rate being insufficient to recover between successive disturbances?

If most of the fungal community is destroyed, the biomass of fungi will take a certain time to recover. If disturbance events occur too frequently, there will be insufficient time for full recovery of fungal biomass to occur before the next disturbance event occurs. We were interested to ascertain how sensitive fungal biomass (estimated indirectly through qPCR) is to disturbance events. We were also interested in determining through qPCR how disturbance affects the relative abundance of bacteria and fungi in the soil. Bacteria usually make up the vast majority of soil living biomass (Curtis et al., 2002; Gans et al., 2005; Torsvik et al., 2002; Tringe et al., 2005; Fierer et al., 2007), and are widely regarded as being potentially faster growing than fungi (Pietikainen et al., 2005). If this is so, a more frequently disturbed soil should have a lower relative abundance of fungi compared to bacteria – with bacteria likely playing a more important biogeochemical role in the more disturbed soil.

5) Are there apparent effects of the removal of plant roots from the soil system?

Plant roots are a major source of carbon to soil organisms (Hutsch et al., 2002), and also the living roots interact with soil biota in various types of relationship (Bardgett et al., 1999). For example, ectomycorrhizal fungi are hosted and supported by living roots, although they also

grow out into the soil (Read 1991). Plant parasites depend upon living plant tissues for survival (Gurevitch et al., 2006). As this experiment involved the removal of all living plant roots, and the incubation of control pots of soil undisturbed for 6 months afterwards, we were interested in comparing the fungal community 'without roots' with the original state with living roots present. This could give some perspective on the true role of living roots in maintaining the soil fungal community.

6) Are the functional guilds of fungi also changed with disturbance frequency?

Although OTUs provide an estimate of diversity in a habitat (Moyer et al., 1994), they provide little information on function other than what may be gleaned from the taxonomic name of the better known fungi (Nguyes et al., 2016). We were interested in determining the functional categories ('guilds', *sensu* (Simberloff and Dayan 1991)) of the identified fungi as the disturbance frequency changed. This involved pairing of high-throughput sequencing and the better automation of parsing fungi by ecological guild using FUNGuild (Nguyen et al., 2016).

2.2. Materials and Methods

2.2.1. Soil microcosm

The study was conducted on soil derived from a weed-overgrown garden (major plant species *Plantago major, Artemisia vulgaris, Taraxacum sp., Digitaria sp., Poa annua*) on the campus of Seoul National University. This is a typical weedy herbaceous community of very widespread plants, typical of moist temperate climates across the northern hemisphere. Overall ground cover by plants was around 90%. The soil we sampled is also a very typical moist temperate-zone brown earth soil in terms of TOC, TN, pH, texture, etc., as would commonly be found in a well-drained overgrown flowerbed, fallow field, along the edges of a dirt path, etc (Table 1).

A total of 30 kg of soil from the top 10 cm was taken across an area of several square meters of the garden. Large roots and stones were removed, and the soil was then thoroughly homogenized by sieving through a 2-mm mesh.

Table 1. Chemical properties of initial and disturbed soils.

	pH	TOC (%)	Total C (%)	Total N (%)	Total P (mg/kg)
Initial	4.77 ± 0.16	2.80 ± 0.11	1.50 ± 0.02	0.12 ± 0.005	186 ± 1.1
0	4.99 ± 0.17	2.97 ± 0.04	1.74 ± 0.11	0.14 ± 0.016	184.0 ± 11.8
0.125	5.01 ± 0.26	2.55 ± 0.35	1.48 ± 0.04	0.13 ± 0.002	159.9 ± 12.0
0.25	5.00 ± 0.30	2.83 ± 0.04	1.36 ± 0.14	0.13 ± 0.027	168.8 ± 6.3
0.5	4.69 ± 0.23	2.64 ± 0.39	1.83 ± 0.17	0.15 ± 0.008	186.1 ± 4.7
1	4.61 ± 0.28	2.57 ± 0.11	1.42 ± 0.25	0.12 ± 0.023	197.3 ± 26.5

TOC represents total organic carbon. The highest disturbance frequency (7days treatment) was set to 1.0.

2.2.2. Disturbance and growth regimen

The ‘disturbance’ event in this experiment was the autoclaving of 90% of the moist soil weight in each pot. At each ‘disturbance event’, the pot contents were tipped out, gently but thoroughly mixed, and a small proportion (10% by weight) of this ‘live’ soil was held back from autoclaving. Then, a moist weight equivalent to 90% of the original contents of the pot was added from a mixed pool of autoclaved soil (see description below on the autoclaving procedure and DNA destruction). The 10% of soil held back was gently mixed into this added autoclaved soil, in order to ‘re-seed’ the soil system with a living biota. This type of disturbance produces ‘a nonspecific mass mortality event’ (*sensu*. Buckling) in each disturbance regime, and has frequently been used in experimental microcosm studies (Buckling et al., 2000; Wertz et al., 2007). Every pot (whether undergoing 90% sterilization by weight or not) was internally mixed/aerated by a separate sterilized trowel every week for consistency with other aspects of the treatment (**Fig. 2**).

Though all pots in all treatments underwent physical mixing of the soil (the contents of each pot being tipped out into a sterile bag and thoroughly mixed, before being placed back into the pot) every week, ‘disturbance’ as a variable in this study is the ‘mass mortality’ event of losing 90% of living biomass. We set five different levels of disturbance frequency: every 7 days, 14 days, 28 days, 56 days, and no disturbance. The highest disturbance frequency (7 day treatment) is described in our results as 1.0 (disturbance events per week) and the no disturbance setting is 0.0 (disturbance events per week). Because this experiment was originally designed to study bacterial ecology (Kim et al., 2013), the highest disturbance frequency (7 days treatment) was determined based on estimated mean turnover times of soil bacteria (Baath 1998; Rousk and Baath 2011). While very few studies have measured fungal growth rates in soil (Rousk and Baath 2011), soil fungi are known to have a wide range of potential growth rates and they

appear to have longer turnover times than soil bacteria (Baath 1998; Rousk and Baath 2011). Rousk and Bååth (2007) reported 20-40 days of fungal turnover time in plant material amended soil at 22℃ incubation temperature (Rousk and Baath 2007). Given that ergosterol measurement normally underestimates the fungal growth rate (Baath 2001) and our pots were kept at a slightly higher temperature (24℃) than the experiment of Rousk and Baath (22℃), the 6 month experiment would have covered multiple turnover times of the 'mean' of the fungal community - with disturbance frequencies varying from several disturbances per mean fungal biomass turnover period (every 7 days disturbance events) to fewer than one disturbance per mean fungal turnover period (56 days disturbance events).

In terms of the balance between disturbance and biomass increase rates of fungi, our disturbance frequencies (7 days, 14 days, 28 days, 56 days, and no disturbance) seem roughly equivalent in ecological terms to the observed ranges of disturbance frequencies impacting macro-organisms in forest, grassland, scrub or coral reef system, where disturbance cycles may occur on times ranging from every few months to every two or three centuries, depending on the particular system and the part of the system being observed (Wertz et al., 2006). Humpback diversity curves in relation to disturbance frequency for plants and corals typically cut across between one and several life cycles for the plants/corals in question (Wertz et al., 2006). For example, a fire disturbance event once a year is equivalent to several life cycles for ruderals, but a fraction of a life cycle for a slow growing shrub. A fire disturbance once every 15 years is equivalent to multiple life cycles of slower growing shrubs. With reference to this literature, our range of disturbance frequencies for fungal communities in a soil system seems roughly appropriate.

In this experiment, the free-draining pots were kept in a growth chamber in darkness at 24℃ and all were watered every three days with sterile distilled water to maintain a moderately damp

soil. The experiment was continued for 6 months and soil samples for DNA extraction were taken from the center (in terms of depth and distance from edges) of each pot. Each week, the positions of all pots within the growth chamber were randomized.

Throughout the six month experiment, all soil-filled pots were kept in the dark within the chamber. Each pot of soil had another empty pot upside down over it, forming a lid that was sealed around the edges with tape, to avoid as much as possible any additional recruitment from direct cross contamination by dust from adjacent pots. Five holes (each 0.5cm in diameter) were drilled in the top of each pot - sufficient to allow aeration but unlikely to allow soil dust to travel between pots. Both additional recruitment from air and cross contamination were checked by most probable number (MPN) method on soils taken from additional pots, which contained sterilized soils only for the test period: no sign of such contamination was found as the soil remained sterile on testing by culture. In the growth chamber, 15 pots (15cm x 15cm x 13 cm, ca. 500g of soil each) were arranged in a completely randomized design with three replicates (**Fig. 3**).

As a result of the mortality of all cells in the sterilized soil, recolonization and recovery of the microbial community would have been largely based upon both utilization of dead microbial cells, and soil nutrients and carbon still remaining from the initial contents of the garden soil. This was intended to simulate a typical secondary succession system in nature, whereby much of the nutrient material for succession is release by the organisms killed in the disturbance event.

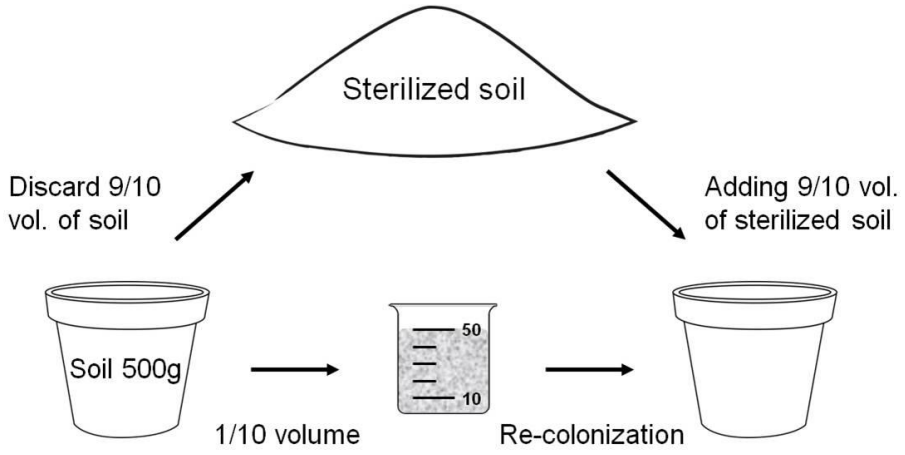

Figure 2. Soil disturbance treatment. 1/10 volume of soils taken from the original pot were thoroughly mixed with new 9/10 volume of sterile soils.

Figure 3. Soil microcosm setup. 15 pots with three replicates per treatment were placed in 24℃ growth chamber

2.2.3. Autoclaving and validation of sterility and DNA destruction

To prepare sterilized soil, the original sieved soil was sterilized by autoclaving twice successively for 90 minutes at 121 ºC, to provide a large pool of sterile soil for 'restocking' pots following each disturbance event. Soil was autoclaved in a steam chamber in small (1.5kg) quantities in specialized autoclave bags. The soil was bagged in sterile packaging and frozen to ensure its continuing sterility until it was used to refill pots in the experiment. Soil sterility was determined by enumeration of heterotrophic bacteria by the most probable number (MPN) technique, as described in studies by Wertz and colleagues (Wertz et al., 2007; Schirmer et al., 2015). No colonies were observed.

Soil DNA destruction in the autoclaved soil was checked by soil extraction of the autoclaved soil after the second autoclaving using the Power Soil DNA extraction kit (MO BIO Laboratories, Carlsbad, CA, USA). Only a small quantity (< 5ng/ µl) of DNA could be extracted in this way. The extract failed to give any detectable amplification for bacterial 16S rRNA gene or fungal internal transcribed spacer (ITS) region 1 primers, presumably because any DNA fragments remaining were too small. This validated that the autoclaved soil would not give any contaminating amplification of the ITS1 region.

2.2.4. PCR amplification and Sequencing of ITS1 region

Soil DNA was extracted using the Power Soil DNA extraction kit (MO BIO Laboratories, Carlsbad, CA, USA) as directed by manufacturer's instructions, with an additional incubation step at 70℃ for 10 min followed by 15 min of bead beating. Extracted DNA was amplified using primer pair ITS1-F (5'-GAACCWGCGGARGGATCA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') targeting ITS1 region. Polymerase chain reactions (PCR)

were performed in 50 µl reactions using the following temperature program: 95℃ for 10 min; 30 cycles of 95℃ for 30 s, 55℃ for 30 s, 72℃ for 30 s, and 72℃ for 7 min. The PCR products were purified using the QIAquick PCR purification kit (Qiagen) and D NA quality is measured by Nanodrop. 50ng of purified PCR product for each sample were combined in a single tube and sent to Celemics Inc. (Seoul, Korea) for sequenci ng using paired end (2 x 150 bp) HiSeq2000 system (Illumina, USA). Initial quality-filtering was performed following the error reducing strategy suggested by Schirmer (Masella et al., 2012). Briefly, quality-trimming (Sickle) and error correction (BayesHammer) were carried out and paired-end reads were assembled using PANDAseq 2.9 (Bengtsson-Palme et al., 2013) with a minimum overlap of 10 bp. Fungal ITS1 region was extracted using ITSx 1.0.11 (Wang et al., 2007). Putative chimera was detected with denovo UCHIME algorithm and chimera-free sequences were clustered into OTUs (defined at a 97% sequence similarity cutoff) using VSEARCH 1.9.0 (Rognes, https://github.com/torognes/vsearch/). The resultant sequences were taxonomically assigned against the UNITE+INSD fungal ITS reference data set using naïve Bayesian classifier with a confidence threshold of 80% (Meyer et al., 2008). All the ITS1 sequences used in this study are deposited to the metagenomic- RAST server (Beals 1984) under the project Pot disturbance fungal community (http://metagenomics.anl.gov/?page=MetagenomeProject&project=18397).

2.2.5. qPCR for fungal ITS1 region

To estimate fungal abundance by quantifying fungal ITS1 copies, quantitative real-time PCR was performed on an Applied Biosystems 7300 Real-Time PCR System (ABI, USA). The 20µl qPCR reactions contained 10µl of SYBR Green Master Mix (Bio-Rad, USA), 1µl of each 10mM forward and reverse primers, 1µl of template DNA, and 7µl of distilled water. Fungal

DNA was amplified using the same primers used for HiSeq sequencing under the following conditions: an initial denaturation step at 95 ℃ for 15 min followed by 35 cycles of 95 ℃ for 1 min, 55 ℃ for 1 min, and 72 ℃ for 1 min. All reactions were done in triplicate.

2.2.6. Statistical analyses

For comparing the level of operational taxonomic unit (OTU) richness and diversity between samples, sequences were randomly subsampled to the standardized number (n=9,432 reads) and the average number of OTUs per sample was used for diversity calculations. Each OTU was assigned to a functional guild (e.g. ectomycorrizal fungi, plant pathogenic fungi and saprotroph fungi) using the FUNGuild database (Nguyen et al., 2016). This is currently the largest database for assigning fungal genera to one of several functional guilds based on a community-annotated database of fungal taxa with known or suspected ecological functions. FUNGuild assigns function based on matches at the genus and species level along with a confidence level: we only considered probable and highly probable confidence score guild assignments. Non-metric multidimensional scaling (NMDS) was generated based on pairwise Bray-Curtis dissimilarities (Clarke and Gorley 2006) between samples using PRIMER v6 (Pianka 1974). One-way analysis of variance (ANOVA) and Tukey HSD test for post-hoc analysis were used to find means that are significantly different from each treatment groups. All graphs including ordination plot and heatmap were generated using picante and vegan packages in R (http://www.R-project.org).

2.3. Results

2.3.1. Effect of disturbance frequency on fungal abundance by qPCR

We first investigated the extent to which total fungal abundance changed in response to a history of different frequencies of disturbance events. Fungal ITS1 copy numbers were most abundant at the highest and intermediate disturbance levels (every 7, 14 and 28 days of treatment) and remained least abundant at low disturbance frequency (every 56 days) and with no disturbance (**Fig. 4**). There were no significant differences in total fungal abundance between the 7 days, 14 days and every 28 days disturbance treatments (ANOVA, $p>0.05$).

We also compared the ratio of total copy numbers of fungal ITS1 and bacterial 16S rRNA gene (**Fig. 5**). The ratio of fungi to bacteria was highest in the more frequently disturbed treatments (every 7 days and 14 days), and lowest in less frequently disturbed treatments (every 28 and 56 days).

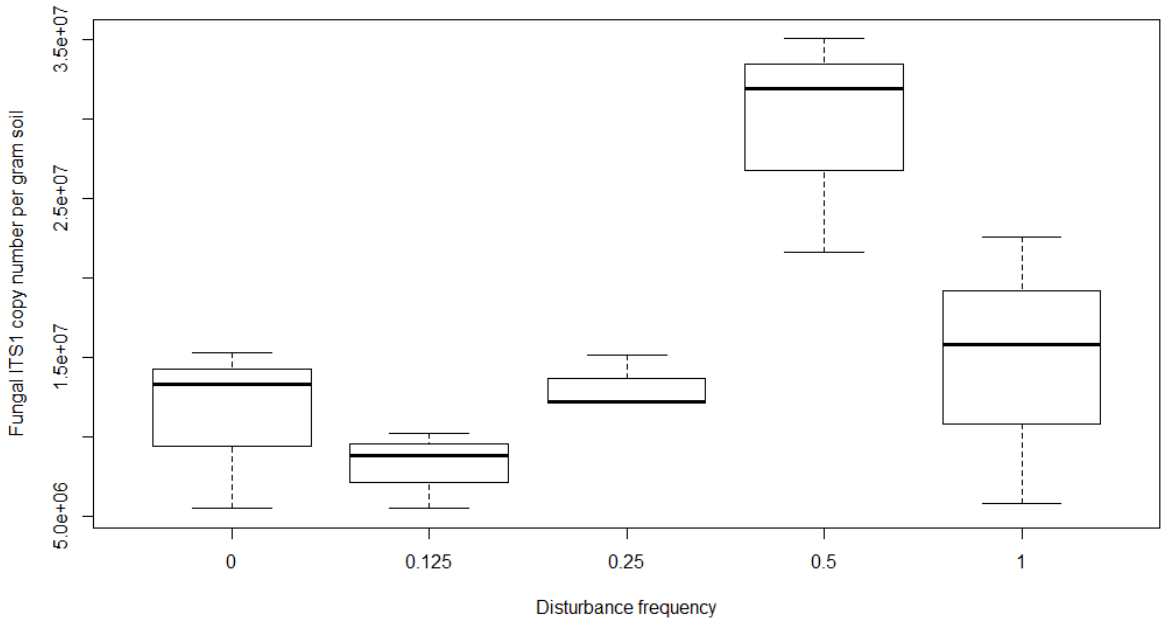

Figure 4. Changes of fungal ITS1 copy numbers with disturbance frequency. The highest disturbance frequency (7 days treatment) was set to 1.0.

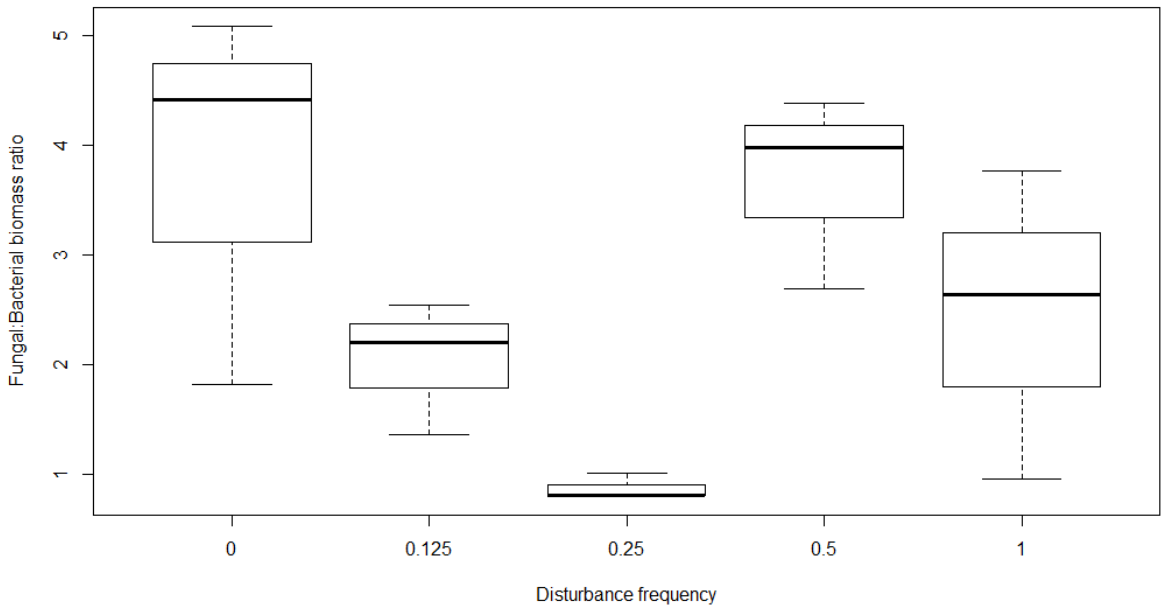

Figure 5. Changes of soil fungal: bacterial biomass ratios with disturbance frequency._The highest disturbance frequency (7 days treatment) was set to 1.0.

2.3.2. Fungal diversity and community composition in relation to disturbance frequency

We obtained a total of 1,992,105 sequences after quality checking and trimming processes, with an average number of 66,403 sequences per sample (ranging from 9,432 to 174,431). To correct for differences in number of reads, all samples were subsampled to the level of the smallest number of reads found in any sample (9,432 reads). OTU richness and Shannon index had a statistically significant (all $p < 0.05$) tendencies to decrease as the disturbance frequency increased. Shannon index are shown here as a representative of these trends (**Fig. 6**)

An NMDS plot was generated using pairwise Bray-Curtis dissimilarities between samples to understand how fungal community structure changes with disturbance level (**Fig. 7**). The highest disturbance frequency (7 days treatment) was set to 1.0. The NMDS plot showed that fungal communities were clustered significantly across disturbance frequency (ANOSIM: Global R=0.846, $p < 0.001$; **Fig. 7**).

We further investigated the dominant fungal OTUs in each sample, to observe the major changes at each disturbance setting. The thirty most abundant OTUs from all samples averaged were combined. The log transformed relative abundances of OTUs were used to produce the heatmap depicted in **Fig 8**.

Overall, most of these dominant fungal OTUs in the initial soil samples at the start of the experiment (before the aging/disturbance treatment) were also present in both fresh/undisturbed and aged/disturbed soil. However, the relative abundances of these OTUs were changed, depending on the history of disturbance (**Fig. 8**). Some OTUs (e.g. 49, 76, 69, 34 and 53) were only abundant in the initial soil. By contrast, OTUs 36, 10 and 16 were only abundant in the aged/disturbed soils – especially the soil treatment with most frequent

disturbance (every 7 days). OTUs 75, 53, 11, 07 and 17 were most abundant in relatively undisturbed soil (every 56 days and no disturbed soil) (**Fig. 8**).

There was however an exception to the distinct patterns in OTU abundances between treatments, in the form of the three most abundant OTUs, which were the most abundant in all treatments including the initial conditions (**Fig. 8**). These three OTUs (1, 5 and 14) were identified as: *Trichosporon_dehoogii*, *Gibberella_intricans* and *Umbelopsis isabellina*. Despite the fact that soil chemical properties (pH, C, N) stayed constant regardless the history of disturbance, the result shows that overall fungal diversity was strongly affected by disturbance frequency.

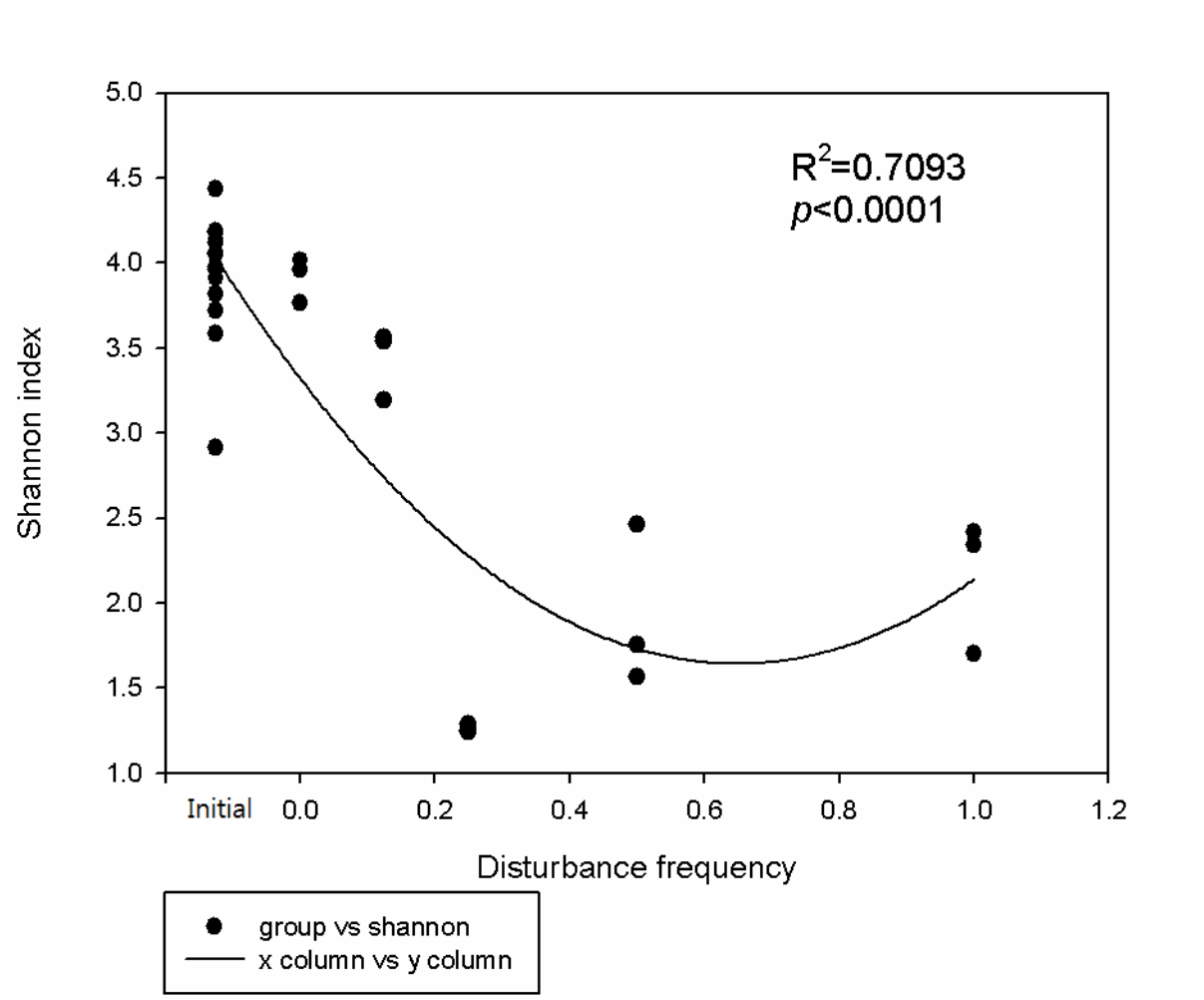

Figure 6. Observed Shannon diversity index of fungal community differing in disturbance frequency. The highest disturbance frequency (7 days treatment) was set to 1.0.

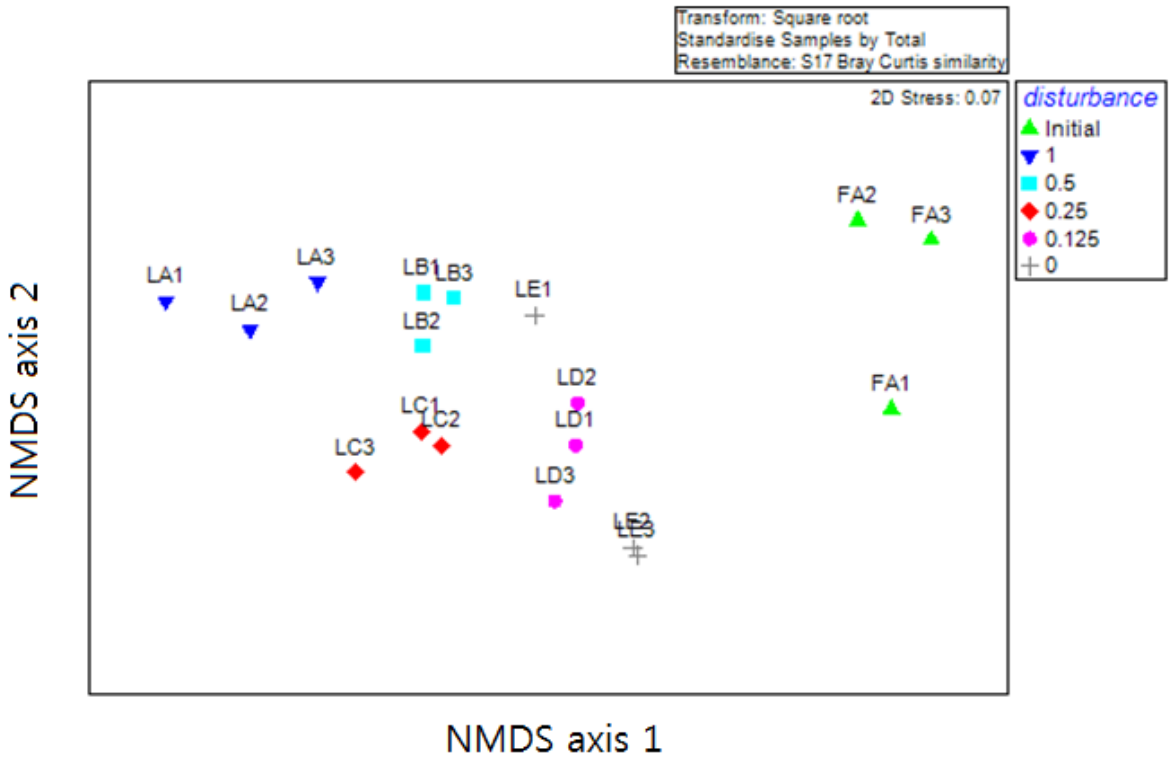

Figure 7. NMDS plot showing fungal community structure under different disturbance frequencies. Most abundant OTUs are left, in order of relative abundance. All samples were used after log pretreatments transformation.

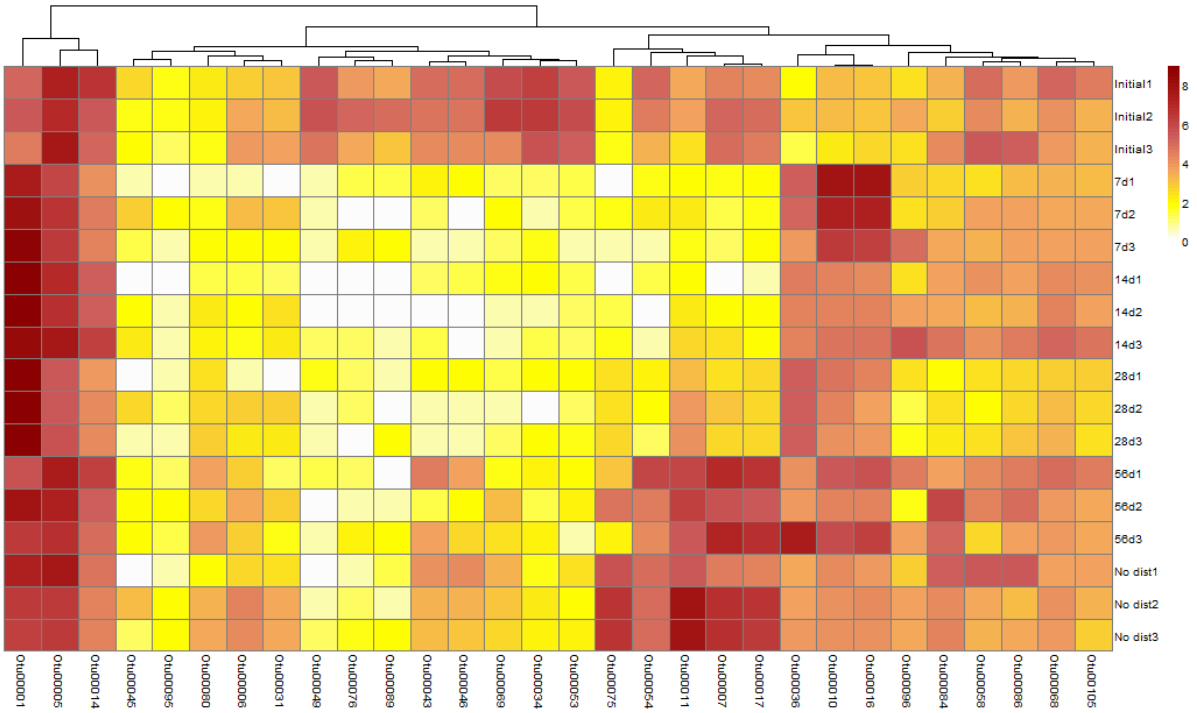

Figure 8. Heatmap displaying the relative abundance of dominant OTUs changing with disturbance. Three most abundant OTUs (1, 5 and 14) were identified as *Trichosporon_dehoogii*, *Gibberella_intricans* and *Umbelopsis isabellina*, respectively.

2.3.3. Phylum-level pattern of fungal community in relation to disturbance frequency

Each fungal phylum showed a distinct pattern in relative abundance in relation to disturbance frequency (**Fig. 9**). *Basidiomycota* were significantly more abundant in the more frequently disturbed soils rather than the less often disturbed ones (p <0.05), while *Ascomycota* showed the opposite trend – becoming less abundant in the more frequently disturbed treatments. *Zygomycota* were more abundant at the highest disturbance frequency (every 7 days treatment).

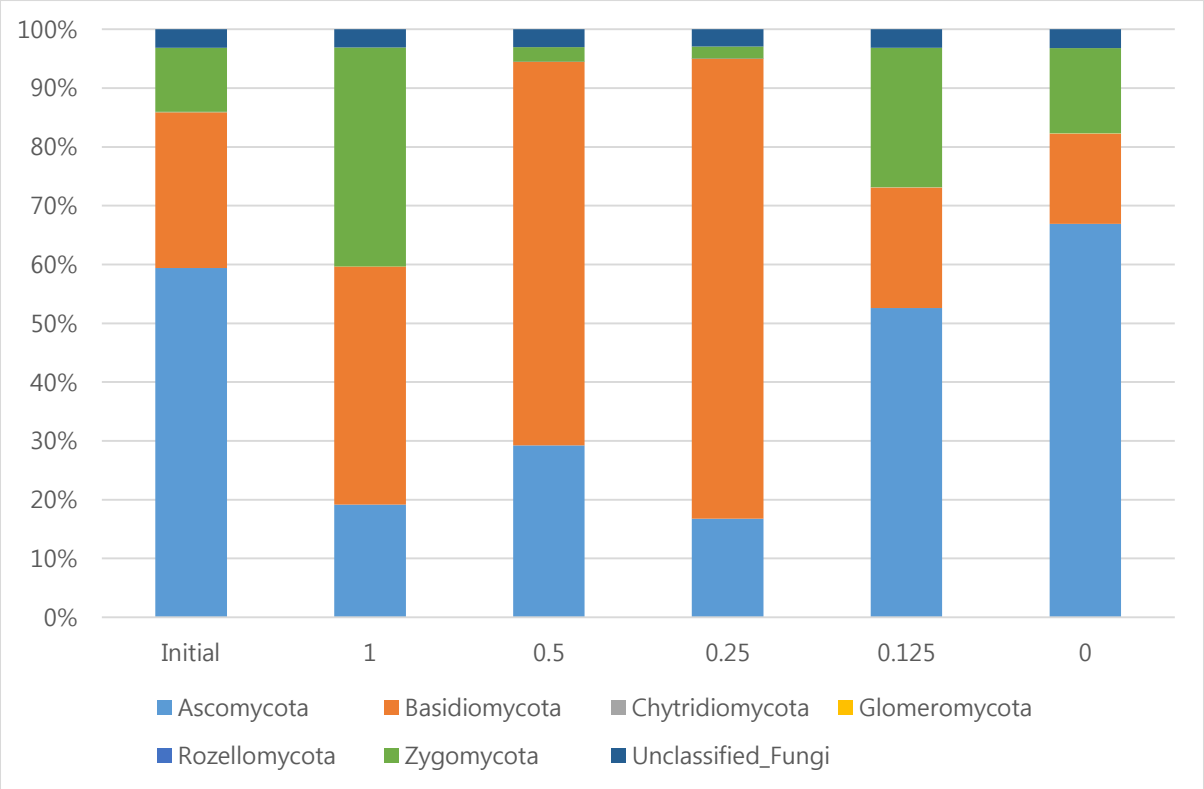

Figure 9. Relative abundance patterns of fungal phyla with disturbance frequency. The highest disturbance frequency (7 days treatment) was set to 1.0.

2.3.4. Ecological relevance of functional groups of fungal community in relation to disturbance frequency

Ectomycorrhizal (EcM) groups of fungi showed a distinct pattern in relative abundance in relation to disturbance frequency (**Fig. 10**). In the initial conditions of this experiment, these EcM groups were significantly more abundant compared to all treatments after 6 months (regardless of frequency of disturbance) (p <0.05). Undefined root endophytes and undefined saprotrophs were also most abundant at initial stage of the experiment, and relatively less abundant in the aged/ disturbed treatment - especially at the intermediate disturbance frequency (every 14 and 28 days). The relative abundance of plant pathogen groups showed no pattern, with no relationship to age or disturbance frequency of sample.

Figure 10. Relative abundance of fungal functional groups (guilds) OTU richness with disturbance frequency level. The highest disturbance frequency (7 days treatment) was set to 1.0.

We also investigated the OTU richness separately in different trophic fungal groups with respect to different disturbance frequency level (**Fig. 11**). Saprotrophic fungi followed similar relative abundance patterns to the undefined saprotroph groups shown in Fig. 7. They were relatively less abundant in intermediate disturbance frequency (every 14 and 28 days). The abundance of pathotropic groups showed no pattern with respect to treatments. Symbiotroph – containing trophic groups (Pathotroph-symbiotroph and symbiotroph groups) were most abundant in the initial stage of the experiment, declining in relative abundance in all treatments by the end of the experiment (p<0.05).

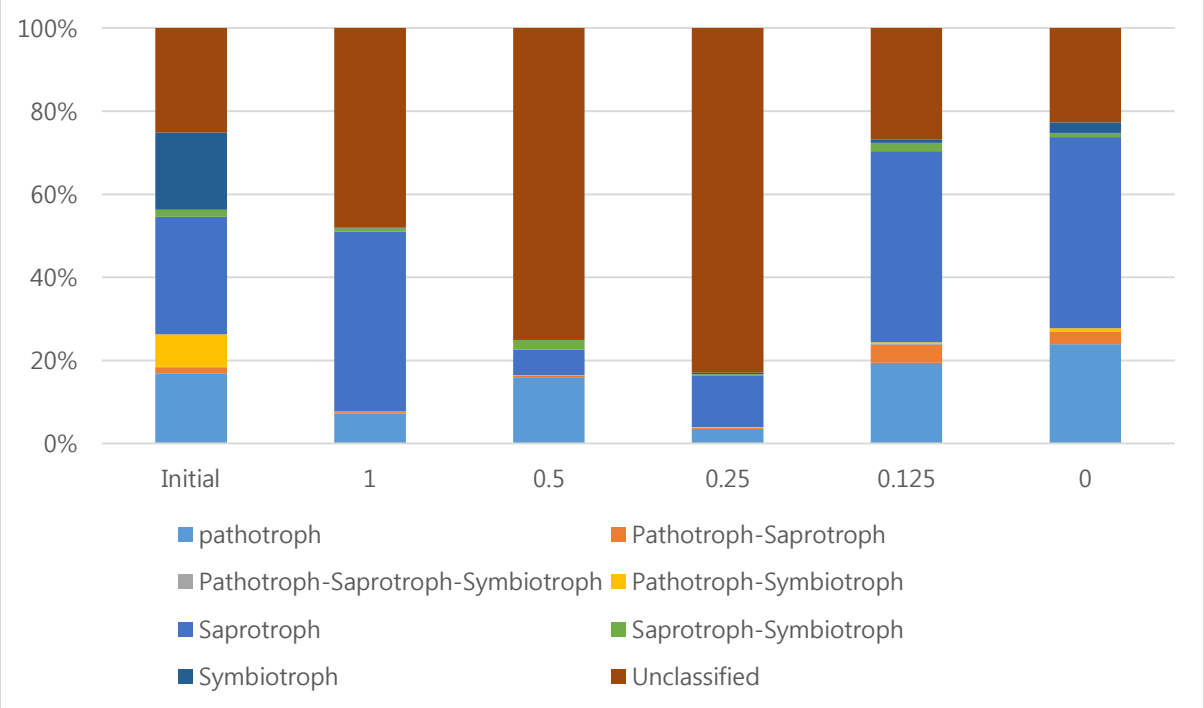

Figure 11. Relative abundance of trophic mode of fungal groups OTU richness with disturbance frequency level. The highest disturbance frequency (7 days treatment) was set to 1.0.

2.4. Discussion

How does disturbance affect overall diversity of soil fungi? Is there any evidence of an 'intermediate disturbance effect' on diversity, as is sometimes seen for larger organisms?

Fungal diversity, expressed as Shannon diversity index, declined with increasing disturbance frequency across the range of frequencies used here. These results are analogous to those found when the soil bacterial community was analyzed from the same experiment (Kim et al., 2013).

Thus, no 'humpbacked' diversity curve was observed on this experiment. This is in contrast with many studies on plants, corals, algae and microbial ecological systems where diversity is compared at a range of disturbance frequencies (Connell 1978; Molino and Sabatier 2001; Ikeda 2003). It is unclear whether widening the range of frequencies of disturbance would have produced such a humpbacked curve, but what this declining trend does seem to make clear is that the remarkably high diversity of soil fungal communities is sensitive to physical disturbance.

By analogy with the view of Grime (Grime 2006) as applied to plant communities, the declining diversity we observed might be explicable in terms of the demands on population survival and the need for rapid growth and reproduction at high disturbance, where fewer lineages have successfully managed to evolve the complex adaptations needed – or such rapid growth and life cycle completion is incompatible with the particular niche that they occupy. It is important to emphasize, that by 'survival' we in fact mean 'survival of population' as a viable entity, rather than 'survival of individual organisms'. There is no sign of the opposite effect, whereby disturbance causes a release from interspecific competition, increasing the diversity. This may indicate that at least in the soil community microcosm studied here, in the undisturbed state competition is not of great enough importance in limiting

the number of species which can survive together, to allow any increase in diversity with disturbance. This in itself may have implications for understanding the limits on the importance of competition in community structuring of soil fungi. Empirically then, it appears that niche exclusion is not the predominant factor in limiting fungal diversity in these soils, because the opportunity for stochastic colonization does not increase diversity. This may be because either because interspecific competition is not intense - because species are already mostly existing in discrete niches with reduced interspecific competition - or because neutral coexistence with extensive niche overlap (Wilson and MacArthur 1967) is important.

Do particular taxa of soil fungi each tend to respond differently to differing disturbance frequencies (e.g. by being more disturbance tolerant or sensitive)?

There were major differences in relative abundance of fungal phyla between the various disturbance treatments, and also compared to initial conditions at the setup of the experiment. In the initial conditions, *Ascomycota* were the most abundant phylum in terms of reads, followed by *Basidiomycota*. After 6 months, comparing the undisturbed control with the treatments subject to varying degrees of disturbance, *Ascomycota* were relatively less abundant in the more frequently disturbed treatments, with an increase in *Basidiomycota.* However, in the most frequently disturbed treatment (disturbance every 7 days) *Zygomycota* had become much more abundant.

These patterns suggest that as we had expected, there are phylogenetically conserved ecological strategies amongst fungi on the broad taxonomic level. Empirically, in this soil system the Ascomycota tend to behave as a relatively 'K' selected group (Pianka 1970; Ngugi and Scherm 2006), less able to tolerate higher frequencies of disturbance. *Basidiomycot*a, by contrast appear more 'r' selected, with increased abundance under more frequently disturbed

conditions (Pianka 1970). *Zygomycota* emerge as the most 'r' selected group (Ngugi and Scherm 2006), being abundant only in the most frequently disturbed treatments. Alternately, if we think in terms of the abundance of resources released by each disturbance event from the killed soil biota, the 'K' selected and 'r' selected forms could be regarded as 'oligotrophs' and 'copiotrophs' respectively, by the terminology of Fierer (Fierer et al., 2007).

It is interesting that the greater abundance of *Basidiomycota* in the more frequently disturbed pots is in opposition to the generally perceived pattern in fungal ecology, that they are a relatively slow-growing and more K-selected group compared to the other fungal phyla (Watkinson 2009; Jasper et al., 1989). Part of the reason why *Basidiomycota* are generally regarded by microbial ecologists as slow growing and disturbance sensitive, is the importance of some basidiomycetes as lignin decomposers in logs and leaf litter, where they do indeed grow more slowly and reach peak abundance later than Ascomycota or *Zygomycota* (Jasper et al., 1989). Adding to the impression that *Basidiomycota* are relatively disturbance sensitive is the role of many important *Basidiomycota* as ectomycorrhiza in forests. In these two senses – as wood decomposers and EcM – the *Basidiomycota* are indeed particularly disturbance sensitive, being less abundant in forests with a history of logging (Kerfahi et al., 2014; Chagas-Neto et al., 2008). Our observations here call into question whether a blanket 'K-strategist' paradigm is appropriate for the *Basidiomycota*. However, it is important to bear in mind that in this experiment only one basidiomycete genus, *Trichosporon* dominated in the 7, 14 and 28 days treatments (comprising over 90% of total *Basidiomycota*). The various species of this genus are able to assimilate a wide range of carbohydrates and carbon sources and to degrade urea (Amos 1966). The different context of our experiment – lack of decomposing wood, and lack of living plant roots - may reveal a hitherto less widely recognized tendency of certain *Basidiomycota* to play a more copiotrophic or 'r'-selected role in soils undergoing frequent disturbance.

In contrast, *Ascomycota* are seen as better able to grow fast to exploit nutrient-rich copiotrophic environments. Although they were abundant in all our experimental treatments, in this particular system they appeared relatively slow growing and 'K' selected overall.

In fungal ecology, the *Zygomycota* are generally seen as being particularly copiotrophic, and their greater abundance in our experimental treatments at highest disturbance frequencies (presumably exploiting abundant dead material left in the soil after autoclaving) fits with this view. The high abundance of *Zygomycota* in the most frequently disturbed (every 7 days) treatment is in a significant part due to the genus *Umbelopsis*. This genus was originally described as a *deuteromycete* (Arx 1982) and later treated as a *zygomycete* related to either the *Mucoraceae* or *Thamnidiaceae* (Mucorales) (Evans 1971).

Is there a predictable fungal community structure that is associated with each disturbance frequency? Does a greater role of randomness take over at higher disturbance frequencies, due to stochastic colonization of resources from small founder populations?

At the OTU level, as we predicted there were strong effects of disturbance regime on fungal community structure. In an NMDS we found distinct and predictable fungal communities that depended on disturbance frequency (**Fig. 7**). A heat map (**Fig. 8**) shows clear differences in composition amongst the most abundant 30 OTUs, with differences mainly showing up in terms of frequency rather than presence or absence. A notable exception was the three most abundant OTUs which were remarkably constant in all the treatments.

In general, the differences in OTU composition and abundance between the treatments are difficult to detect by eye when compared on the heat map diagram (**Fig. 8**), with many of the same OTUs present throughout. It seems that the differences in community structure that show

up on the NMDS are actually the overall effect of many rather subtle differences. However, there are signs of characteristic OTUs which are absent in some treatments but abundant in others. For instance OTUs 10, 16, 36 (*Penicillium skrjabibii*, *Umbelopsis isabellina* and *Umbelopsis dimorpha*) were only present or abundant in the soils that had been subject to disturbance events during the 6 month experiment, being absent from the aged control (**Fig. 8**). These three taxa belong to the *Ascomycota* and *Zygomycota* respectively (*Penicillium skrjabibii* - *Ascomycota*/ *Umbelopsis isabellina* and *Umbelopsis dimorpha* – *Zygomycota*). Their dominance may occur because nutrients from dead cells are more abundant in the most frequently disturbed soils, allowing these to grow relatively fast in a nutrient rich system. *Umbelopsis* is a common saprotrophic genus, found in a range of moist nutrient rich soils and in animal dung (Harper and Webster 1964). *Penicillium* is an abundant and diverse genus in soils and on rotting plant material, although it seems that nothing is specifically known of the ecology of *Penicillium skrjabibii*.

The production of distinctive communities by different disturbance regimens suggests that soil fungal niches may be quite finely divided in relation to disturbance frequency or time since the last disturbance. In the high disturbance frequency treatments, the increased abundance of OTUs that are rare at lower disturbance frequencies can be explained by a trade-off between competitive ability and disturbance tolerance, which is one of general assumptions in ecology (Tilman 1988). This may involve the ability to grow fast without interference competition in early succession after disturbance, as opposed to reliance on antagonistic reactions between fungal species (Lenssen et al., 2004). However it is still controversial whether weak competitors really are generally more tolerant of disturbance than strong ones (Violle et al., 2010; Agrios 1988).

It is a matter of speculation as to what exact ecological parameters the different OTUs are

segregating themselves in relation to, when they respond to disturbance history. Possible factors may include the ability to grow rapidly on labile substrates (e.g. hydrolysable polysaccharides, proteins and nucleotides) left over after the death of all living cells in the autoclave, versus the ability to grow on more recalcitrant substrates such as the soil humus, lignin and cellulose that remain in the soil from when it was still present in the garden. It is to be expected that the more labile substrates will be utilized more quickly after each disturbance event, followed by a shift towards reliance on recalcitrant substrates later on.

The existence of distinct niches in relation to disturbance history suggests a mechanism by which part of the very high OTU diversity of soil might be maintained. There are of course an almost limitless number of subtly different ways in which disturbances could occur in nature (e.g. scale, degree of mixing, whether with or without sterilization of part of the soil, temperature conditions after disturbance, water content), and it is possible that each of these might produce its own distinctive fungal community. Any soil system is continually subject to some sort of disturbance, for example from soil movement under rain or frost, trampling by animals, burrowing by small soil animals and root growth. The fungal diversity that is observed in natural soils (as well as variation between one soil sample and another) may be maintained by the variety of these disturbances that occur. The overall result we found here - of discrete predictable communities produced by different disturbance regimes - was also found in the case of bacteria in the same experiment (Kim et al., 2013). Together, these findings emphasize a strong degree of niche differentiation in relation to disturbance in both bacteria and fungi, which may help to explain the coexistence of a vast diversity within the soil biota.

Despite the predominant pattern of differences between the initial starting community, and the final communities after 6 months at the conclusion of the experiment, three fungal OTUs did not follow the pattern of being abundant only in either low or high disturbance

environments (**Fig. 8**). Even though the degree of dominance is different between treatments, these three species (*Umbelopsis isabellina, Gibberella intricans* and *Trichosporon dehoogii*) which were abundant in the original garden soil, were also predominant in aged and disturbed soils irrespective of disturbance frequency, indicating that those three OTUs rapidly re-colonize the new habitat after disturbance and yet also survive in a more competitive stable environment. *Umbelopsis* and *Trichoporon* are discussed previously. *Giberella* is generally known as a very diverse genus of plant parasites (Lekberg et al., 2011) although in this case it must be living as a saprotroph. The occurrence pattern shown by these three genera, of high abundance in both stable and frequently disturbed conditions seems to stand in contradiction with Tilman's (Tilman 1988) generalization that rapid recolonization ability and persistence in stable competitive conditions are mutually exclusive, at least in plants. It is not clear why these three fungal taxa are able to remain predominant under both disturbed and undisturbed conditions. Possible explanations are that in these particular cases organismal traits behind competitive ability and disturbance tolerance might not be closely related, or that these OTUs play an unknown role in soils that is not affected by interactions with other soil fungi. The abundance of these three OTUs may also reflect the fact that within the soil there is also a continuous process of growth, death and turnover of living cells on a microscopic scale, and it may be these 'micro-successional' niches that they are exploiting. It is worth noting, however, that while all three of these OTUs are abundant in every disturbance treatment, the heat map shows differences in their relative abundance between high and low disturbance treatments (**Fig . 8**). Overall, these three OTUs are very much the exception to the patterns dominating the rest of the community. It is clear that in general despite the three most abundant OTUs which do not change much in abundance, more frequent disturbances shift the fungal communities such that most of the more common OTUs of undisturbed soils are either much less abundant or much more abundant that they are at low disturbance frequencies.

Compared to the initial conditions, which represent soil taken from a system with abundant plant roots, the 'aged' samples – whether from disturbed or undisturbed pots – also have a fairly discrete OTU composition. Most lower level taxa that were abundant in the original fresh garden soil were no longer dominant in any of the aged and disturbed soil treatments. Instead, fungal species which were rare in the initial soil became dominant after 6 months, with different sets of OTUs occurring and becoming abundant depending on disturbance frequency. However, the same three most abundant OTUs in the experimental pots were also the most abundant in these initial samples.

We had predicted that there would be greater dispersion in the NMDS points in the more frequently disturbed treatments, compared to the less frequently disturbed ones. This was because we anticipated that there would be a greater role of stochasticity produced by a history of frequent population bottlenecks and re-expansions when disturbance was frequent, producing a greater range of community compositions. However, we did not observe any increase in dispersion of communities in the more frequently disturbed treatments (Tripathi et al., 2016). This contrasts with the results we found for the same system when we studied bacterial communities (Kim et al., 2013), where there was a clear increase in dispersion of communities on the NMDS at higher frequencies of disturbance – suggesting a greater role of stochasticity with more disturbance. It is unclear why fungal communities may be less susceptible to stochastic effects than bacteria. One possible reason could be that overall fungal diversity is much lower than for bacteria, so that niche redundancy is less (Rousk and Baath 2011). This may mean that following a population bottleneck, a particular niche for fungi tends to remain unfilled until the OTU that occupies it has expanded its population. In bacteria, by contrast, it may be more of a lottery as to which of many suitable OTUs expands its population to exploit a resource first.

Do fungi overall become less abundant in the soil at higher disturbance frequencies, due to their growth rate being insufficient to recover between successive disturbances?

It is interesting that fungal abundance (as measured by fungal ITS1 copy number per gram of soil) in this experiment ended up being relatively greater at intermediate levels of disturbance (14 days treatments), and was least at the lowest frequency of disturbance (56 days and no disturbance treatments), and at high disturbance frequencies (7 and 28 days). This corresponds to the results we found for the same system when we studied bacterial communities, where intermediate disturbance frequencies had the greatest abundances of bacteria (Allison and Martiny, 2008). There are no obvious soil parameter differences that might explain the differences in abundance between different disturbance regimes, which is unsurprising because we derived sterilized soil anew from a common pool for all pots equally at each disturbance event. However, it is possible that at intermediate disturbance frequencies the release of organic nutrients from dead cells killed in autoclaving promotes a short-lived burst of fungal cell growth that would not be apparent in the lower frequency disturbance treatments due to longer time since the last disturbance - while at the highest disturbance frequencies there is insufficient time for buildup of fungal populations to exploit the available resources.

From the qPCR results of bacteria and fungi, we compared the ratio of gene copies between fungi and bacteria. Bacterial copy numbers were relatively most abundant (compared to fungi) at the intermediate disturbance frequency. Conversely, then, fungal copy numbers were relatively fewer at intermediate disturbance frequency. While absolute abundances of bacteria and fungi cannot be compared, due to differences in inherent primer activity, metagenome studies apparently always show dominance by bacteria in soils (el Zahar Haichar et al., 2008). Nevertheless, we regard these changes in ratio of bacterial and fungal copy numbers as

potentially instructive for understanding their shifting ecological roles in natural systems – with fungi as a group being favored by either the highest or the lowest disturbance frequencies.

Are there apparent effects of the removal of plant roots from the soil system?

An additional aspect of this study, quite apart from the effects of disturbance history, is the influence that removal of living plant roots from a soil might have on microbial communities. There is abundant evidence that much microbial life is associated with living roots and their exudates into the soil (Kim et al., 2014). In other experiments, the identity of the plant species growing in a soil have been shown to have effects on the bacterial soil community of the rhizophere (Smalla et al., 2001; Fitter and Garbaye, 1994).

In the present study, the aged samples without disturbance during six months may be seen as simply representing a soil with all the plant roots removed. Comparing these with the 'initial conditions', on the day the soil was obtained from the garden, reveals certain changes in the soil fungal community in phylum level. The NMDS clustering of the initial vs undisturbed samples reveals these changes (**Fig. 7**). However, broadly speaking the changes are not large, compared to the effects of different disturbance treatments for example. The heat map of the most abundant OTUs also reveals some differences of the undisturbed controls from initial conditions, but an overall similarity in terms of continuing presence and broadly similar levels of abundance for most OTUs (**Fig. 8**). It appears that at least on the timescale of a few months, living roots may not be very important for maintaining most of the fungal community found in bulk soil, although of course with rhizoplane fungi (not sampled here) there is presumably a very important role of living roots.

Are the functional guilds of fungi also changed with disturbance frequency?

Using the FUNGuild database (Nguyen et al., 2016), to classify the fungi in our experiment by ecological guild (**Fig. 10**) and trophic mode (**Fig. 11**), we found various interesting patterns.

One unexpected result was the persistent presence of EcM groups of fungi by the end of the experiment (**Fig. 10**). While not abundant by comparison with the initial conditions of this experiment, a range of EcM families and species occur in all the treatments. The persistence of many fungal species reported in the literature to be exclusively EcM (Wang et al., 2011), in our experiment after 6 months in the absence of plant roots, suggests that in fact these may not be truly obligate EcM but instead able to persist to some extent saprotrophically. For example the supposedly obligate EcM species *Russula lepida* (Nelson et al., 1994), is present in all treatments after 6 months at similar levels of abundance. One possible explanation for the persistence of 'obligate' EcM species is the presence of inactive spores left over from the original garden soil, providing a source of DNA showing up in the analysis. However, in the more frequently disturbed treatments there would be a considerable dilution (by a factor of 10 each time) of the original garden soil by sterilized garden soil free of amplifiable DNA, to the extent that after several disturbances the fungal spores should be several orders of magnitude less abundant than before. Their persistence at higher levels than this suggests that certain of these 'obligate' EcM fungi do actually grow and persist at low levels in a root-free fungal community – offering a novel dimension to their ecology.

Another interesting feature of the community differences observed in this experiment is the persistent presence of groups classified by the FUNGuild as plant pathogens (**Fig. 10**). Most plant-pathogenic fungi in our system belong to the Ascomycetes and the Basidiomycetes, comprising for example *Fusarium* and *Phoma* as amongst the most abundant genera. While

OTUs classified as plant pathogens were most diverse and abundant in the soil at the start of the experiment, they remained important in all treatments even after 6 months, in the absence of living plants. Clearly, these cannot be plant pathogens active after several months both in the absence of living plants and maintaining large populations after multiple dilution events, so this must be due to errors in trophic guild classification. At least some plant pathogen genera are known to contain saprobes, for example *Fusarium* (Puri et al., 2015). This demonstrates a limitation in the FUNGuild database which requires correction.

As might be expected, undefined root endophytes were abundant at the initial stage of the garden soil. Nearly 10% of the OTUs in the initial conditions were categorized by FUNGuild as undefined root endophytes. After the 6 months of disturbance, almost none of these endophytes were remaining in any treatment, demonstrating that indeed these do seem to be obligately dependent on the presence of roots. Root endophytes are defined as endosymbionts that live within a plant for at least part of their life cycle without causing apparent disease (Grime 1973), so it is unsurprising that endophytes become less common in the absence of plant roots.

Comparing different disturbance frequencies, undefined saprotrophs became relatively less abundant at the intermediate disturbance frequency (every 14 and 28 days). It is unclear why the undefined saprotrophs become less abundant under intermediate disturbance – and maybe reflect biases in knowledge of the particular taxa abundant in this system.

Using the alternative 'trophic group' classification available under FUNGuild software gave a similar set of categories and relative abundances for classified groups (**Fig. 11**), but with a much higher proportion of unclassified reads. In the intermediate disturbance treatments, the majority of reads are unclassified by trophic group – reflecting the limitations in the database regarding the more common fungi present in this treatment. Of the classified reads, nearly 20%

in the initial conditions of the fresh garden soil were symbiotrophs, and these unsurprisingly reduced to less that 1% in the aged/disturbed treatments. However, pathotrophs remain at around 10% of reads in most treatments after 6 months, suggesting some misclassification of groups that are actually saprotrophs. Nevertheless, in all treatments the largest category is saprotrophs.

In the higher frequency disturbances (every 7 and 14 days), a clear majority of the OTUs were unclassified by guild/trophic type – although this presumably reflects lack of attention to these fungi in the database, perhaps due to many of them being uncultured forms.

Does this experiment have relevance for understanding natural systems?

As we pointed out in our earlier paper on soil bacteria in this same system, any experimental attempt to represent 'disturbance' responses in ecology is to some extent an artificial abstraction, that will miss some aspects of reality of natural disturbance effects. In nature, disturbance events bring about death of most of the organisms they impact. These events are associated with nutrient release and the two processes (death of population and nutrient release) are treated as if inseparable in most of the ecological literature (Huston 1983; Bruns 1995).

It remains a moot point whether the changes in community structure we observed as associated with frequency of disturbance (e.g. mass death of most cells), are really more a result of a crash in population followed by recovery, or the frequent availability of bursts of nutrients released after dead cells break down following soil sterilization. Actually, the ability to survive disturbance may well depend upon the ability to both exploit these nutrient bursts and increase population size rapidly before the next disturbance event: both are an integral part of the 'r' strategy discussed in the ecological literature for plants and other organisms (Huston 1983).

As a microcosm system, our experiment must also differ in some ways from 'natural' disturbance, for example the complete sterilization of most the soil volume, following by a thorough mixing-in of unsterilized soil. The complete removal of roots from previous plant cover is also different from a natural system. However, in a general way, our experimental system can be said to resemble physical turning over of a soil and mixing as a result of (for example) large herbivore activity, flood scour and sediment deposition, or wind throw of trees and other plants – as well as agricultural environments where ploughing, weeding, planting and harvesting take place (Bruns, 1995). Sterilization of the surface soil followed by microbial recolonization - analogous to our study - could also result from a natural forest or grassland fire. Patchy sterilization of almost the entire soil could result from volcanic heating. It is evident from field observations of burned-over or volcanically active areas (pers. observations by JMA) that often in nature these mixing and/or sterilization processes do indeed vary on the scale of a few centimeters, and within soil volumes of a few hundred cubic centimeters, comparable to our microcosms. Thus, the conditions we have created here in the laboratory do not seem so very far removed from plausible reality, and it is reasonable to suppose that useful lessons may be learned from what is observed in our microcosms.

CHAPTER 3. Soil pH rather than elevation dominates bacterial phylogenetic community assembly on Mt. Norikura, Japan.

3.1. Introduction

A major aim of microbial ecology, as in ecology in general, is to understand the processes that control the composition of microbial communities (Nemergut el al., 2013). While microbial ecology has a long history, until recently progress was frustrated by the difficulty of discerning microbial community composition. Modern molecular methods have led to a vast improvement in the quality of data obtainable on microbial communities. The 16S rRNA gene has become a useful tool for microbial ecologists to study soil bacterial diversity (Dunbar et al., 2002; Torsvik and Ovreas, 2002). In its early stages, work concentrated on the empirical description of diversity and patterns (Nannipieri et al., 2003; Fierer et al., 2007; Roesch et al., 2007). Now, as the subject matures, attention is turning more towards explaining these patterns in terms of fundamental ecological mechanisms (Hanson et al., 2012; Besemer et al., 2012; Dumbrell et al., 2010). Even though many studies have been conducted on bacterial community structure, most of these studies only concentrate on variation in diversity and do not consider patterns in phylogenetic relatedness (Bohannan and Hughes, 2003; Fierer and Jackson, 2006). Phylogenetic measures can reveal differences in the composition of two communities that would appear identical using standard measures of species richness and composition (Martin, 2002).

There is a great deal of interest at present in the importance of phylogenetic structuring: the tendency for co-occurrence in communities of sets of lineages that are more closely related than by chance - or conversely less closely related that expected by chance (Harvey and Pagel, 1991). Phylogenetic structure of communities has long been thought of as important. Darwin (1859) suggested that closely related taxa are more likely to interact intensely with each other than with more distantly related taxa. Patterns in phylogenetic species co-occurrence or clustering can indicate that more closely related taxa share traits important for their persistence

in a particular environment (Webb et al., 2002). Such habitat filtering in relation to phylogenetic affinities is crucial, and might be more important than competition (Tofts and Silvertown, 2000; Webb, 2000; Kembel and Hubbell, 2006). In contrast to being enriched in closely related sets of lineages, a community could instead be composed of taxa that are more distantly related than by chance, as a result of current or past competitive exclusion between similar (and thus closely related) taxa and/or as a result of convergent evolution in traits important for persistence in a given environment (Cavender-Bares et al., 2004; Kembel and Hubbell, 2006). However, few studies have so far been done on microbes to test for such patterns.

Another related topic that has attracted much attention recently is the relative importance of deterministic versus stochastic processes in microbial community structuring (Stegenet al., 2012; Wang et al., 2013). Older studies found that the assembly of microbial communities was influenced by deterministic processes (Torsvik et al., 2002), in which predictable biotic/abiotic factors determine the presence or absence and relative abundance of microbial species (Lozupone and Knight, 2007; Fierer and Jackson, 2006). However, more recent studies have suggested that stochastic process involving the vagaries of birth, death, dispersal and colonization also play a major role in shaping microbial community assembly (Caruso et al., 2011; Peay et al., 2010). It has long been debated whether the microbial community composition is governed by a balance between determinism and stochasticity in various ecosystems, but the topics remains understudied (Wang et al., 2013; Stegen et al., 2013; Langenheder and Szekely, 2011; Zhou et al., 2014). In this paper we investigated such questions in the context of elevational gradients.

The effect of elevational gradients on plant and animal diversity has been extensively documented over the past century (Bryant et al., 2008; Kluge and Kessler 2010; Graham et al.,

2009). In recent years, a number of studies have been conducted on trends in soil bacterial diversity with elevation in mountainous regions (Bryant et al., 2008; Singh el al., 2012; Singh et al., 2014). It is unclear whether there is any consistent trend in soil bacterial community structuring patterns along the elevational gradients. Recently, Wang and collegues studied trends in phylogenetic structuring of soil bacterial communities with elevation on Mt Laojun, China (Wang et al., 2012). They found closer phylogenetic relatedness at higher elevations on the mountain, which might due to the environmental filtering (e.g. lower temperature or frequent temperature fluctuations) increased towards higher elevations.

In the present study, we were interested in testing whether the community pattern found in the previous study might be more widely true of elevational gradients in the mid latitudes - in which case it might hint at a rule relating to elevation gradients and possibly disturbance gradients in general. We were also interested in studying patterns in the role of determinism versus stochasticity along elevation gradients, as an indicator of the potential importance of disturbance gradients towards more extreme environments in affecting this aspect of community assembly. Wang et al. did not study this aspect in their elevation gradient study, but recent work by Dini-Andreote et al. suggests that along the successional gradient of a salt marsh chronosequence (Dini-Andreote et al., 2015), the more recent history of disturbance followed by colonization in younger sediments may lead to a greater role of stochasticity compared to older sediments from the same chronosequence. However, Tripathi et al. (submitted) suggest that gradients in soil pH can better explain the relative influence of stochastic and deterministic processes in various successional environments.

In the present paper, we tested two main hypotheses regarding community structuring patterns of bacteria along elevational gradients, based on both the findings of Wang et al. (2012) on Laojun, and the findings of Dini-Andreote et al., (2015) on the salt marsh chronosequence.

We tested the hypothesis of 1) stronger phylogenetic clustering in the upper reaches of the mountain 2) a stronger role of stochasticity at higher elevations due to frequent frost heave and rain wash disturbance in the less vegetated environment with poorer soil development, and frequent freezing and thawing. Our work focused on a mountain in temperate Japan, Mount Norikura. Like Laojin, Norikura is an extinct andesitic volcano, last active during the late Quaternary.

3.2. Materials and Methods

3.2.1. Site description and Vegetation

Mt. Norikura is an extinct volcano in central Japan, reaching 3,026 meters above sea level (masl) with a mean annual temperature (MAT) of 8.5˚C at 1000 masl and mean annual precipitation (MAP) around 2,100 mm. It has a cool temperate monsoon climate at its base at 1,000 masl with MAP at a maximum in summer months.

Norikura is of Quaternary age, with most of the mountain above 1000m having a late Quaternary volcanic cover around 15,000 years old (Nakano et al., 1987). This is silica-rich andesitic material and tends to give acidic soils (Nakano et al., 1995). The lower parts of the mountain below around 1,000 masl are older Quaternary granite (Miyajima and Takahashi, 2007; Takahashi et al., 2003). The lower slopes of Norikura are covered by typical Japanese cool temperate forest, with a mix of mostly deciduous angiosperm trees and evergreen conifers (Miyajima and Takahashi, 2007; Miyajima et al., 2007). This gives way to boreal-type conifer and birch forest in mid-elevations, with the forest cover thinning out above this level. The upper parts of Norikura are open pine scrub with a variety of small woody shrubs and herbaceous plants such as grasses occupying open ground. Vegetation surveys have shown that there is a

steady decrease in tree and herbaceous plant species richness with elevation (Miyajima et al., 2007). The vegetation cover above 1,500 masl is little disturbed by humans, except in some areas right at the summit (we avoided sampling these areas). Vegetation below 1500 masl consists mostly of mature secondary forest >60 years old (Miyajima et al., 2007).

Dominant species at each elevational gradients were described below: at 800 masl (deciduous broad-leaved *Zelkovaserrata, Juglans mandshurica* var. *sachalinensis,* and *Lindera praecox*), at 1,400 masl (deciduous broad-leaved *Quercus crispula*, *Castanea crenata*, and *Betula platyphylla* var. *japonica*), at 1,600-2,000 masl (evergreen conifer *Abies veitchii* and *Tsuga diversifolia*), and at 2,200-2,500 masl (deciduous broad-leaved *Betula ermanii* and *Sorbus matsumurana*) (Miyajima and Takahashi, 2007; Miyajima et al., 2007) (Fig. 12-16).

Figure 12. Photos of sampling sites on Mountain Norikura at -700 masl. Soils from 11 elevational isoclines of the mountain, separated by about 200m were collected.

Figure 13. Photos of sampling sites on Mountain Norikura at -1,000 masl. Soils from 11 elevational isoclines of the mountain, separated by about 200 m were collected.

Figure 14. Photos of sampling sites on Mountain Norikura at -1,700 masl. Soils from 11 elevational isoclines of the mountain, separated by about 200 m were collected.

Figure 15. Photos of sampling sites on Mountain Norikura at -2,300 masl. Soils from 11 elevational isoclines of the mountain, separated by about 200 m were collected.

Figure 16. Photos of sampling sites on Mountain Norikura at -2,700 masl. Soils from 11 elevational isoclines of the mountain, separated by about 200 m were collected.

3.2.2. Soil Sampling

Sampling was carried out over 7 days from late July to early August 2014. Samples were collected on the eastern slope along the Ecoh line and hiking trail. In total, 55 samples were collected from 11 elevations on the mountain, along elevational isoclines separated by ~200 m of elevation. At each elevational level, five separate samples spaced 100 m apart in a line were taken. Within each individual sample, five soil cores were combined to make a composite sample. The soil cores were 10 cm in diameter and 10 cm deep and were taken at four corners and central point of the quadrat of each sample. Samples were transported to the laboratory at Shinsu University within 5 hours of being gathered. At the laboratory, the contents of each bag were gently mixed, then gently passed through a 5 mm sieve.

At each altitudinal sampling band, five samples were taken in a line paralleling the contours at 100m intervals horizontally. We sampled a cylinder of topsoil (below any purely organic A layer) 5cm deep and 10cm in circumference, at each corner of the square and in the center, and mixed all five cores together into the same bag. Replicate quadrat samples from the same elevation levels were stored separately in different sealed sample bags in drinks coolers at ambient, to minimize temperature changes on the way down the mountain before they could be deposited in the freezer.

Sampling completed within a single day, along a broad transect in late July when bacterial activity at all elevations can be expected to be at its maximum. Both sets of samples were frozen at -80˚C within 24 h of sampling, and stored there until sieving and extraction could take place. Mt. Norikura was sampled on its eastern slope along the Ecoh line and hiking trail. However, all samples were taken at least 50m from any trail, to minimize human effects.

3.2.3. DNA extraction, PCR amplification and sequencing

The DNA extraction procedure is as follows: DNA was extracted from each of the collected sieved regolith/ soil samples using the MOBIO Power Soil DNBA extraction kit (MOBIO Laboratories, Carlsbad, CA, USA) as directed by the manufacturer. The extracted DNA was amplified using the primer pair targeting the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene. Polymerase chain reactions were performed in 50-µl reactions under the following conditions: denaturation at 94 ℃ for 2 min, followed by 25 cycles of amplification at 94 ℃ for 30 s, 57 ℃ for 30 s and 72 ℃ for 30 s, followed by a final extension of 72 ℃ for 5 min. PCR products were analyzed by electrophoresis in 1 % agarose gels and were purified using Wizard SV Gel and PCR Clean-up System (Promega, USA). The resulting 16S rRNA gene was sequenced at Macrogen (Macrogen, INC., Seoul, Korea), using paired-end (2*300nt) Illumina MiSeq sequencing system (Illumina, USA) for Norikura samples.

3.2.4. Sequencing processing and taxonomic analysis

The sequenced data generated from MiSeq sequencing and paired-end sequences were assembled using PANDAseq software (Masella et al., 2012). After assembly, all the sequence data were processed separately using Mothur platform (Schloss et al., 2009). The sequences were aligned against the EzTaxon-aligned reference (Chun et al., 2007), and further filtered to remove gaps. Sequences were de-noised using the ‘pre.cluster’ command in Mothur implementation of pseudo-single linkage pre-clustering algorithm from Huse and colleagues (Huse et al., 2010). Putative chimeric sequences were detected and removed via the Chimera Uchime algorithm contained within Mothur (Edgar et al., 2011) in de novo mode, which first splits sequences into groups and then checks each sequence within a group using the more abundant groups as reference. The operational taxonomic units (OTUs) were clustered using

average neighbor clustering algorithm with a threshold of 97% sequence similarity. The entire singleton OTUs were removed from all dataset prior to analysis. All the 16S rRNA gene sequences were classified against Ez-taxon (Kim et al., 2012) using the naïve Bayesian classifier implemented in mother (at 80% bootstrap cutoff with 1000 iterations) (Wang et al., 2007). All the 16S rRNA sequence data are available under the project Norikura bacterial phylogenetic clustering patterns on MGRAST server (http://metagenomics.anl.gov/mgmain.html?mgpage=project&project=71454f126d6d67703739383636) (Meyer et al., 2008).

3.2.5. Phylogenetic analysis

All samples were standardized by random subsampling to 6,356 sequences per sample. A maximum likelihood tree was constructed from all aligned sequences of representative OTUs using Fast tree (Price et al., 2010). We firstly calculated environmental-optima for all OTUs to test the phylogenetic signal with respect to all environmental variables by following the Stegen et al's procedure (Stegen et al., 2012). After that, between-OTU differences in environmental optima were calculated as Euclidean distances using normalized optima of environmental variables. Mantel correlograms were used to measure the correlation to measure the correlation coefficients between differences in environmental optima and phylogenetic distances (Wang et al., 2013; Stegen et al., 2013), and Bonferroni correction test using 999 permutations were evaluate significance of these correlations.

Soil bacterial diversity was estimated using Faith's phylogenetic diversity (PD) because Shannon and other diversity indices (e.g. Ace or Chao) do not explain the evolutionary history or phylogeography of bacterial community (Faith, 1992). To access to relationship of elevation and soil pH with Faith's PD indices of the bacterial community, we fitted a linear and quadratic

regression (Sigma plot v10).

To analyze the phylogenetic community assembly, I measured nearest taxon index (NTI) using ‘phylocom comstruct’ command in Phylocom software (Webb et al., 2008). The NTI describes degree of phylogenetic clustering between taxa of a given sample with respect to the regional pool of taxa. Positive values of NTI reflect phylogenetic clustering (species are more related) and negative values reflect phylogenetic over-dispersion (species are less related). The βNTI measures the pattern of turnover in phylogenetic composition between samples (Kembel and Hubbell 2006; Stegen et al., 2012; Dini-Androte et al., 2015). It can be used to estimate the relative influence of various community structuring processes. For example, homogenous ecological selection for more closely related lineages occurring together more often than by chance, heterogeneous selection whereby more closely related lineages co-occur less often than by chance. Moreover, dominance of stochastic processes whereby the chance effects of dispersal, evolution and population extinction – rather than niche structuring and competitive effects - play a predominant role in bringing about the composition of the community. I calculated the β-mean nearest taxon distance (βMNTD) using ‘comdistnt’ function (abundance.weighted = TRUE) of Picante R package to calculate the turnover in phylogenetic community composition. Developed null modeling approaches in previous study was adapted in this study to disentangle the community assembly processes (Dini-Andreote et al., 2015; Wang et al., 2013; Stegen et al., 2013; Stegen et al., 2015). β-nearest taxon index (βNTI) which is the difference between observed βMNTD and mean of null distribution of βMNTD normalized by its standard deviation was calculated. The βNTI values for all possible pairwise comparisons within, values >-2 and <+2 indicate the dominance of stochastic process (Stegen et al., 2012; Stegen et al., 2013). However, the βNTI values <-2 or >+2 indicating the dominance of homogenous (less than expected phylogenetic turnover) or variable selection (more than expected phylogenetic turnover), respectively (Dini-Androte et al., 2015).

The phylogenetic community structure turnover was quantified using the β-mean nearest taxon distance (βMNTD) as follows:

$$\beta\text{MNTD} = 0.5\left[\sum_{i_k=1}^{n_k} f_{i_k} \min\left(\Delta_{i_k j_m}\right) + \sum_{i_m=1}^{n_m} f_{i_m} \min\left(\Delta_{i_m j_k}\right)\right],$$

Where f_{i_k} is the relative abundance of OTU *i* in community *k*, n_k; the number of OTUs in *k* and $\min\left(\Delta_{i_k j_m}\right)$ is the minimum phylogenetic distance between OTU *i* in community *k* and all OTUs *j* in community $\min\left(\Delta_{i_m j_k}\right)$. The R statistical package comdistnt (abundance.Weighted = TRUE; package picante) function was used to calculate the βMNTD. To infer on the ecological process driving the phylogenetic structure, we used a βMNTD null model approach that generates an expected level for a stochastic dominance. The null value for βMNTD was obtained by recalculating the βMNTD for each iteration of the null model randomly that shuffles OTUs across the tips of the phylogeny that provides βMNTD null distribution (Stegen et al., 2012). The β-nearest taxon index (βNTI) was used to determine direction and magnitude of deviation between an observed βMNTD value and the null βMNTD distribution using the formula below:

$$\beta\text{NTI} = (\beta\text{MNTD}_{obs} - \overline{\beta\text{MNTD}_{null}})/sd(\beta\text{MNTD}_{null}),$$

A separate null model was used to calculate the βNTI for all pairwise comparisons (Dini-Andreote et al., 2015). Both NTI and βNTI values were also calculated for dominant bacterial taxa such as *α-Proteobacteria*, *β-proteobacteria*, *γ-Proteobacteria*, *Acidobacteria* and *Actinobacteria*.

3.2.6. Statistical analysis

Community composition was analyzed by non-metric multidimensional scaling (NMDS) using the standard 'metaMDS' function in the Vegan package in R (Oksanen et al., 2007). The 'envfit' function in the vegan package was then used to fit the environmental parameters on the NMDS ordination, which produces vectors that point in the direction a variable is most rapidly changing and that have lengths proportional to the strength of the correlation between the ordination and a given variable; significance was calculated using 999 permutations.

To check relationships between Faith's phylogenetic diversity (PD)/ nearest taxon index (NTI) and elevation/soil pH, we used linear and quadratic regression analyses to assess the relative influence of stochastic and deterministic assembly processes along the elevation and soil pH, we compared all possible pairwise comparisons of βNTI values within certain elevation and pH range. To further evaluate the variation in community assembly processes along gradients of elevation and soil pH, βNTI values were regressed against Euclidean distance matrices of elevation and soil pH. We assessed statistical significance of the resulting comparison by Mantel tests with 999 permutations using the 'mantel' function of Ecodist R package (Goslee and Urban, 2007). All these statistical analyses were also performed for dominant bacterial taxa *α-Proteobacteria*, *β-proteobacteria*, *γ-Proteobacteria*, *Acidobacteria* and *Actinobacteria*

3.3. Results

3.3.1. Bacterial community composition

We obtained in total of 369,479 quality sequences for all soil samples, which ranged from 6,356 to 9,480 sequences). To correct for differences in number of reads, all samples were subsampled to the level of the smallest number of reads found in any sample (6,356 reads). A total of 16,381 unique OTUs were identified and were assigned to more than 48 bacterial phyla. Among the identified groups, *Acidobacteria* (23%) were the most abundant phylum across the 11 elevation gradient soils and *α-proteobacteria* were the second most abundant, accounting for 21% of all sequences (**Fig.17**). The NMDS ordination plot showed that bacterial community compositions were segregated by elevational gradient. The *envfit* function in R showed that pH (R^2=0.6136, p<0.001) and NH_4- (R^2=0.1394, p<0.05) were statistically significant as a structuring factor in the bacterial community composition in the different elevation level (**Fig. 18**). The physicochemical characteristics including total carbon (TC), total nitrogen (TN), soil pH and ammonium were measured directly for the 55 samples (Table 2).

Table 2. Measured soil properties of samples collected along the elevational range on Mt. Norikura.

Sample	Elevation(m)	pH	Total N (%)	Total C (%)	NH_4-N (mg/L)
N1	2934	6.05	0.07	1.07	5.90
N2	2946	5.63	0.04	0.44	6.83
N3	2946	5.89	0.01	0.15	6.52
N4	2944	5.81	0.02	0.16	3.26
N7	2740	5.58	0.05	0.58	3.72
N8	2738	5.50	0.06	0.72	5.12
N9	2736	5.53	0.09	1.61	4.42
N10	2731	5.82	0.13	2.27	7.45
N11	2522	4.91	1.71	27.15	52.77
N12	2518	5.56	0.31	5.28	14.90
N13	2520	5.60	0.85	14.57	18.16
N14	2521	5.23	0.93	16.66	33.52
N17	2351	4.43	1.42	23.94	38.41
N18	2337	4.54	1.02	19.66	4.66
N19	2358	4.44	1.80	36.90	14.67
N20	2378	4.62	2.11	33.38	42.60
N21	2027	4.66	1.60	43.27	10.86
N22	2090	4.20	1.70	42.78	47.49
N24	2057	4.84	1.51	44.02	42.68
N25	2070	4.57	1.40	41.76	61.92
N26	1677	4.70	1.38	31.25	13.27
N27	1691	4.86	1.67	30.57	34.30
N28	1696	4.40	1.61	32.12	44.00
N29	1700	5.11	1.02	21.43	24.44
N31	1492	5.00	0.87	14.36	6.52
N32	1487	5.22	0.85	15.51	16.06
N33	1492	4.96	0.96	16.51	24.06
N34	1500	5.15	0.80	14.11	21.34

N35	1489	4.76	0.91	16.39	20.56
N36	1319	5.35	0.69	12.88	11.87
N37	1321	5.42	0.60	12.90	14.90
N38	1321	5.59	0.58	11.07	15.52
N39	1320	5.80	0.55	10.09	9.54
N40	1318	5.41	0.82	14.95	14.20
N42	1105	6.07	1.44	21.28	18.62
N43	1095	6.21	2.04	34.05	31.43
N44	1103	6.41	0.62	8.74	6.29
N45	1105	6.40	0.99	14.94	8.92
N46	917	6.31	0.84	10.98	4.66
N47	960	6.30	1.46	22.11	13.19
N48	991	6.00	0.80	10.10	3.72
N49	990	5.37	0.75	8.72	5.59
N50	984	5.88	1.17	15.32	7.45
N51	744	6.01	0.40	3.89	3.26
N53	744	6.40	0.96	12.56	5.35
N54	741	5.26	0.68	6.89	4.19
N55	750	5.30	0.74	7.45	2.56

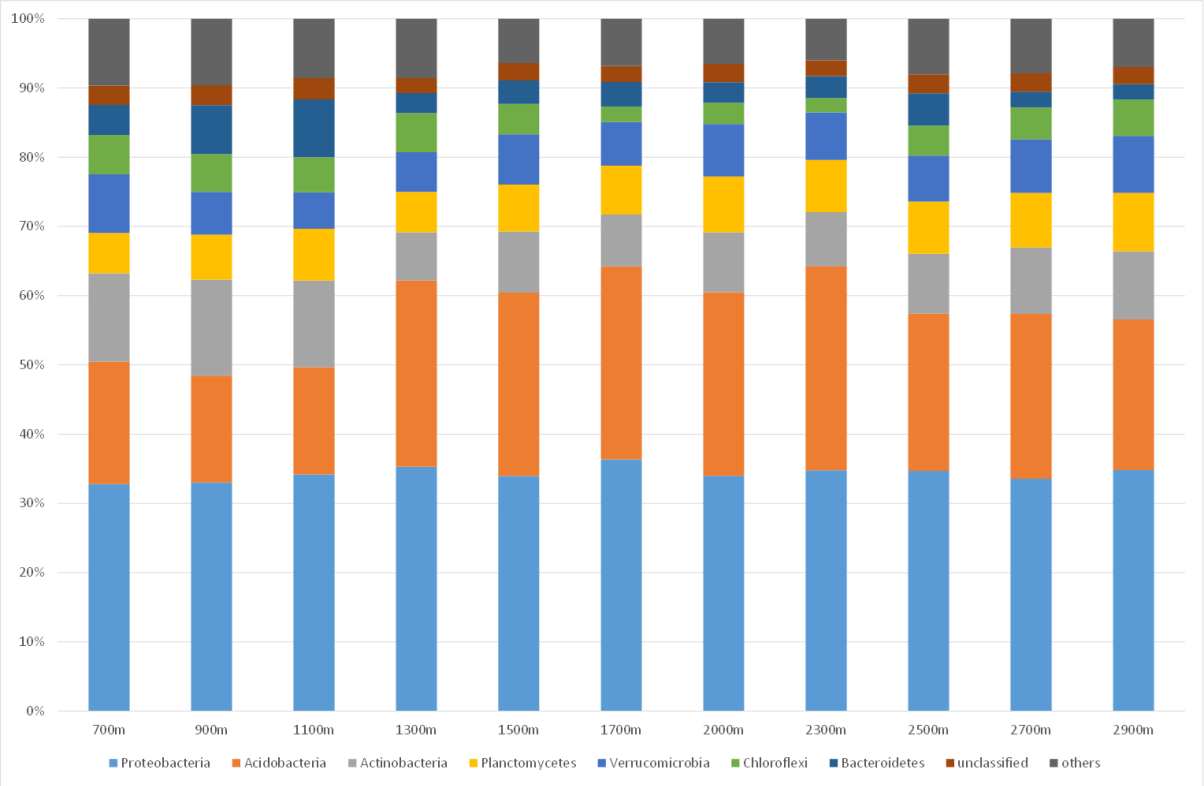

Figure 17. Relative abundance patterns of bacterial phyla with elevation gradients.

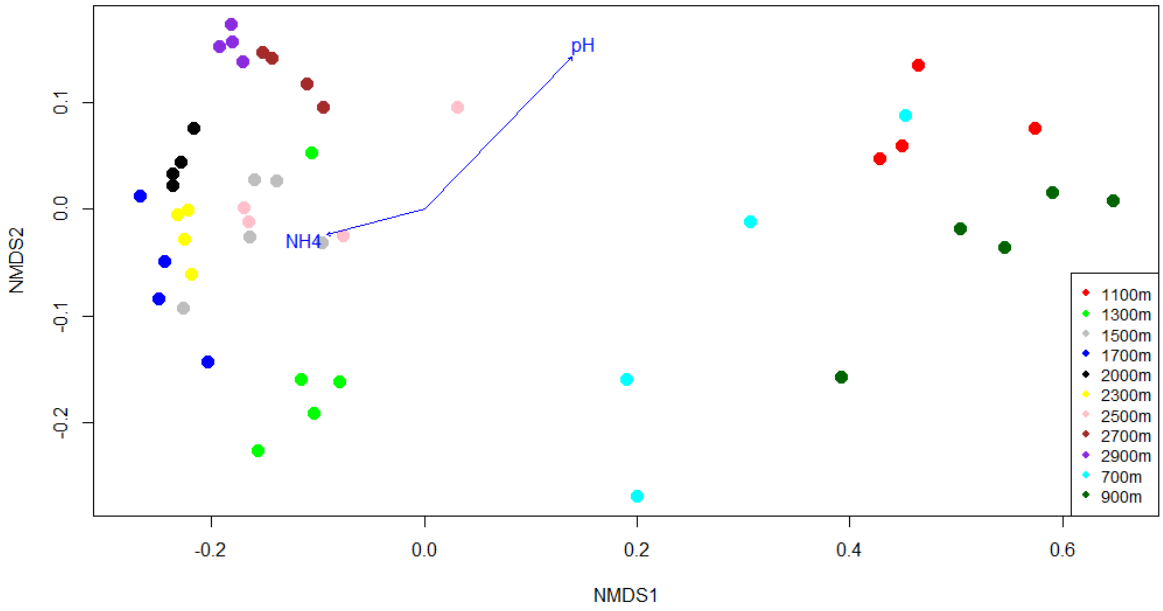

Figure 18. Non-metric multidimensional scaling (NMDS) ordination plot of bacterial communities based on pairwise Bray-Curtis distances. A vector overlay of the significantly correlated variables is shown on the plot.

3.3.2. Phylogenetic signal and Phylogenetic diversity

The phylogenetic signal using the Mantel correlogram showed significant correlations over short phylogenetic distances (p<0.05, **Fig. 12**). This relationship indicates that closely related bacterial OTUs have similar habitat preferences including all environmental variables (occupying similar niches). For this reason, it is possible to calculate the phylogenetic turnover only among nearest relatives (e.g. NTI and βNTI), which support the most appropriate ecological inferences for this study.

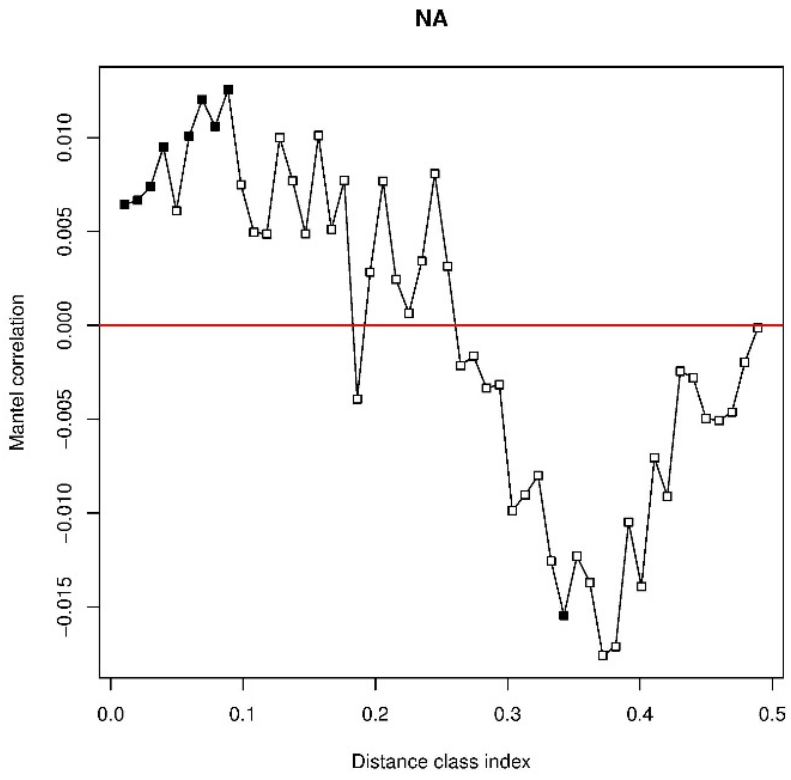

Figure 19. Mantel correlogram between the pairwise distance of OTU niche distances and phylogenetic distance in Norikura. Significant correlation (p<0.05, solid square) phylogenetic signal in species ecological niches.

Regression analyses of elevational gradients on phylogenetic diversity (PD) and nearest taxon index (NTI) provide contrasting results for bacteria overall, and particular dominant phyla or classes. The phylogenetic diversity showed a concave unimodal pattern with elevation in overall bacteria (R^2=0.2230, p=0.0015) (**Fig. 20(a)**) and dominant phyla except *α-Proteobacteria* (R^2=0.1848, p=0.0015) (**Fig. 20(c)**). Phylogenetic diversity of *α-Proteobacteria* exhibited a linear pattern with elevation. Relationship between elevation and phylogenetic diversity was significantly correlated in all dominant phyla or classes (**Fig. 20(c)**, **Fig. 20(e)**, **Fig. 20(g)**, **Fig. 20(I)**, and **(Fig. 20(K)**).

In contrast, NTI results followed a convex unimodal pattern in relation to elevation for all bacteria combined (R^2=0.31, p<0.0001) (**Fig. 20(b)**). The trend in community assembly in relation to elevation gradient shows a maximum in phylogenetic clustering in the mid-elevations. However, there are distinct patterns in individual phyla or classes of bacteria. NTI results shows that *Acidobacteria* (**Fig. 20(J)**) and *Actinobacteria* (**Fig. 20(L)**) are significantly correlated with elevation while *α- proteobacteria*, *β-proteobacteria* and *γ- proteobacteria* did not show significant trends (**Fig. 20(d)**, **Fig. 20(f)** and **Fig. 20(h)**).

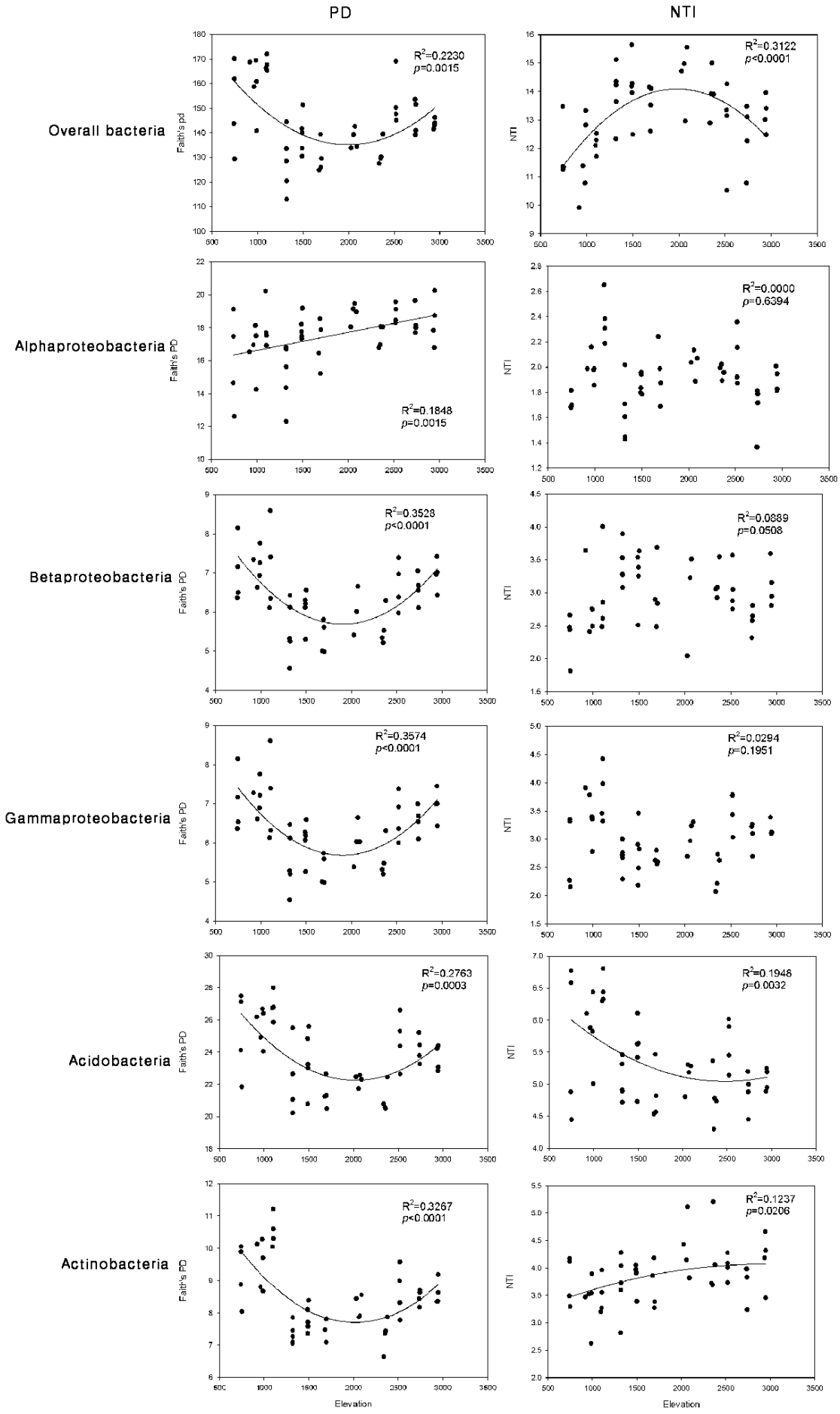
PD
NTI
Overall bacteria
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Acidobacteria
Actinobacteria
Faith's pd
Faith's PD
NTI
R2=0.2230
p=0.0015
R2=0.3122
p<0.0001
R2=0.1848
p=0.0015
R2=0.0000
p=0.6394
R2=0.3528
p<0.0001
R2=0.0889
p=0.0508
R2=0.3574
p<0.0001
R2=0.0294
p=0.1951
R2=0.2763
p=0.0003
R2=0.1948
p=0.0032
R2=0.3267
p<0.0001
R2=0.1237
p=0.0206
Elevation
Elevation

Figure. 20. Regression analyses of phylogenetic diversity (PD) and nearest taxon indices (NTI) measures of bacterial phyla and classes with elevational gradients. Shown are overall bacteria (a-PD; b-NTI), *α-Proteobacteria* (c-PD; d-NTI), *β-proteobacteria* (e-PD; f-NTI), *γ-Proteobacteria* (g-PD; h-NTI), *Acidobacteria* (i-PD; j-NTI) and *Actinobacteria* (k-PD; l-NTI).

Phylogenetic diversity (PD) increased linearly with increased soil pH for all bacteria combined (r^2=0.4603, p<0.0001) (**Fig. 21(a)**) and all dominant phyla except *α-Proteobacteria* (**Fig. 21(c)**). Phylogenetic diversity exhibited no pattern in relation to pH in *α-Proteobacteria*.

However, the NTI decreased linearly in relation to increasing pH (**Fig 21(b)**), showing that the bacterial community was phylogenetically more clustered in lower pH soils compared to higher pH soils (r^2=0.40, p<0.0001). When analyze the five dominant bacterial taxa separately, each showed distinct patterns that did not agree with the overall pattern for all bacteria combined (**Fig. 21(d)**, **Fig. 21(f)**, **Fig. 21(h)**, **Fig. 21(j)** and **Fig. 21(l)**). The results of Mantel test for phylogenetic diversity showed that R^2 values were higher in relation to soil pH than elevation in all cases except for *α-Proteobacteria* (**Fig.20** and **Fig. 21**). Also, the results of Mantel test in nearest taxon index (NTI) showing that R^2 values were higher in relation to soil pH than elevation in all cases except *β-proteobacteria* and *Actinobacteria* (**Fig. 20** and **Fig. 21**). This indicates that soil pH is the better predictor of PD rather than elevation.

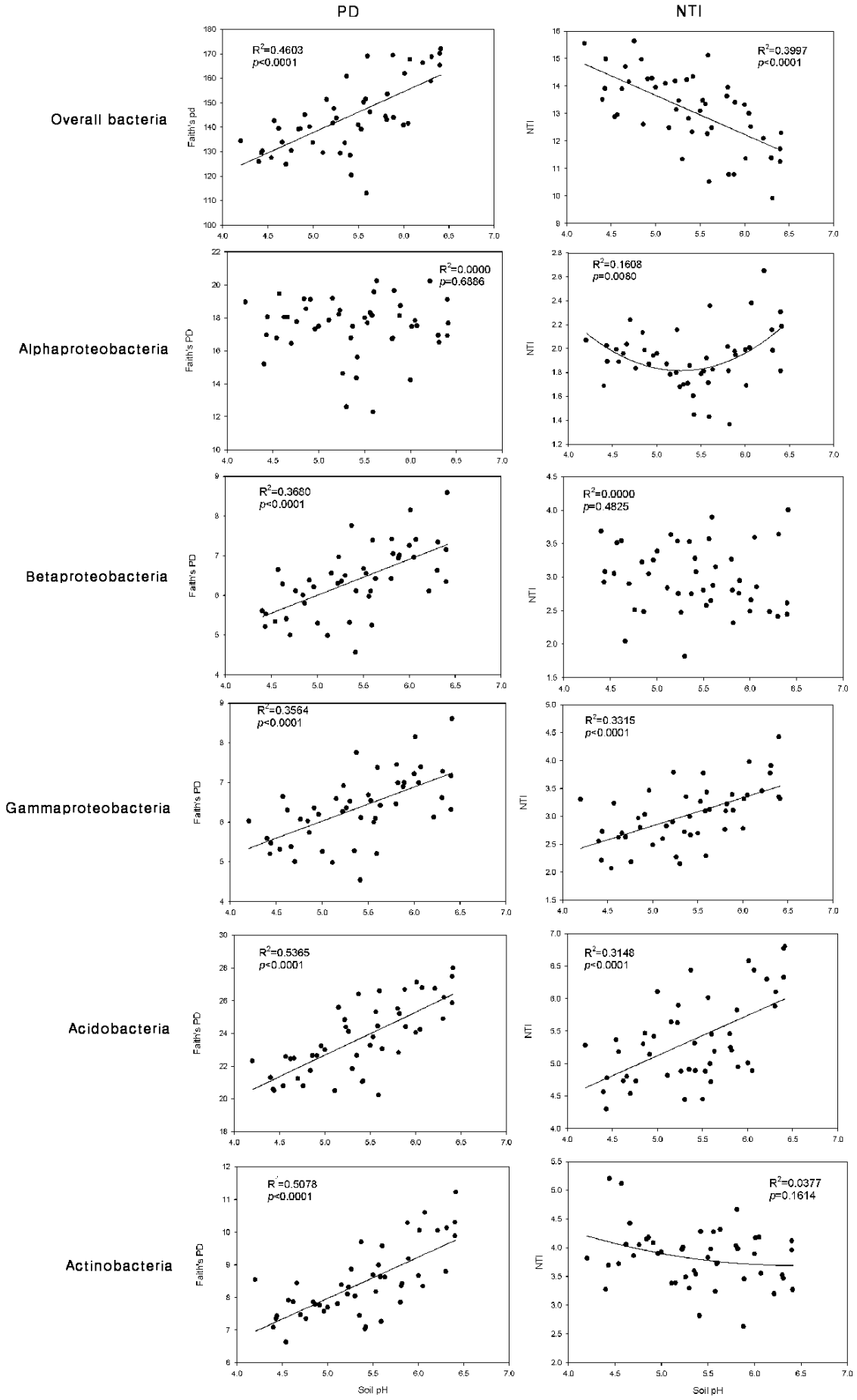
PD
NTI
Overall bacteria
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Acidobacteria
Actinobacteria
Faith's pd
Faith's PD
NTI
Soil pH
R²=0.4603
p<0.0001
R²=0.3997
p<0.0001
R²=0.0000
p=0.6886
R²=0.1608
p=0.0080
R²=0.3680
p<0.0001
R²=0.0000
p=0.4825
R²=0.3564
p<0.0001
R²=0.3315
p<0.0001
R²=0.5365
p<0.0001
R²=0.3148
p<0.0001
R²=0.5078
p<0.0001
R²=0.0377
p=0.1614

Figure. 21. Regression analyses of phylogenetic diversity (PD) and nearest taxon indices (NTI) measures of bacterial phyla and classes with soil pH gradients. Shown are overall bacteria (a-PD; b-NTI), *α-Proteobacteria* (c-PD; d-NTI), *β-proteobacteria* (e-PD; f-NTI), *γ-Proteobacteria* (g-PD; h-NTI), *Acidobacteria* (i-PD; j-NTI) and *Actinobacteria* (k-PD; l-NTI).

All βNTI values from pairwise comparisons were significantly correlated to changes in elevation and soil pH in overall bacteria, as well as in dominant bacterial taxa (**Fig. 22**). In pairwise comparisons of overall bacteria, βNTI was significantly correlated to changes in elevation (**Fig. 22(a)**). Increasing differences in elevation were associated with the shifting relative influence of deterministic and stochastic processes ($r=0.32$, $p<0.0001$). Also, in Pairwise comparisons of overall bacteria βNTI values were significantly correlated to changes in soil pH (**Fig. 22(b)**). Increasing differences in soil pH were associated with the relative influence of deterministic and stochastic processes ($r=0.55$, $p<0.0001$). We found that relatively higher correlation of overall bacteria's βNTI and changes in soil pH than elevation (**Fig. 22(a)** and **Fig. 22(b)**). Also dominant bacterial taxa showed stronger correlations (higher Mantel's R^2 values) to changes in pH than elevation except for *γ-Proteobacteria* (**Fig. 22**). The results indicates that soil pH is the better predictor of βNTI values, which present the relative roles of stochastisity and deterministic process, rather than elevation.

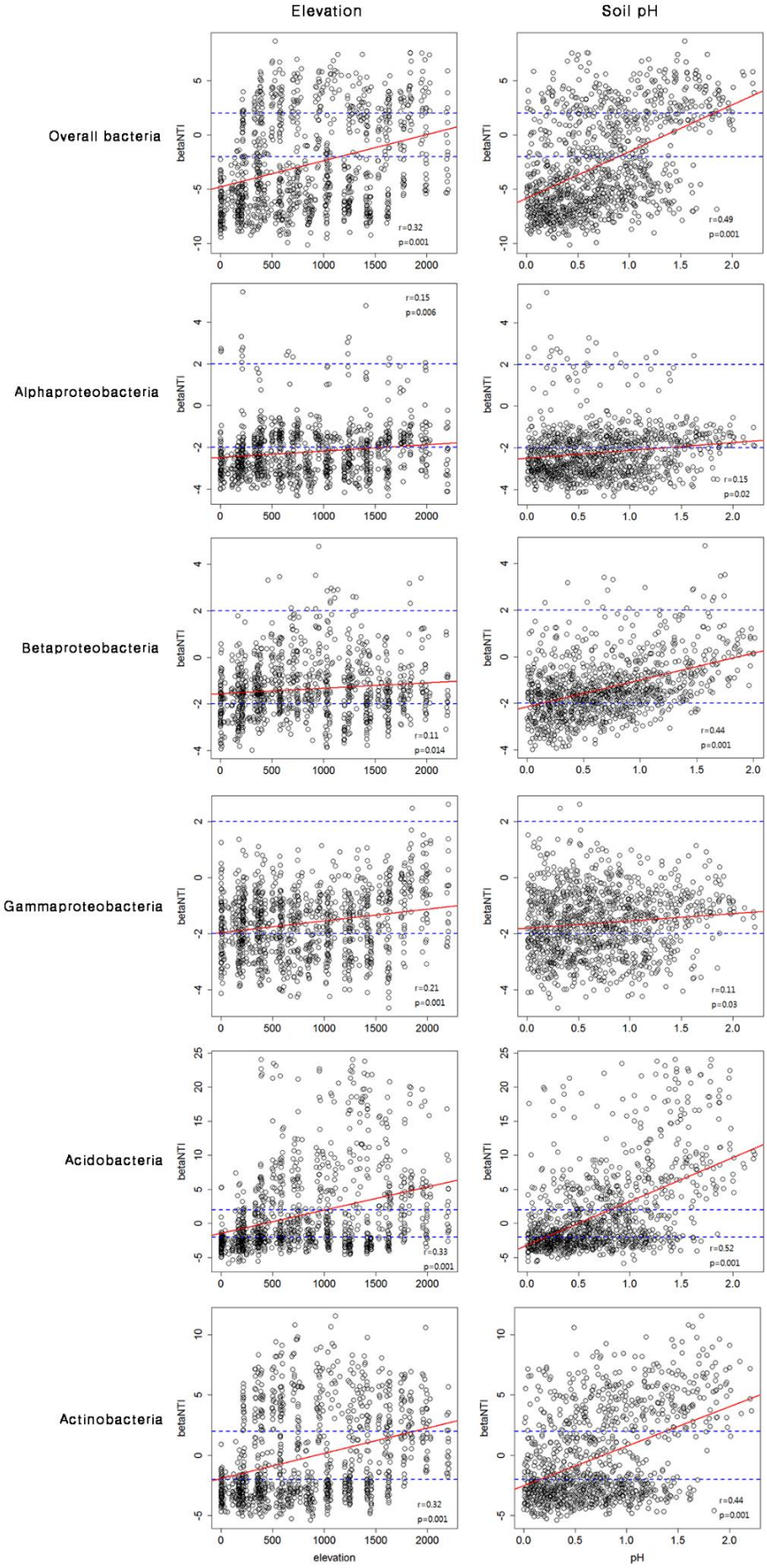
Elevation
Soil pH
Overall bacteria
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Acidobacteria
Actinobacteria
betaNTI
r=0.32
p=0.001
r=0.49
p=0.001
r=0.15
p=0.006
r=0.15
p=0.02
r=0.11
p=0.014
r=0.44
p=0.001
r=0.21
p=0.001
r=0.11
p=0.03
r=0.33
p=0.001
r=0.52
p=0.001
r=0.32
p=0.001
r=0.44
p=0.001
elevation
pH

Figure 22. The relationship between βNTI and change in elevation and soil pH of overall bacteria/ dominant bacteria phyla or classes. Horizontal dashed blue lines indicate upper and lower significance thresholds at βNTI = +2 and -2, respectively. Shown are overall bacteria (a-elevation; b-soil pH), *α-Proteobacteria* (c-elevation; d-soil pH), *β-proteobacteria* (e-elevation; f-soil pH), *γ-Proteobacteria* (g-elevation; h-soil pH), *Acidobacteria* (i-elevation; j-soil pH) and *Actinobacteria* (k-elevation; l-soil pH).

3.4. Discussion

The elevational diversity gradient is one of the most fundamental patterns in larger organisms' biogeography (Lomolino, 2001; Bryant et al., 2008). However, much evidence shows that microbes do not follow the classic patterns see in plants and animals (Bryant et al., 2008; Fierer et al., 2011). Analyses of phylogenetic relatedness with elevation may provide insights regarding the mechanisms (e.g. environmental filtering or competition) that shape the bacterial community (Webb et al., 2002; Kembel and Hubbell, 2006; Cavender-Bares et al., 2009).

Here we tested the hypotheses of 1) stronger phylogenetic clustering in the upper reaches of a mountain and 2) stronger role of stochastic rather than deterministic processes, due to frequent frost heave and rain wash disturbance, in the upper reaches.

Hypothesis 1. Stronger phylogenetic clustering in the upper reaches of a mountain.

Our results did not support the hypothesis that stronger phylogenetic clustering would prevail in the more open terrain towards the top of the mountain. The overall bacteria NTI results instead showed a significant convex trend along the elevational gradient of Mt. Norikura (**Fig. 20(b)**). The trend in community assembly in relation to elevation gradient shows a maximum in phylogenetic clustering in the mid-elevations. In contrast, there was a closer negative linear trend in relation to soil pH (**Fig. 21(b)**), and the trend of NTI with elevation appears to be due to the higher correlation between elevation and soil pH in Mt. Norikura. The zone of lowest soil pH in the mid-elevations can thus explain the stronger phylogenetic clustering in this part of the mountain **(Fig. AF1**).

In Wang et al's recent study, by contrast, there was an increase in phylogenetic clustering with increasing elevation (Wang et al., 2012). Since both mountains (Norikura and Laojun) show a similar general elevational zonation in vegetation, with a forested lower part that opens up to alpine tundra above, the contrasting results clearly suggest that the hypothesized mechanism does not prevail in structuring communities on high mountains in general. The contradictory results might be due to the differences in soil conditions that relate to the underlying geology, and it is possible that this instead is the prevailing factor that affects phylogenetic structuring (see below).

Bacterial phylogenetic diversity (PD) also responded strongly to elevational gradients and soil pH. The PD followed a unimodal pattern in relation to elevation for overall bacteria and five dominant bacterial taxa except α-proteobacteria (**Fig. 20**). However, in relation to soil pH there was a positive linear trend as phylogenetic diversity increased as soil pH increased in overall bacteria and dominant bacterial taxa, except *α-proteobacteria*. Also, the *envfit* function in R support that pH was the strongest structuring factor in the bacterial community composition in Norikura (**Fig. 18**).

As was earlier found by Tripathi et al (submitted) for successional environments, low soil pH is associated with stronger phylogenetic clustering compared to higher soil pH. It has been widely noted that soil pH is overwhelmingly the strongest predictor of soil bacterial diversity (Fierer and Jackson, 2006; Lauber et al., 2009; Tripathi et al., 2012), as is also the case here on Norikura (**Fig. 21**). The taxonomic homogeneity of the lower soil pH environments found here and by Tripathi et al would seem to represent the constraints on lineages colonizing this environment, presumably due to the physiological challenges of maintaining a near-neutral intracellular soil pH in a relatively acidic environment (Fierer and Jackson, 2006, Tripathi et al., 2012). Such constraints on lineage colonization may also be key in producing the lower

diversity of low pH soils (Fierer and Jackson, 2006; Lauber et al., 2009; Tripathi et al., 2012).

From both PD/NTI values against elevation and soil pH, Mantel test showed that there were stronger correlations with soil pH than elevation, for overall bacteria and most cases for particular dominant bacterial taxa. This might indicate soil pH is the better predictor of PD and NTI rather than elevation.

Hypothesis 2. Stronger role of stochasticity in the upper reaches of each mountain.

It is generally known that the microbial community assembly is influenced by both deterministic and stochastic factors (Dumbrell et al., 2010l Ofiteru et al., 2010; Langenheder and Szekely, 2011). Here we investigated the relationship between βNTI and elevational gradients to infer changes in the relative influences of deterministic and stochastic assembly processes in overall bacteria and dominant bacterial taxa respectively. Also contrary to our predictions, the βNTI results did not show any clear trend with elevation (**Fig. 22(a)**, **Fig. 22(c)**, **Fig. 22(e)**, **Fig. 22(g)**, **Fig. 22(I)** and **Fig. 22(k)**).

We also found that the bacterial community in all elevational ranges influenced dominantly by homogeneous selection (βNTI<-2) (**Fig. AF2**). The βNTI results suggests then that stochasticity does not have a stronger role in the open landscapes and more unstable soils towards the top of the mountain. From the results, we can infer that elevation is not the best predictor of the relative importance of stochastic and deterministic processes. The βNTI values across the whole dataset lumped together indicate a predominance of homogeneous selection (**Fig. AF2**).

Instead, we found that overwhelmingly the strongest predictor of βNTI on Mt. Norikura was soil pH, and that over small ranges in soil pH, bacterial communities were largely influenced by homogenous selection in overall bacteria and dominant bacterial taxa (**Fig. 22(b)**, **Fig. 22(d)**, **Fig. 22(f)**, **Fig. 22(h)**, **Fig. 22(j)** and **Fig. 22(l)**). Larger changes in soil pH led to dominance of variable selection. Increasing differences in soil pH between pairs of samples were thus associated with the shifting relative influence of deterministic and stochastic processes. A Mantel test showed that there was stronger correlation between βNTI and pH than for elevation in overall bacteria and dominant taxa except *γ-Proteobacteria* (**Fig. 22**).

Overall, we found that environmental filtering involving pH strongly determines elevational trends in bacterial community composition, and that stochasticity also has an important role. What is unclear, however, is why pH should dominate bacterial community composition in this way. Tripathi et al. (2012) have suggested that the stronger physiological constraints on survival in low pH environments, with greater energy costs associated with homeostasis, restrict the possibilities of multiple species surviving in the same niches. In such a constraining environment, there are either fewer evolved species in existence that are viable in each niche (leading to less stochasticity), or exacting physiological demands that must be met in order to remain viable in competition – impeding neutral coexistence (and thus leading to less stochasticity).

Chapter 4. General Conclusions

Microbes are not only make up a large proportion of the biological diversity of the terrestrial ecosystem but also are a fundamental component of nutrient cycling and productivity. Although microbes have long existed on earth since 3.8 billion years, it has only been 400 years since humans first realized that they are all around us. Due to their small size, microbe's life history, spatial distribution, and ecological function are still poorly understood. Comparing to larger organisms, the patterns of microbial community structure are still unknown and the full breadth of microbial diversity was poorly documented. This study investigated how fungal communities respond to disturbance frequency in a laboratory setting, as well as the extent to which ecological processes or other environmental factors contribute to structuring bacterial communities in elevational ecosystems.

At first, I tested the intermediate disturbance hypothesis on soil fungal communities. The effect of disturbance on soil fungal community was investigated in a soil microcosm study. The extent of soil fungal diversity in experimental ecosystem and how they respond to changes of disturbance frequency was investigated. Fungal diversity decreased with increasing disturbance frequency rather than showing a hump-shaped relationship as hypothesized. Community structure shifted with disturbance suggesting that 'randomness' influences the community more strongly in more frequently disturbed conditions. Studies on microcosms can reveal the effects of disturbance or to determine the ecological role of microorganisms. Different fungal phyla showed different responses to disturbance: *Basidiomycota* were significantly more abundant in the more frequently disturbed soils rather than the less often disturbed ones, while *Ascomycota* showed the opposite trend- becoming less abundant in the more frequently disturbed treatments. *Zygomycota* were more abundant at the highest disturbance frequency. From the results, the soil fungal community composition and abundance responded strongly to a history of different frequencies of disturbance was founded. The response of fungal community composition to disturbance frequency was to a large extent

predictable, with replicates with the same disturbance history clustering closely on an ordination. What this experiment makes clear is that there can be a strong element of predictability in soil fungal community development. The results suggesting a considerable degree of niche differentiation in relation to disturbance history, may go some way to explaining how thousands of fungal OTUs are able to coexist in soil without out-competing one another. The relative abundances of OTUs were changed, depending on the history of disturbance. OTUs 49, 76, 69, 34, and 53 were only abundant in the initial soil. By contrast, OTUs 36, 10, and 16 were only abundant in the aged/disturbed soils-especiaaly the soil treatment with most frequent disturbance. OTUs 75, 53, 11, 07, and 17 were most abundant in relatively undisturbed soil. However three most abundant OTUs (1, 5, and 14), identified as *Trichosporon dehoogii*, *Gibberella intricans*, and *Umbelopsis isabellina* were abundant in all treatments including the initial conditions. There are clearly distinct niches and strategies that differ between particular OTUs, and in a more general way between higher level taxa, and guilds/trophic groups. Whether these niche differences are absolutely essential in maintaining coexistence is however an unknown. It is also clear that the diversity of the soil fungal community is easily decreased by disturbance. Under the range of conditions explored here, disturbance of the soil only decreases diversity.

What processes and factors operate in shaping microbial phylogenetic communities in the mountain ecosystem was further examined in Mt. Norikura in Japan. This study clarify the processes that govern microbial phylogenetic community in the Mt.Norikura in Japan. Among the identified groups, *Acidobacteria* were the most abundant phylum across the all elevational gradients and *α-Proteobacteria* were the second most abundant phylum. The phylogenetic diversity (PD) showed a concave unimodal pattern with elevation in overall bacteria and dominant phyla except *α-Proteobacteria*. However, nearest taxon index (NTI) results followed a convex unimodal pattern in relation to elevation for all bacteria combined. Phylogenetic

diversity (PD) increased linearly with increased soil pH for all bacteria combined and dominant phyla except *α-Proteobacteria*. The Nearest taxon index (NTI) decreased linearly in relation to increasing soil pH showing that the bacterial community was phylogenetically more clustered in lower pH soils compared to higher pH soils. There is clearly an important role of deterministic processes in bacterial assembly within its mountainside. This study finds a different trend from the earlier study and suggests that there may be no consistent trend in phylogenetic community structuring of bacteria with elevation. All β-Nearest Taxon Index (βNTI) values were significantly correlated to changes in elevation and soil pH in overall bacteria, as well as in dominant bacterial taxa. Increasing differences in elevation were associated with the shifting relative influence of deterministic and stochastic proceeses. There were no indication of the hypothesized trend towards a greater role of stochasticity in community structuring towards the higher elevations of the mountain. However, in lower soil pH environments there is a greater role of determinism compared to near-neutral pH environments. Increasing differences in soil pH were associated with the relative influence of deterministic and stochastic processes. I found that relatively higher correlation of overall bacteria's βNTI and changes in soil pH than elevation. Also dominant bacteria taxa showed stronger correlations to changes in soil pH than elevation except for γ-Proteobacteria. This suggests that we may be beginning to discover certain fundamental, widespread rules of community structuring of soil bacteria. It is important to test whether this holds true pattern in other environments, including other examples of environmental gradients.

Overall, it appears that patterns of diversity and community composition of microbial in experimental and mountain ecosystems are somewhat different from those observed in larger organisms (e.g. plant and animals). In experimental ecosystem, no 'humpbacked' diversity patterns was observed that is contrast with many studies on larger organisms. Also, bacterial phylogenetic diversity was not follow the elevational diversity patterns that of plants and

animals. Soil pH rather than elevation dominates bacterial community assembly processes. Deterministic process dominates bacterial phylogenetic community assembly in all elevational range of Mt. Norikura. Disturbance and elevation were the most important factors influencing species diversity and community composition. Together these results provide a baseline ecological framework with which to pursue future research on both ecosystems.

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APPENDIX

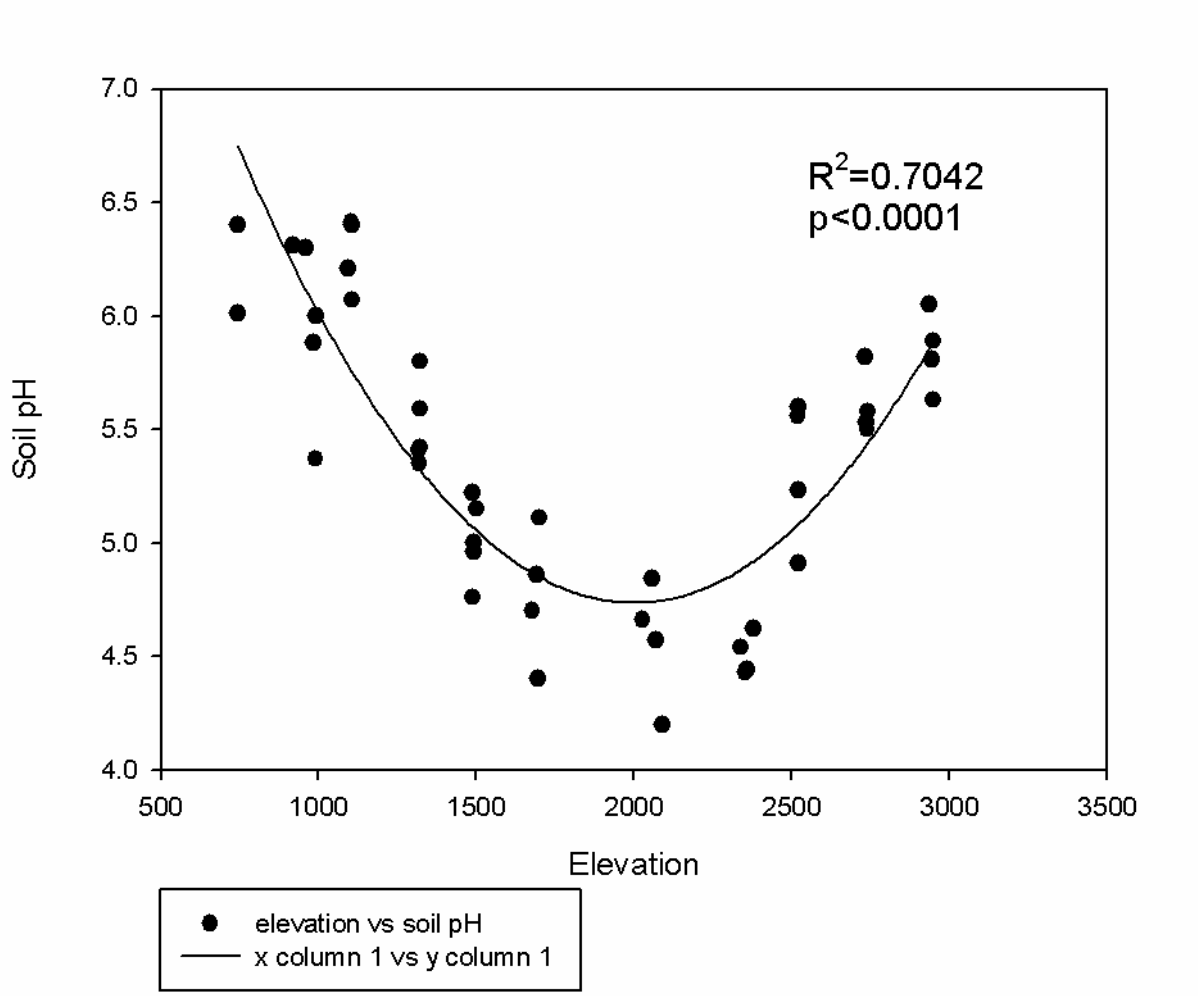

Figure. AF1. Relationship between elevation and soil pH on Norikura.

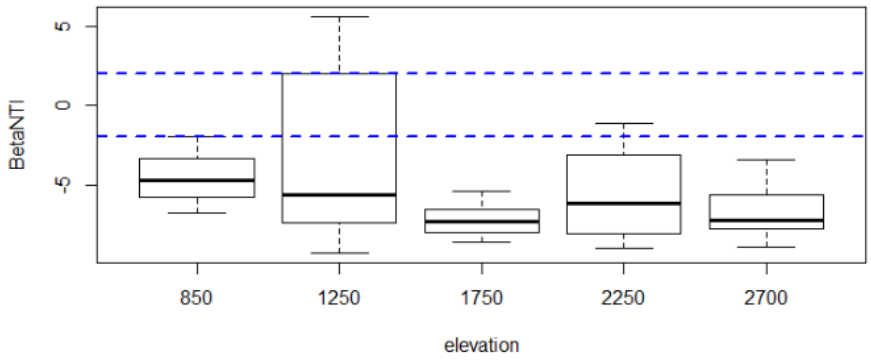

Figure. AF2. Patterns of βNTI across elevational gradients on Norikura. Horizontal dashed blue lines indicate upper and lower significance thresholds at βNTI = +2 and -2, respectively.

국문초록 (Abstract in Korean)

미생물은 지구의 생물다양성 측면에서 중요한 부분을 차지하고, 육상생태계에 존재하는 수많은 서식처들에서 많은 종들이 발견되고 있다. 이렇게 다양한 미생물 종이 존재함에도 불구하고 자연에서 이들의 생태학적 역할이나 다양성에 관한 연구는 많이 부족한 실정이다. 본 연구에서는 미생물 군집형성에 영향을 주는 요인들을 알아보기 위해 인위적인 실험환경과 산림 생태계에서 각각 1) 교란빈도와 2) 환경 구배가 토양 미생물 다양성에 미치는 영향과 미생물 군집형성에 영향을 끼치는 그 외의 환경적 요인들에 대해 알아 보았다.

우선 실험실 조건에서 교란빈도가 토양 진균(fungi) 군집에 미치는 영향에 대해 알아보았다. 교란(disturbance)은 생태학적인 측면에서 중요한 역할을 차지함에도 불구하고 실험실 조건에서 토양 진균 군집이 교란에 받는 영향은 식물과 동물과 같은 상대적으로 큰 생물체에 비해 적게 연구되었고, 식물과 동물에서 오랜 기간 동안 연구 되어온 것에 비하여 토양 진균 군집의 교란에 대한 민감성에 관한 연구는 많이 이루어 지지 않았다. 본 연구에서는 토양 microcosm에서 진균 군집의 구조와 다양성이 물리적 교란의 빈도에 따라 어떻게 변화하는지를 알아보았다. Microcosm을 통해 전체의 90%가 살균된 토양에서 남은 10% 만을 다음 세대로 옮기는 물리적 교란을 서로 다른 빈도로 처리함으로써, 교란 주기에 따른 진균 군집의 영향을 Illumina Hiseq 시퀀싱을 통하여 살펴보았다. 교란빈도가 증가함에 따라 진균 종 다양성은 감소하였지만, 식물에서 보여진 ‘humpback’ 패턴이 나타나지 않았다. 반면 교란 빈도에 따라 특정 그룹이 늘어나기도 하지만 반대로 몇몇 그룹은 감소하는 양상을 보였다. 전체적인 토양 진균 군집구조는 물리적 교란에

민감하게 반응할 뿐 아니라 그룹에 따라서 교란의 빈도에 다른 양상을 나타낸다는 것이 관찰되었다. 또한 물리적 교란의 빈도에 따른 확연한 OTU-level 구성이 나타난다는 것이 확인 되었고, 기능에 따른 진균의 그룹들 (guilds)이 교란 빈도에 따라 변화 하는 것이 관찰 되었다. 교란 빈도에 따른 군집 구성의 변화는 토양 진균이 교란 빈도에 따라 서로 다른 생태적 지위 (niche)를 차지한다는 것을 나타낸다.

다음으로 인위적인 환경조건뿐만 아니라 실제 자연환경에서 어떠한 환경 요인들이 미생물 군집을 조절하는지 알아보았다. 이를 위해 일본의 Mt. Norikura 에서 세균 군집의 다양성과 패턴이 환경 요인의 구배(environmental gradients) 에 따라 어떻게 달라지는지 조사하였다. 또한 군집의 형성과 계통적 다양성 그리고 세균 군집이 결정론적 혹은 확률적인 과정에 의해 구분되는지 알아 보았다. 이를 위한 토양 샘플들은 Mt. Norikura 에서 서로 다른 고도에서 채취 된 후 Illumina Miseq 시퀀싱을 통해 분석되었다. Mt. Norikura 에서의 계통적 군집형성은 해발 고도와 높은 연관성을 나타내지 않았다. 계통적 군집형성은 토양 pH와 강하게 연관되어 산성 (acidic)의 토양에서 더욱 강한 군집형성을 나타냈다. 이러한 양상은 토양의 pH가 천이 (succession) 환경에서 미생물 군집형성에 강한 영향을 미친다는 기존의 연구결과와 일치한다. 토양 pH와의 높은 연관성은 이것이 세균군집 구성뿐만 아니라 다양성에 영향을 미치는 중요한 요인이라는 것으로 설명이 가능하다.

주요어 : 진균 다양성, 토양 microcosm, intermediate disturbance hypothesis, 교란, 세균, 토양 pH, 군집 구성, 계통적 다양성, 계통적 군집 형성

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