Nitrogen cycling driven by soil microbial communities in exotic black locust plantations and native oak forests in the drylands of East Asia

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1. General Introduction

1-1. Introduction

Nitrogen (N) is the most limiting nutrient in many terrestrial ecosystems (Binkley and Hart, 1989; LeBauer and Treseder, 2008), that influences the productivity, composition, and function of forests (LeBauer and Treseder, 2008; Tanner et al., 1998; Wright et al., 2011). Plants primarily use soil inorganic N, such as ammonium N and nitrate N, and can also use organic N as a significant resource in soils with low N availability (Jones and Kielland, 2002; Näsholm et al., 1998; Nordin et al., 2001). Soil inorganic N is produced by microbial communities through decomposition and mineralization of organic matters. By associating with soil microbes, especially mycorrhizal fungi, plants can access various soluble organic compounds such as amino acids and peptides (Abuzinadah et al., 1986; Bajwa and Read, 1985; Finlay et al., 1992). In addition, the difference in N cycling have significant impacts on terrestrial carbon (C) storage, because soil N availability often controls the decomposition of soil organic matter (SOM) and plant litters (Allison et al., 2010; Perakis and Sinkhorn, 2011; Schimel and Weintraub, 2003), and forest productivity (Norby et al., 2010). Thus, belowground microorganisms have a very important role in the forest N cycling, and further in ecosystem function such as terrestrial C sink.

Soil microbes include algae, protozoa, fungi, bacteria and archaea (prokaryote), and parts of groups in the soil microbes strongly accelerate the N cycling (Paul, 2007). The series of N transformation processes in the soil can be briefly divided into three steps (Fig. 1). The first step is degradation, which produces small-molecule organic N from large-molecule organic N such as the SOM. The second step is mineralization, in which the small-molecule organic N compounds are mineralized and converted to ammonium N. The third step is nitrification, in which ammonium N is converted to nitrate N. Fungi and prokaryotes are the main drivers of the degradation and mineralization steps, respectively (Moore et al., 2003). In the nitrification step, ammonia-oxidizing bacteria and archaea are important because they perform the rate-limiting process (Isobe et al., 2015; Kowalchuk and Stephen, 2001). Many reports suggest that differences in soil fungal and prokaryotic communities contribute to the difference in forest N cycling (Fraterrigo et al., 2006; Hawkes et al., 2005; Phillips et al., 2013). The analysis of environmental DNA through the use of specific gene markers such as species-specific DNA barcodes have been rapidly developed in last two decades. The analysis can tell us the abundance and community composition of millions of microbes living in a gram of soil (Fierer and Jackson, 2005; Shokralla et al., 2012). Furthermore, new tools to refer to the big data can predict from environmental DNA what ability the microbial community has for driving the soil N cycling (Langille et al., 2013; Nguyen et al., 2016).

Soil fungal and prokaryotic communities are sensitive to various environmental factors, and they change their function to drive the N cycling in response to the environment. For example,

soil pH is a strong factor controlling microbial community composition (Xiong et al., 2012; Fierer et al. 2006). Low availability of water and organic matters causes the high sensitivity of microbial biomass and structure to moisture and nutrient level (Barnard et al., 2013; Hu et al., 2014; Liu et al., 2009). As a result, the N transformation process slows with high soil pH, low water and substrate availability (Pathak and Rao, 1998; Zhang and Zak, 1998). Aboveground plant species are also a primary determinant factor for the soil microbial community and soil N cycling through biological symbiosis (Averill and Hawkes, 2016; Cheeke et al., 2016; Phillips et al., 2013). The response of the N transformation steps to the environmental factors will differ between the steps, as nitrification is suggested to be more sensitive to environmental change than mineralization because nitrifying microbes are less diverse than ammonifying microbes (Isobe et al., 2019). Thus, the analyses of environmental properties, microbial composition (especially, functional composition) and its performance on soil N cycling enable us to understand the mechanism behind the N cycling. These analyses are also useful to more accurately predict how the N cycling will respond to current rapid environmental changes, such as the anthropogenic land-use change and climate change, mediated by the change in soil microbial community.

Dryland (dry sub-humid, semiarid, arid and hyperarid) ecosystems occupy over 5.06×10^9 ha (41%) of land globally (Zhang et al., 2010), and they are under high risk of desertification due to the land-use change and climate change (Bestelmeyer et al., 2015). East Asia has drylands in central and western China and in Mongolia. Loess Platea is located in central China, and the size is 6.27×10^7 ha, which is mostly under dryland climate. Rainfall patterns are expected to change dramatically in the near future, especially on drylands; drought events will likely increase in frequency, the areal extent of dryland zones will expand (IPCC, 2014). Loess Plateau is also predicted to experience an increase in evapotranspiration rate and frequency of drought events in the near future (Li et al., 2012; Sun et al., 2019). In Loess Plateau, a large scale of land-use change, such as cut-down of natural vegetation, has also occurred from 1930s to 1950s. Exotic tree species to this region, black locust (*Robinia pseudoacacia*), were largely planted in the 1960s and the plantations are currently very common in this region (Du et al., 2011), although the climax species in this region is the oak (*Quercus liaotungensis*) (Yamanaka et al., 2014). The combination of climate change and land-use change can have substantial impacts on this region. Exotic monoculture plantations are suggested to be more vulnerable to climate change than natural mixed forests (Afreen et al., 2011). The wilting of the top of the crown was observed in the black locust plantations but not in the oak forest (Tateno et al., 2007), which suggests that the black locust plantations are suffering from the water limitation and future rainfall change will have critical impacts on the plantations.

Rainfall pattern changes are expected, but it is still not clearly predicted how rainfall

pattern change determines soil N cycling (Homyak et al., 2017). In drylands, water is usually the most limiting resource for net primary production (NPP), but N is also a co-limiting factor for NPP (Austin and Sala, 2002; Eskelinen and Harrison, 2015). Further, when drought periods are protracted, N is a greater limiting resource for NPP than water (Ren et al., 2017). Phosphorus (P) is also a limiting resource for plant growth in some ecosystems, but N is usually more limited in drylands than P. In fact, leaf N:P ratios of black locust and oak in this region were <14 (11.1 and 9.2, respectively; Tatsumi et al. unpublished), which is in the range of N limitation rather than P limitation (Koerselman and Meuleman, 1996). Many studies have examined the response of soil N cycling to rainfall (Bowles et al., 2018; Chen et al., 2017, 2013; Ladwig et al., 2015; Landesman and Dighton, 2010). However, most studies have focused on only single parts of the N transformation process or have not provided detailed information on microbial N drivers. A comprehensive study that includes whole steps of soil N transformation and relevant microbes is necessary to more accurately predict the responses of soil N cycling (and further C cycling) to the future rainfall patterns change.

The introduction of exotic species was often reported to totally change the soil N cycling compared to in natural forests (Hawkes et al., 2005; Kolbe et al., 2015; Xu et al., 2018). Black locust forest was also suggested to have significantly different N cycling from the oak forest, but the mechanism is still unknown (Tateno et al., 2007). Black locust is known as a N-fixing tree, but my preliminary survey showed black locust trees in this region do not actively fix N (see Chapter 4). In this study, I focused on the fact that black locust is symbiotic with a different type of mycorrhizal fungi from the oak. The forest mycorrhizal type is getting more attention as a potentially large factor controlling N and C cycling (Averill et al., 2014; Averill and Hawkes, 2016; Phillips et al., 2013). The black locust is symbiotic with arbuscular mycorrhizal (AM) fungi (Yang et al., 2014), although the native oak is symbiotic with ectomycorrhizal (ECM) fungi (Zhang et al., 2013). ECM fungi can produce many hydrolytic and oxidative extracellular enzymes (Chalot and Brun 1998, Courty et al. 2010, Kohler et al. 2015 but see Pelliter and Zak 2018), and obtain small organic N-bearing molecules from SOM, leaving behind a relatively C-rich substrate (Averill et al., 2014; Orwin et al., 2011). As a result, ECM fungi are thought to limit the amount of N available for free-living microbes, and to slow free-living microbial SOM decomposition (Averill and Hawkes, 2016; Fernandez and Kennedy, 2016; Gadgil and Gadgil, 1971). On the other hand, AM fungi lack the saprotrophic ability and are believed to use inorganic rather than organic N (Hodge and Storer, 2014; Read and Perez-Moreno, 2003; Smith and Read, 2008; Smith and Smith, 2011). As a result, AM fungi do not strongly limit the amount of N available for free-living microbes, and therefore do not prevent SOM decomposition by saprotrophic microbes. The difference in SOM decomposition rate are thought to cause different soil C storage (lower in AM than ECM forests),

and in fact, the black locust forest had lower C storage than the oak forest in this region (Tateno et al., 2007). However, it is still a hypothesis and direct tests for the N competition between ECM fungi and free-living microbes are still lacking (Averill and Hawkes, 2016; Fernandez and Kennedy, 2016). For understanding how the black locust plantation causes different N cycling from the oak forest, it is an important approach to test the N competition hypothesis, which assume N is competed between ECM fungi and free-living microbes. In the hypothesis, it is unknown which form of N is competed for and which microbial groups compete for the N with ECM fungi. To reveal that, it is necessary to compare every steps of soil N transformation and relevant microbes between the AM (black locust) forest and the ECM (oak) forest. The knowledge will improve our understanding of the effects of AM and ECM forests on the biogeochemical cycling, further contributing to design landscape with more desirable ecosystem function.

Moreover, natural succession to the oak forest has not observed in the black locust plantations in this region. Plantations of exotic trees may modify native flora (Harrington and Ewel, 1997). The black locust forest had lower plant diversity than the oak forest in this region (Otsuki et al., 2005), and the slow natural succession also occurred in other black locust plantations of Central Europe (Vítková et al., 2015). Exotic trees suppress a growth of native plants in many ways, such as an allelopathic effect (Murrell et al., 2011; Orr et al., 2005), an increase in pathogens (Mangla et al., 2008), and a disruption of belowground mutualism (Stinson et al., 2006). It also

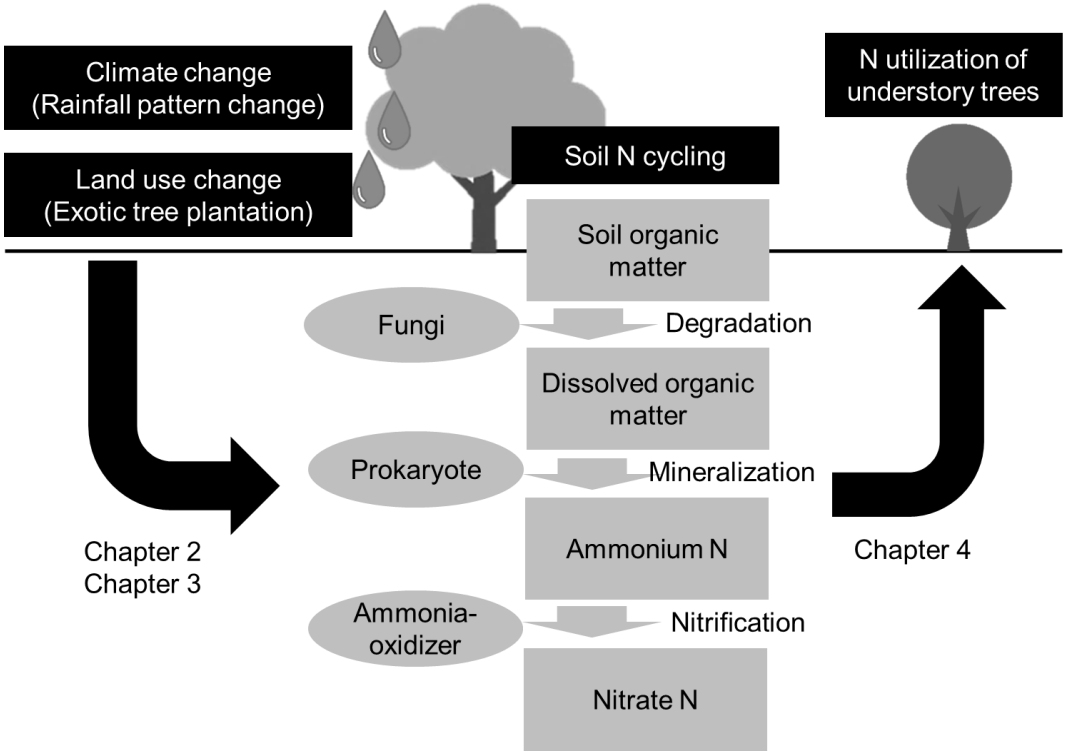

Fig. 1. Outline of this study (what I will reveal in each chapter)

can be possible that the characteristic soil N cycling in the black locust forests suppresses the oak growth because soil N availability controls the recruitment and growth of plant species (Averill et al., 2018; Clark and Tilman, 2008), although the mechanism has not been revealed yet. I hypothesized the canopy trees affect the N utilization of understory trees by changing the soil N cycling. Plants were reported to change their N utilization such as soil N source and/or uptake rate in response to soil N availability (Dijkstra et al., 2015; Warren, 2009), although some plants did not change (Andersen et al., 2017; Ashton et al., 2010; Russo et al., 2013). In addition, black locus forest and oak forest would provide different mycorrhizal fungal inoculum to the understory trees, because black locust and oak are symbiotic with different type of mycorrhizal fungi as described above. Mycorrhizal fungi are known to have different performance in transferring N to host plants depending on taxonomy (Mensah et al., 2015; Veresoglou et al., 2011). I expected the black locust forest changed the N utilization of the understory trees compared to the trees in the oak forests, and this can be a potential reason of the slow succession. The understanding of effects of canopy trees on understory trees would contribute to develop an effective method to convert exotic forests into native forests.

To reveal the effects of the climate change and land-use change on soil N cycling and plant N utilization, I used soil DNA metagenomic analysis and N isotopic analysis. For simplification, as described above, I briefly divide the series of N transformation processes in the soil can be divided into three steps: 1) degradation of high-molecular-weight organic matter, 2) mineralization, and 3) nitrification (Fig. 1). Each step is primarily driven by fungi, prokaryotes and ammonia-oxidizing prokaryotes, respectively. Further, by using the natural abundance of stable isotope of N (δ^{15}N), we can reveal the plant N use process (Koba et al., 2003; Templer et al., 2007). The δ^{15}N of soil organic and inorganic N, and plant tissues are naturally determined in response to soil N cycling and plant N uptake. For example, the δ^{15}N signature of leaves correlates with those of soil ammonium vs. nitrate taken up by plants (Falkengren-grerup et al., 2004; Kahmen et al., 2008). Thus, by integrating the method above together with conventional chemical analyses of plants and soils, it becomes possible to understand how soil microbial communities respond to the environmental factors and change their function to drive the N cycling and affect plant N uptake process.

1 - 2 . Objectives and the dissertation outline

In this series of studies, my hypotheses and questions are that: How and in what mechanism (1) the soil N transformation steps change with aridity levels in the forests, (2) the soil N transformation steps of the black locust forest differ from those of the oak forest, and (3) the N

utilization of the native understory trees differs between the black locust forest and the oak forest. To answer the first question, in Chapter 2, I examined the three steps of soil N transformation process with the soil microbial community, in three black locust forests occurring along a natural aridity gradient. For the second question, in Chapter 3, I studied the soil N transformation steps and its microbial drivers in the black locust forests and the oak forests, focusing on the difference in the mycorrhizal type. Finally, to answer the third question, in Chapter 4, I examined N utilization, such as the soil N source and associating mycorrhizal fungi, of the native plants co-existing in the black locust forest and the oak forest. Further, I will discuss the interaction between the effects of these environmental changes on the N cycling, and the terrestrial C sequestration. I will also discuss the potential that the black locust plantation changes the native plant ecosystem. These knowledges will contribute to understand the best land management in this region, such as how we can promote natural succession in the current exotic plantations, what plant species should be used for establishing plantations in order to maximize ecosystem function such as to increase terrestrial C storage.

2. The steps in the soil nitrogen transformation process vary along an aridity gradient via changes in the microbial community

2-1. Introduction

Studies of N cycling responses to rainfall pattern changes are essential for a better understanding of soil dynamics in an era of rapid climate change. Many studies have examined the mechanism of soil N dynamics responses to rainfall (Bowles et al., 2018; Chen et al., 2017, 2013; Ladwig et al., 2015; Landesman and Dighton, 2010). However, most have focused on only single parts of the N transformation process or have failed to provide information on microbial N drivers. A comprehensive study that includes steps of soil N transformation and relevant microbes will contribute significantly to a deeper understanding of the mechanism underlying the responses of soil N dynamics to rainfall pattern change. The series of N transformation processes in the soil can be divided into three steps as introduced in the Chapter 1. Soil N transformation is a step-by-step process; substrate quantity and quality can limit downstream transformations (Norman and Barrett, 2014; Updegraff et al., 1995). Changes in an upstream step can affect the subsequent downstream step by acting as a bottleneck, i.e., a step that delays the progress of subsequent steps. For example, in cold ecosystems, the mineralization step is limited by temperature and can act as a bottleneck (Rustad et al., 2001; Schimel et al., 2004). The nitrification step can also act as a bottleneck when the pH is low (Ste-Marie and Paré, 1999). Thus, each step may react differently to changes in rainfall patterns. Our understanding of the total soil N dynamics process will be improved by dividing the chain of events into separate steps that can be examined in detail.

The responses of soil N dynamics to rainfall pattern changes are mediated by soil microbes (Bowles et al., 2018; Chen et al., 2017). Microbes also fall into three categories of step drivers, corresponding to each N transformation steps, as introduced Chapter 1. A focus on microbial composition improves understanding of the mechanism of soil N dynamics because the abilities and strategies for N transformation vary among microbial taxa. For example, among fungi, saprotrophs and mycorrhizal species decompose organic matter primarily to obtain C and N, respectively. Mycorrhizal fungi receive C from their host plants (Lindahl et al., 2007; Smith and Read, 2008). The soil bacteria may be categorized as copiotrophs, which readily decompose labile compounds, and oligotrophs, which decompose recalcitrant compounds (Fierer et al., 2007). These two bacterial groups also differ in their ability to produce extracellular enzymes, including N-acetylglucosaminidase and chitinase, which decompose monomers and polymers, respectively (Zimmerman et al., 2013). Ammonia-oxidizing bacteria and archaea also have different oxidization capabilities: nitrification rates tend to correlate with ammonia-oxidizing bacterial

abundance, but not with archaeal abundance, likely because ammonia-oxidizing bacteria are competitive at high ammonium concentrations (Di et al. 2009; Chen et al. 2013; Banning et al. 2015), although not in acidic soil (Isobe et al., 2015; Prosser and Nicol, 2008).

Many studies have examined the ways in which soil physicochemical properties influence the microbes driving soil N processes. The low soil moisture, low organic matter content, and high pH in drylands limit fungal and bacterial growth (Frey et al., 1999; Maestre et al., 2015) and change community compositions by reducing the abundances of copiotrophic and ammonia-oxidizing bacteria (Adair and Schwartz, 2008; Xiong et al., 2012). However, under such stressful conditions, microbial groups with high drought tolerance or enhanced abilities in decomposition of recalcitrant nutrient would flourish, as observed in Actinobacteria and Verrucomicrobia (Bouskill et al., 2013; Cederlund et al., 2014; Iwaoka et al., 2018). Thus, a comprehensive view of soil N dynamics will emerge from studies that link information on each step of soil N transformation, their drivers, the soil microbial community, and soil physicochemical properties. With a such a broad view, we can better predict how rainfall pattern shifts will alter the soil conditions that regulate microbial communities and the chain of N transformation processes.

Natural aridity gradients provide appropriate candidate conditions for studies aiming to predict the effects of rainfall pattern changes on soil N dynamics. Along an aridity gradient, soil moisture content changes with other soil properties, such as organic matter content, as a function of rainfall history, unlike in shorter-term artificial rainfall experiments. Soil N availability, N transformation rate, and microbial abundance tend to increase with rainfall toward the wetter end of an aridity gradient (Adair and Schwartz, 2008; Austin and Sala, 2002; Burke et al., 1997). However, this is not always the case, as mineralization rate, nitrification rate and N availability have been reported not to change with rainfall (Feral et al., 2003; Meier and Leuschner, 2014; Smith et al., 2002). Contradictions among studies have arisen because the underlying mechanisms are not understood. In this study, I examined three black locust forests occurring along a natural aridity gradient on the Loess Plateau in northeastern China. Differences in vegetation, including variability in canopy trees, strongly influence soil N dynamics (Finzi et al., 1998; Phillips et al., 2013); I therefore compared the same vegetation type along the aridity gradient.

I examined the process of soil N dynamics changes (mediated by shifts in the soil microbial community) along the aridity gradient. I aimed to use the data from this study to predict responses to rainfall pattern alterations in the time of global climate change. I studied all three steps of N transformation, the associated microbial communities, and soil physicochemical properties along the aridity gradient. My fundamental hypothesis was that inhibition of a single step in the transformation process would cause a bottleneck in the soil N cycling process. Such bottlenecks would be key elements in the N limitation of forest productivity in arid ecosystems

during future climate change.

2-2. Materials and Methods

2－2－1. Study sites and soil sampling

I conducted the study in three black locust forests spanning the southern to central sectors of the Loess Plateau. The three stands were located in mountainous areas of Shaanxi Province, China: (i) near Yongshou County (Huiping; 34°48N, 107°59E, 1395 m elevation), (ii) near Yan'an City (Mt. Gonglushan; 36°25N, 109°32E, 1320 m elevation), and (iii) near Ansai Country (Zhifanggou; 36°45N, 109°15E, 1175 m elevation), which I refer to hereafter as the BL-Wet, BL-Med (BL-Medium), and BL-Dry sites, respectively. The vegetation type has been classified as a forest–steppe transitional zone; the mean annual rainfall decreases gradually from southeast to northwest (Yamanaka et al., 2014). The reference evaporation rate increases from south to north in my research region (Li et al., 2012). Annual rainfall and mean annual air temperature at the sites were as follows: 606 mm and 10.8°C at the BL-Wet site (Li et al., 2015), 514 mm and 10.2°C at the BL-Med site (Otsuki et al., 2005), and 449 mm and 8.8°C at the BL-Dry site (Qiu et al., 2012). The area has hot summers and cold winters; heavy precipitation occurs in summer. Although the precipitation gradient in this study, only 150 mm in mean annual precipitation, is smaller than those in other studies (Adair and Schwartz, 2008; Austin and Sala, 2002; Feral et al., 2003; Meier and Leuschner, 2014), 150 mm is a critical difference in this area, the arid boundary of a forest. The rainfall amount at the BL-Dry site was 74 % of that at the BL-Wet site; 30% exclusion of rainfall in semi-arid forests has been shown to reduce forest production (Ogaya and Penuelas, 2007). My gradient enables a comparison of the effects of precipitation under similar environmental conditions of mean annual temperature and soil type.

At all three sites, forest canopies were closed and occupied by >90% black locust, with understory covers of shrubs and herbaceous species. Tree basal area, the amounts of organic layer and litter quality (C: N ratio) decreased along the aridity gradient (Table 1). In each forest, I established four plots (20 × 20 m), each >30 m distant from all neighboring plots. All plots had similar slopes (flat land or gentle slopes near ridges) because topography considerably affects soil and N contents in this region (Tateno et al., 2017). Soil samples (0–10 cm deep) were taken from four locations in each plot. The sampling locations were *ca*. 10 m distant from one another. The four soil samples were combined into a single sample. Samples were taken in September 2015, and in April, June, and September 2016.

Table 1. Tree density, total basal area, diameter at breast height (DBH), height, and litter amount. All trees within >363 m^2 were measured, and all litter in >1.44 m^2 was collected to be used for the calculation of one site. Black locust trees with DBH > 5 cm were measured. Average tree DBH and heights are shown as means ± SDs.

	BL-Wet site	BL-Med site	BL-Dry site
Tree density (trees ha^{-1})	1225	2400	1490
Total basal area (m^2 ha^{-1})	26.4	25.2	21.6
Average tree DBH (cm)	15.5 ± 5.9	11.0 ± 3.2	11.6 ± 7.1
Maximum tree DBH (cm)	38.1	19.0	32.5
Average tree height (m)	15.8 ± 3.5	9.8 ± 2.5	13.1 ± 2.8.
Maximum tree height (m)	21.5	14.2	18.4
Litter amount (g m^{-2})	1405	1321	945

2－2－2． Measurement of soil physical and chemical properties

Soil water content was determined after samples were dried at 105°C for >3 days. Soil pH was measured with a pH meter (D-51; HORIBA, Kyoto, Japan) in a 2:5 soil/water suspension. After soil samples were ground, total C and N contents were measured using the dichromate oxidation and Kjeldahl methods, respectively. Soon after soil collection and incubation at 25°C for >6 days without freezing soil samples, I extracted dissolved C and N with 2 M KCl in a 1:10 soil/extractant ratio. The extracts were stored in a freezer until further analysis. The total extractable organic C (EOC) and N (EON) contents in the extracts were measured with a TOC-$L_{CPH/CPN}$ + TNM-L device (SHIMADZU, Kyoto, Japan) (Shimadzu, 2013). I measured the amounts of ammonium and nitrate N in the extracts with a Bran + Luebbe AutoAnalyzer III device (BLTEC, Tokyo, Japan) using a colorimetric procedure. I also measured the amount of nitrite N in the extracts which was from soils collected on two occasions from the same sites. Soil EON content, reportedly correlated with protein abundance that can be degraded by prokaryotes (Wang et al., 2013; Yu et al., 2002), was calculated as the difference between the concentrations of total extractable N and inorganic N (i.e., ammonium + nitrate N). Net N mineralization and nitrification rates in the soil were calculated as the differences in inorganic N and nitrate N, respectively, before and after incubation. I report N content per kilogram of dry soil. SOM quality and extractable organic matter quality were examined based on the total organic C: N and EOC: EON ratios, respectively.

2－2－3． Soil DNA extraction and quantification by real-time quantitative PCR

DNA was extracted from 0.25-g soil samples using the MoBio Powersoil DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions. The real-time

quantitative polymerase chain reaction (qPCR) was run in a Light Cycler Nano thermal cycler device (Roche Diagnostics K.K., Mannheim, Germany) with the SYBR Green I intercalating dye. Fungal ITS, bacterial 16S rRNA, archaeal 16S rRNA, ammonia-oxidizing bacterial *amoA*, and ammonia-oxidizing archaeal *amoA* genes were targeted to estimate the population size of each soil microbial group. Fungal ITS abundances were determined using the universal primer sets ITS1F_KYO2 /ITS2_KYO2 (Toju et al., 2012). The qPCR conditions for fungal ITS genes were as follows: initial denaturation at 95°C for 10 min, followed by >30 cycles at 95°C for 1 min, 53°C for 30 s, and 72°C for 1 min (Fierer and Jackson, 2005). The subsequent step in the analysis for 16S rRNA and *amoA* genes has been described in detail by Iwaoka et al. (2018).

2－2－4． Sequence analysis

I amplified the ITS and 16S rRNA genes in the DNA extracts using the primer sets ITS1F_KYO2 /ITS2_KYO2 (Toju et al., 2012) and U519f (Suzuki and Giovannoni, 1996)/U785r (Wang and Qian, 2009), respectively, with the following reaction solution ratio: 44:1:1:4 Platinum PCR Super Mix High Fidelity (Thermo Fisher Scientific)/10 mM forward primer/10 mM reverse primer/10-fold-diluted DNA sample. The subsequent step in the analysis has been described in detail by Iwaoka et al. (2018). Sequencing was performed on an Ion Personal Genome Machine (PGM) (Thermo Fisher Scientific). The processing of the sequencing data for 16S rRNA genes was performed as described by Iwaoka et al. (2018). ITS sequences shorter than 360 bp in length were removed; ITSx was used to extract fungal ITS sequences (Bengtsson-palme et al., 2013). The remaining steps were the same as for 16S rRNA sequences. Sequence data were deposited in the Sequence Read Archive of the National Center for Biotechnology Information under accession number DRA008376.

In my analyses of the fungal and prokaryotic community structures, I standardized the read numbers to 10031 and 8726, respectively, using random pick-up based on the minimum read number. I searched for fungal trophic modes from the fungal taxonomy and predicted specific functions based on the 16S rRNA genes using the FUNGuild database and the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) pipeline, respectively (Langille et al., 2013; Nguyen et al., 2016). To perform PICRUSt searches, the sequences were re-picked at a 97% identity level with reference to the Greengenes database (version 13_05). The OTU table was equalized into 1000 reads and normalized by gene copy number and the metagenome functional profiles were predicted in PICRUSt (ver 1.1.1), generating a table of Kyoto Encyclopedia of Gene and Genomes (KEGG) Orthologs (KOs, Kanehisa & Goto, 2000). I selected the genes necessary for coding enzymes commonly measured to evaluate soil N

cycling (Isobe et al., 2018; Saiya-Cork et al., 2002; Sinsabaugh et al., 2008), i.e., β1,4-N-acetylglucosaminidase (NAG; EC 3.2.1.52), chitinase (EC 3.2.1.14), leucine aminopeptidase (LAP; EC 3.4.11.1), arginase (EC 3.5.3.1), and urease (EC 3.5.1.5).

2－2－5. Statistical analyses

I used one-way analysis of variance (ANOVA) with the sampling date (September 2015/April 2016/June 2016/September 2016) as a random effect to test for significant differences among the sites in soil properties such as water content, pH, total and extractable organic C, N contents and C: N ratios, inorganic N content and net transformation rate, microbial abundance, relative abundances of fungal and prokaryotic groups, and predicted gene abundances. I chose a significance level of $P < 0.05$ for all tests. Prior to ANOVA, I tested for normality and homogeneity of variance in the data using the Kolmogorov–Smirnov and Levene's tests, respectively. The car package in R software (version 3.1.2; R Development Core Team, 2014) was used to perform Levene's test (Fox et al., 2014). When the *P* values for these tests were <0.05, data were square-root or reciprocally transformed prior to ANOVA analysis. Significant pairwise differences between sites were identified by Tukey–Kramer multiple comparison tests, or Steel–Dwass multiple comparison tests when the homogeneity of variance was violated. Correlations were tested using the Pearson procedure or by a generalized linear mixed model with site and sampling

Table 2. Soil physicochemical properties, organic matter quality and quantity and net N mineralization and nitrification rates. Values are means ± SDs. The right side shows *F* and *P* values (***$P < 0.001$) from one-way ANOVA with the sampling date (September 2015/April 2016/June 2016/September 2016) as a random effect, testing for significant among-site differences. Significant values are bolded. Different lowercase letters adjacent to SD values in the table rows indicate significant pairwise differences between sites (Tukey–Kramer or Steel–Dwass test).

	BL-Wet		BL-Med		BL-Dry		*F* value	
Water content (%)	22.5 ± 3.8	a	10.1 ± 3.8	b	9.1 ± 2.8	b	**128.5**	***
pH	7.04 ± 0.72	b	8.07 ± 0.23	a	8.24 ± 0.26	a	**31.9**	***
Total N content (g kg^{-1})	2.59 ± 0.53	a	2.11 ± 0.47	b	1.18 ± 0.53	c	**35.0**	***
Total C content (g kg^{-1})	27.2 ± 6.5	a	22.9 ± 5.1	a	13.1 ± 6.3	b	**23.3**	***
Total C: N ratio	10.4 ± 0.7	a	10.8 ± 0.4	a	10.9 ± 1.0	a	2.0	
EOC content (mg kg^{-1})	247.1 ± 354.3	b	235.7 ± 47.3	a	185.7 ± 30.9	b	0.7	
EOC: EON ratio	11.3 ± 6.2	b	18.8 ± 5.2	a	23.0 ± 5.0	a	**22.5**	***
Net N mineralization rate (mg kg^{-1} day^{-1})	0.66 ± 1.56	a	0.54 ± 0.48	a	0.85 ± 0.54	a	0.9	
Net nitrification rate (mg kg^{-1} day^{-1})	2.11 ± 2.09	a	0.66 ± 0.28	b	0.74 ± 0.41	b	**13.3**	***

date as random effects.

The non-metric multidimensional scaling analysis (NMDS) analysis of community structure dissimilarity based on the Bray–Curtis index was performed using the metaMDS function in the vegan package (Oksanen et al., 2016) of R software. The envfit function in the vegan package was used to identify significant correlations between (i) soil properties and the relative abundances of the fungal trophic groups, saprotrophic fungal orders, and the predicted abundances of genes for N-degrading enzymes and (ii) the NMDS values of points as vectors on the NMDS ordination plot. Permutational multivariate analysis of variance (PerMANOVA) was performed to test the significance of the effect of site on the microbial community, with sampling date as a random effect, using the Adonis function in the R vegan package.

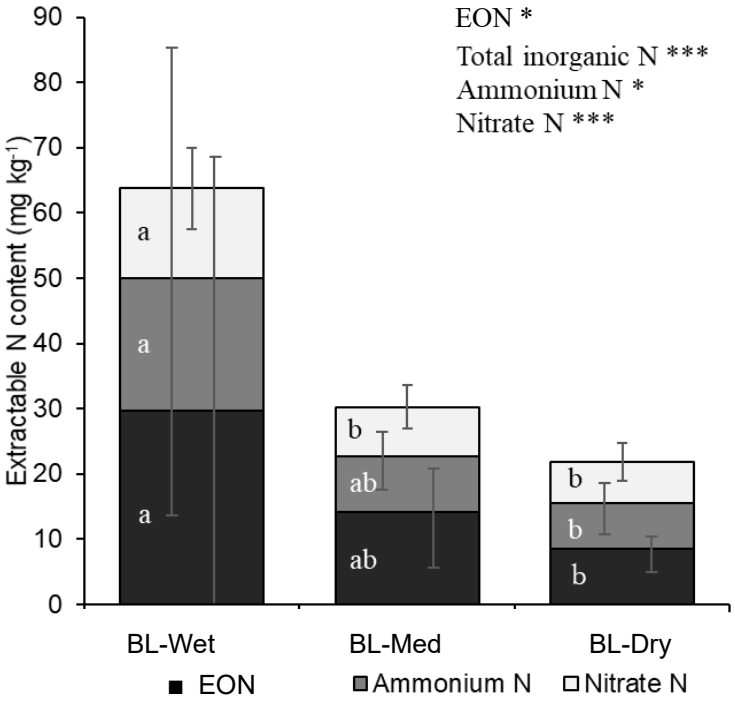

Fig. 2. Extractable N contents in the soils. Values are means ± SDs. Significant differences among sites, identified by one-way ANOVA with the sampling date as a random effect, are indicated in the upper right text panel. *$P < 0.05$, ***$P < 0.001$. Results of Tukey–Kramer or Steel–Dwass tests are shown inside the bars; different lowercase letters indicate significant pairwise differences between sites for each parameter.

Table 3. Logged values of soil fungal ITS and bacterial and archaeal 16S rRNA gene abundances at three sites on an aridity gradient. Values are means ± SDs. The right side shows *F*- value from one-way ANOVA with the sampling date as a random effect, testing for significant differences among sites. Different lowercase letters adjacent to SD values in the table rows indicate significant pairwise differences between sites (Tukey–Kramer or Steel–Dwass test).

	BL-Wet		BL-Med		BL-Dry		*F* value
Fungal ITS	7.61 ± 0.40	a	7.40 ± 0.40	a	7.45 ± 0.42	a	2.4
Bacterial 16S	9.05 ± 0.54	a	8.86 ± 0.54	a	8.79 ± 0.61	a	2.3
Archaeal 16S	6.76 ± 0.40	a	6.76 ± 0.41	a	6.73 ± 0.42	a	0.1

2 - 3 . Results

2 - 3 - 1 . Soil physicochemical properties and organic matter quality and quantity

Soil water content was significantly higher at the BL-Wet site than at the other two sites (Table 2). Soil pH also differed significantly among sites and was lowest at the BL-Wet site. Soil total N content differed significantly among sites and was highest at the BL-Wet site, followed by the BL-Med site. Total C: N ratios did not differ significantly among sites. The EOC: EON ratio differed significantly among sites and was lowest at the BL-Wet site. For soil water content, pH, and EOC: EON ratio, the values for the BL-Med site were more similar to those in the BL-Dry site, but for total N content, the BL-Med site was more similar to the BL-Wet site.

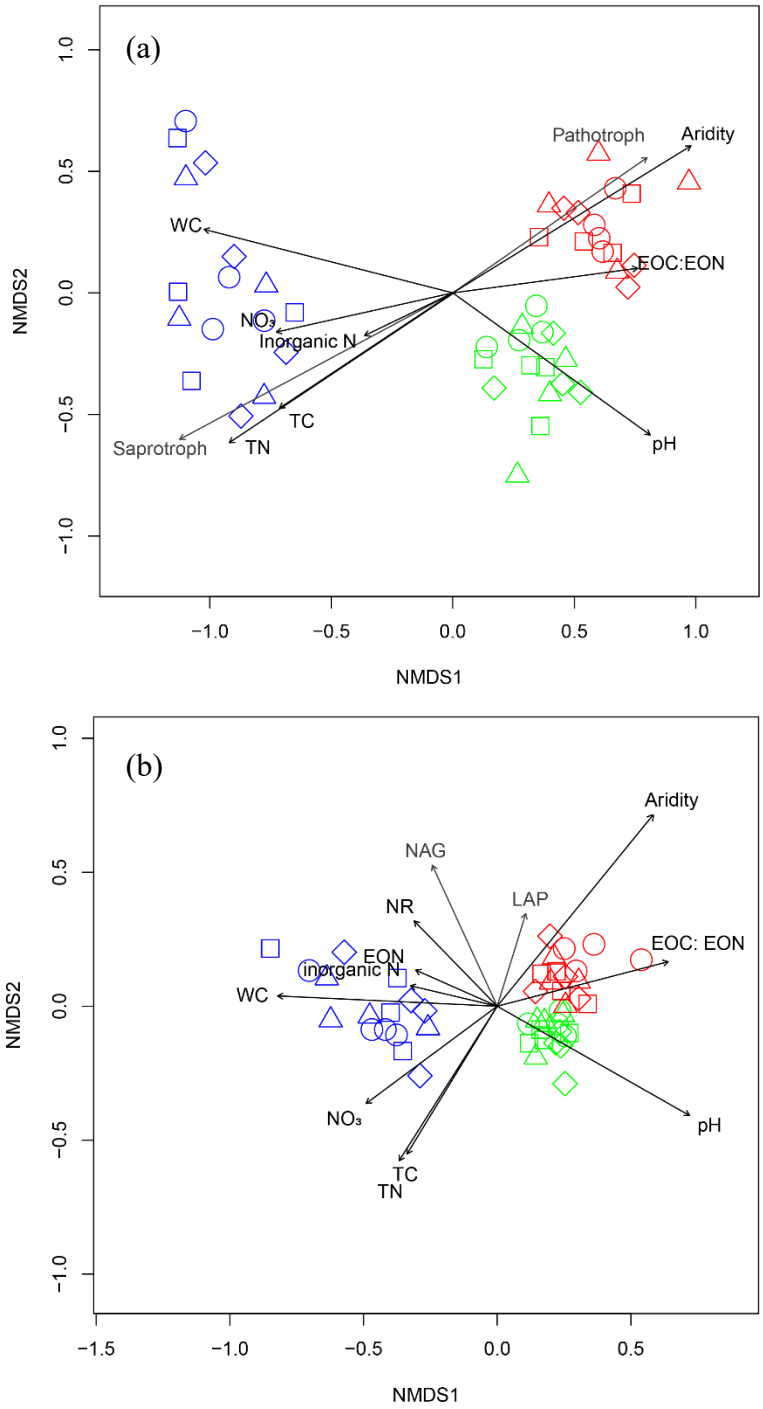

Fig. 3. The NMDS based on (a) fungal and (b) prokaryotic community dissimilarities. Blue, green, and red symbols represent the BL-Wet, BL-Med (medium), and BL-Dry sites, respectively. Circles, triangles, squares, and diamonds represent samples collected in September 2015, April 2016, June 2016, and September 2016, respectively. Only significant vectors are plotted on the ordination. Black vectors represent soil properties. Dark gray vectors represent relative abundances of trophic groups or predicted gene abundances. WC, water content; TN, total N content; TC, total C content; C: N, total C: N ratio; NO_3, nitrate N content; EON, EON content; EOC: EON, EOC: EON ratio.

2－3－2． Extractable N dynamics in soils

EON, ammonium N, nitrate N, and total inorganic N contents differed significantly among sites (Fig. 2). The BL-Wet site had significantly higher EON and ammonium N contents than did the BL-Dry site. The BL-Wet site had significantly higher nitrate N and total inorganic N contents than did the BL-Med and BL-Dry sites. The net N mineralization rate in the soil did not differ significantly among sites (Table 2). The net nitrification rate differed significantly among sites, and was highest at the BL-Wet site. Nitrite N was generally undetectable, with a maximum of 0.008 ppm (< 0.1 mg kg^{-1}).

2－3－3． Soil fungal communities

Fungal ITS gene abundance in the soil did not differ significantly among sites (Table 3). However, the PerMANOVA showed that soil fungal community structure was influenced significantly by site ($F = 11.8$, partial $R^2 = 0.34$, $P < 0.001$, Table S3), and the fungal communities at the three sites along the aridity gradient were separated on axis 1 of the NMDS ordination (Fig. 3a).

The relative abundances of saprotrophic and pathotrophic fungi differed significantly among sites (Fig. 4, Table 4). The relative abundance of saprotrophic fungi was highest and second highest at the BL-Wet and BL-Med sites, respectively. The relative abundance of pathotrophic fungi was highest at the BL-Dry and BL-Med sites. Saprotrophic fungal community composition also changed along the aridity gradient, with an apparent reduction in the relative abundance of

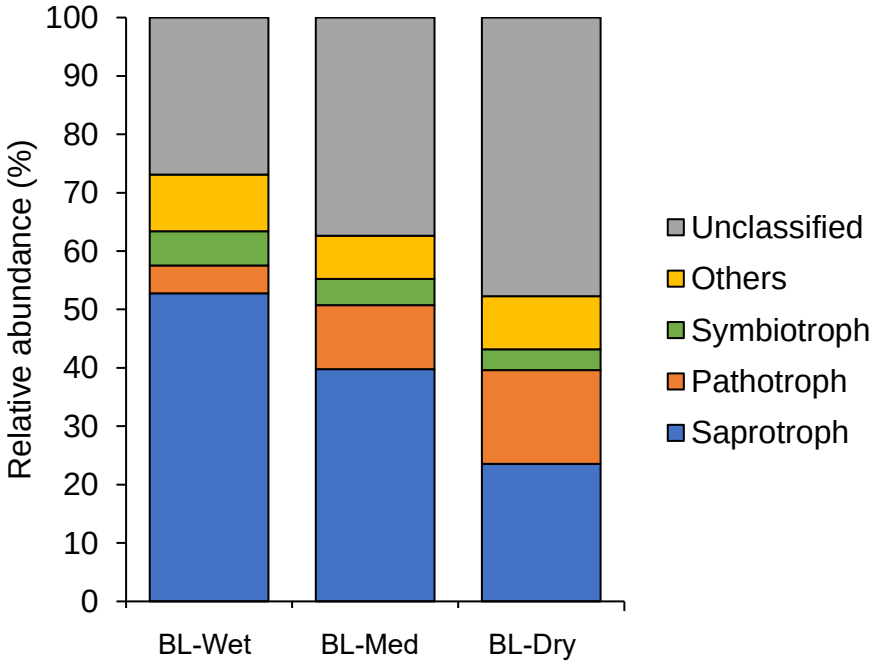

Fig. 4. Relative abundances of fungal trophic groups based on FUNGuild database trophic modes. The values in the bar graphs are the averages. The FUNGuild algorithm frequently outputs multiple trophic modes for single OTUs (e.g., saprotroph - symbiotroph); in these cases, the OTUs were placed in the “Others” category.

Table 4. Relative abundances of the main fungal trophic groups, the main orders and species of saprotrophs, and the results of tests for significant differences among sites. Values are means ± SDs. The right side shows F- and P- values (*P < 0.05, ***P < 0.001) from one-way ANOVA testing for significant differences among sites, with the sampling date as a random effect. Significant values are bolded. Different lowercase letters adjacent to the SD values identify significant pairwise differences between sites for each fungal group (Tukey–Kramer or Steel–Dwass test). The relative abundance of the order Mortierellales exceeded 20%; all members of this order were congeners of Mortierella (family Mortierellaceae). They are separated here at the species level. Unknown species of Mortierella and species with <1% relative abundance were assigned to "Mortierella sp." Orders with <1% relative abundance are removed.

	BL-Wet		BL-Med		BL-Dry		*F* value	
Saprotroph	52.8 ± 10.1	a	39.8 ± 9.9	b	23.6 ± 6.8	c	**43.6**	***
Mortierellales	34.0 ± 13.1	a	17.7 ± 5.6	b	9.3 ± 5.2	c	**47.9**	***
Mortierella camargensis	11.9 ± 5.5	a	5.0 ± 2.0	b	3.0 ± 4.3	c	**28.4**	***
Mortierella amoeboidea	2.7 ± 1.9	a	4.2 ± 1.7	a	3.3 ± 2.5	a	2.9	
Mortierella sp	19.5 ± 12.7	a	8.5 ± 4.2	b	3.1 ± 2.2	c	**31.5**	***
Hypocreales	6.5 ± 2.4	b	12.3 ± 4.4	a	6.4 ± 2.8	b	**15.9**	***
Sordariales	5.2 ± 4.6	a	2.0 ± 1.8	b	0.4 ± 0.5	c	**19.4**	***
Pleosporales	1.2 ± 0.9	b	1.6 ± 1.0	ab	2.5 ± 1.6	a	**4.2**	*
Eurotiales	1.6 ± 0.8	a	1.7 ± 0.8	a	1.4 ± 0.7	a	0.5	
Pathotroph	4.8 ± 3.1	b	11.0 ± 5.1	a	16.1 ± 9.2	a	**14.8**	***
Symbiotroph	5.9 ± 3.9	a	4.5 ± 2.3	a	3.5 ± 2.2	a	2.9	

the order Mortierellales as dryness increased (Table 4). In the NMDS ordination plot, the vectors for the relative abundances of saprotrophic fungi was more highly correlated with the vectors for soil total N content than with those for water content and pH, and they were also strongly negatively correlated with EOC: EON ratio (Fig. 2a).

Table 5. Predicted abundances of genes associated with soil N decomposition (NAG, Chitinase, LAP, Arginase and Urease) enumerated using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) pipeline procedures. Values are means ± SDs. Significant differences among sites, identified by one-way ANOVA with the sampling date as a random effect, are indicated in the upper right corner (***P < 0.001). Different lowercase letters indicate significant pairwise differences between sites (Tukey–Kramer or Steel–Dwass test).

	BL-Wet		BL-Med		BL-Dry		*F* value	
NAG	773 ± 57	a	713 ± 35	b	735 ± 35	b	**9.1**	***
Chitinase	200 ± 29	b	231 ± 12	a	209 ± 19	b	**9.3**	***
LAP	451 ± 34	b	472 ± 26	ab	478 ± 22	a	**5.3**	**
Arginase	202 ± 19	b	222 ± 12	a	200 ± 16	b	**12.4**	***
Urease	372 ± 23	a	384 ± 26	a	382 ± 36	a	1.3	

Table 6. Relative abundances of the main prokaryotic phyla and classes, and results of tests for significant differences among sites. Values are means ± SDs. The right side shows *F*- and *P*- values (**P* < 0.05, ****P* < 0.001) from one-way ANOVA testing for significant differences among sites, with the sampling date as a random effect. Significant values are bolded. Different lowercase letters adjacent to the SD values identify significant pairwise differences between sites for each prokaryotic group (Tukey–Kramer or Steel–Dwass test). Phyla with >10% relative abundance (Proteobacteria, Actinobacteria, and Bacteroidetes) are separated into classes. Phyla and classes with <1% relative abundances are removed.

	BL-Wet		BL-Med		BL-Dry		*F* value	
Proteobacteria	41.4 ± 4.4	a	35.0 ± 5.1	b	40.5 ± 3.7	a	**21.1**	***
Alphaproteobacteria	15.3 ± 2.4	b	18.5 ± 3.2	a	19.7 ± 3.7	a	**12.4**	***
Deltaproteobacteria	8.8 ± 1.4	a	6.6 ± 1.0	c	7.7 ± 1.1	b	**23.2**	***
Gammaproteobacteria	8.6 ± 1.9	a	4.3 ± 1.2	c	6.6 ± 1.9	b	**32.1**	***
Betaproteobacteria	8.4 ± 2.7	a	5.3 ± 1.7	b	6.2 ± 1.9	b	**30.0**	***
Actinobacteria	9.1 ± 4.2	c	21.3 ± 4.6	a	14.7 ± 4.2	b	**61.2**	***
Actinobacteria	3.9 ± 2.4	b	8.6 ± 1.9	a	7.7 ± 2.2	a	**37.8**	***
Thermoleophilia	3.6 ± 1.3	b	9.3 ± 2.5	a	4.9 ± 1.8	b	**64.4**	***
Acidimicrobiia	0.8 ± 0.3	b	1.6 ± 0.4	a	1.1 ± 0.2	b	**27.4**	***
Bacteroidetes	16.1 ± 3.4	a	10.8 ± 2.7	b	13.4 ± 2.6	b	**16.6**	***
Saprospirae	9.4 ± 1.6	a	7.4 ± 1.8	b	8.1 ± 2.6	ab	**5.1**	*
Cytophagia	2.8 ± 1.0	a	1.9 ± 0.6	b	3.1 ± 0.9	a	**12.6**	***
Sphingobacteriia	2.3 ± 1.4	a	0.9 ± 0.5	b	1.5 ± 0.5	b	**8.5**	***
Acidobacteria	8.4 ± 1.4	a	7.8 ± 1.4	a	7.6 ± 1.4	a	1.6	
Gemmatimonadetes	6.0 ± 1.4	a	7.0 ± 1.3	a	6.9 ± 1.4	a	3.0	
Verrucomicrobia	5.9 ± 1.8	a	3.6 ± 1.0	b	3.8 ± 1.4	b	**27.2**	***
Crenarchaeota	2.7 ± 1.9	b	5.6 ± 2.7	a	4.4 ± 1.5	ab	**16.2**	***
Planctomycetes	3.4 ± 0.9	a	3.7 ± 0.5	ab	3.1 ± 0.4	b	**3.8**	*
Chloroflexi	1.3 ± 0.3	b	1.8 ± 0.4	a	1.5 ± 0.4	ab	**13.8**	***
Nitrospirae	1.8 ± 0.5	a	1.0 ± 0.2	b	0.8 ± 0.2	b	**47.1**	***

2－3－4． Soil prokaryotic communities

The soil 16S rRNA gene abundances of bacteria and archaea did not differ significantly among sites (Table 3), but the PerMANOVA showed that the soil prokaryotic community structure differed significantly by site along the aridity gradient ($F = 14.9$, partial $R^2 = 0.36$, $P < 0.001$, Table

S3). The NMDS also identified clear differences in prokaryotic community structures among sites (Fig. 3b).

The predicted relative abundances of genes associated with NAG, chitinase, LAP and arginase differed significantly among sites (Table 5). The relative abundance of NAG was highest at the BL-Wet site, and those of chitinase and arginase were highest at the BL-Med sites, and that of LAP was highest at the BL-Dry site (Table 5). The abundance of urease did not differ among sites. The relative abundances of the dominant copiotrophic bacteria, Betaproteobacteria, Gammaproteobacteria, and Bacteroidetes and its classes (Cleveland et al. 2007; Fierer et al. 2007), were significantly different between sites (Table 6) and were highest in the BL-Wet site. In contrast, the relative abundance of the second-ranking Actinobacteria significantly differed among sites and was highest in the BL-Med site. In the NMDS, the vectors of predicted abundances of genes associated with NAG pointed in directions consistent with the vector for EON content and inorganic N content (Fig. 3b).

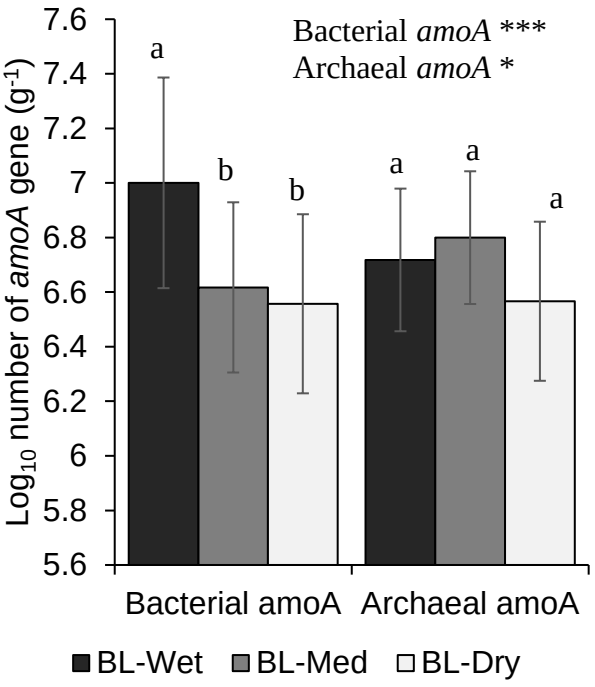

Fig. 5. Logged values of soil bacterial and archaeal *amoA* gene abundances. Values are means ± SDs. Significantly different values among sites, identified by one-way ANOVA with the sampling date as a random effect, are indicated in the upper right sector of the figure (*$P < 0.05$, ***$P < 0.001$). Different lowercase letters above the bars indicate significant pairwise differences between sites (Tukey–Kramer or Steel–Dwass test).

2－3－5． Soil ammonia-oxidizing communities

Abundances of *amoA* genes in the soil differed significantly among sites for bacteria, but not for archaea (Fig. 5). The ammonia-oxidizing bacterial abundances were significantly positively and negatively correlated with soil moisture and pH, respectively (Table 7). Ammonia-oxidizing archaea abundance was not correlated with soil moisture, pH, or ammonium N content.

Table 7. Coefficients for relationships between ammonia-oxidizer abundances and soil properties, based on single correlation tests and a generalized linear mixed model with the site and sampling date as random effects (in parentheses). ***$P < 0.001$. Significant values are bolded.

	Ammonia-oxidizing bacteria	Ammonia-oxidizing archaea
Water content	**0.56** ***	0.07
	(0.51 *)**	(-0.23)
pH	**-0.67** ***	-0.10
	(-0.60 *)**	(0.11)
Ammonium N content	0.06	-0.06
	(0.08)	(0.00)

2-4. Discussion

Each of the soil extractable N contents, i.e., EON, ammonium N, and nitrate N, was reduced with increasing aridity (Fig. 2), which was contrary to my expectation that the content of a specific form of N would remain constant or increase with increasing aridity due to the bottleneck step. The change in net nitrification rate was consistent with the decrease in nitrate N content (Table 2). The net N mineralization rate by laboratory incubation did not differ significantly among sites, although the ammonium N and total inorganic N contents decreased with increasing aridity. N mineralization may be activated after a rainfall event, as suggested in previous studies in which high microbial activity was observed after the rewetting of dry soil (Franzluebbers et al., 1996; Mikha et al., 2005). Therefore, N mineralization activity in the field may have been higher after rainfall, and rainfall frequency increases from the southeast to northwest on the Loess Plateau (Wan et al., 2014); therefore, the BL-Wet site likely had more frequent rainfall and higher N mineralization activity in the field. According to the pattern of soil extractable N contents, all N degradation steps were affected by the aridity gradient. Substrate accumulates when a bottleneck develops in the stepwise degradation process, but I did not detect accumulated forms of N, suggesting that changes maintained a balance, with no evidence of a continuous bottleneck along the aridity gradient.

Fungal abundance did not decrease with increasing aridity (Table 3), although EON content which was primarily produced by fungal community decreased with increasing aridity. However, fungal community composition changed with the aridity level (Figs. 3a, 4). The relative abundances of saprotrophic fungi, especially that of the dominant order Mortierellales, decreased markedly with increasing aridity (Figs. 3a, 4, Table 4). As saprotrophs obtain C energy from decomposition and Mortierella-dominated communities have high C-degrading potential (e.g.,

phenol-oxidase production) (Phillips et al., 2014), the decreases in these groups should reduce the degradation rate and contribute to the increase in the C: N ratio of extractable organic matter. Pathotrophs, which alternatively increased in abundance along the aridity gradient (Fig. 4), acquire energy from host cells (Nguyen et al., 2016); therefore, the group cannot contribute to decomposition.

Reductions in the abundances of saprotrophs such as Mortierellales with increasing aridity may be caused by reductions in the content of SOM. I found a strong positive correlation between saprotroph relative abundances and soil total N contents (Fig. 3a). This finding is consistent with those of other studies, in which increases in saprotrophic fungi and taxa in Mortierellales with addition of organic matter have been observed (Chigineva et al., 2009; Hanson et al., 2008; Kao-Kniffin and Balser, 2008), and is further supported by the apparent decrease in the amount of plant input and SOM content with increasing aridity (Table 4). Moreover, fungal communities are reportedly resilient to changes in water content or pH (Barnard et al., 2013; de Vries et al., 2018; Rousk et al., 2010). Thus, the decrease in organic matter content along the aridity gradient likely reduced saprotrophic fungi, particularly Mortierellales, thereby decreasing the EON content and increasing the EOC: EON ratio. In contrast, it has been reported that soil moisture content determines fungal decomposition activity which may control SOM content (Cregger et al., 2012; Hawkes et al., 2011). However, if fungal degradation decreased at my study sites due to soil dryness, then SOM content would have increased, not decreased, with increasing aridity. Therefore, organic matter content was a limiting factor of fungal degrading ability on the aridity gradient in the current study.

Prokaryotic abundance did not decrease with increasing aridity (Table 3), although ammonium N content, which was produced mainly by prokaryotic community, decreased with increasing aridity. However, community composition changed with aridity, which was also the case for the degradation step (Figs. 3b). The BL-Wet site had the highest NAG production potential for labile compound decomposition, whereas the BL-Med site had the highest chitinase and arginase production potential for recalcitrant compound decomposition (Table. 5). Consistently, the relative abundances of copiotrophs such as the Betaproteobacteria, Gammaproteobacteria, and Bacteroidetes were highest in the BL-Wet site (Table 6). And that of Actinobacteria, which are strongly competitive in the utilization of recalcitrant compounds such as chitin, was highest at BL-Med site (Bastian et al., 2009; Bell et al., 2013; Cretoiu et al., 2013). Active decomposition of labile compounds by abundant copiotrophs at the BL-Wet site and decomposition of recalcitrant compounds by Actinobacteria at the BL-Med site were key elements in the N mineralization step gradient along the aridity gradient.

The BL-Wet site had the highest EON content and the lowest EOC: EON ratio; such

organic matters would be suitable substrates for NAG. The BL-Med site would contain relatively more high-molecular-weight organic compounds such as chitin, the substrate of chitinase, based on that the BL-Med site had relatively low EON contents and a relatively high EOC: EON ratio (similar to the BL-Dry site), but relatively high total C and N contents (similar to the BL-Wet site; Table 2). Increasing pH and decreasing moisture with increasing aridity may reduce NAG production potential by limiting the growth of copiotrophs such as Betaproteobacteria (Armstrong et al., 2016; Lauber et al., 2009; Lennon et al., 2012). However, Actinobacteria are resilient to low moisture and alkaline soil (Barnard et al., 2013; Lauber et al., 2009; Placella et al., 2012). The increase in Actinobacteria in the BL-Med site are difficult to explain without considering organic matter quality and quantity, because the BL-Med site had medium moisture and pH. My findings agree with previous observations that the response of bacterial communities to soil water conditions is mediated by organic matter type and availability (de Vries et al., 2012; Göransson et al., 2013; Placella et al., 2012).

The gradient in nitrification was likely related to the activities of the ammonia oxidizer communities. Ammonia-oxidizing bacterial abundance decreased along the aridity gradient, but the abundances of ammonia-oxidizing archaea did not differ among sites (Fig. 5), as observed in previous studies (Adair and Schwartz, 2008; Chen et al., 2013). The frequently observed positive relationship between the net nitrification rate and ammonia-oxidizing bacterial abundance (Banning et al., 2015; Chen et al., 2013; Di et al., 2009) suggests that nitrification is driven mainly by ammonia-oxidizing bacteria at this study site. However, we did not test differences in archaeal versus bacterial ammonia oxidation activity through addition of octyne as previously described (Taylor et al. 2013).

Ammonia-oxidizing bacterial abundance was significantly positively correlated with soil moisture and negatively correlated with pH, but the abundance of these microbes was not related to ammonium N content (Table 7). The positive correlation between the abundance of this group of prokaryotes and moisture content was consistent with the findings of previous studies (Gleeson et al., 2010; Marcos et al., 2016; Placella and Firestone, 2013), but the negative correlation with pH was not (Hu et al., 2013). Ammonium N content has been shown to determine ammonia-oxidizing bacterial abundance with a 10-fold difference in the ammonium N content (Verhamme et al., 2011; Wang et al., 2011). Hence, the determining factor in ammonia-oxidizing bacterial abundance at this study site was probably soil moisture and not substrate ammonium N, unlike in upstream steps. Soil moisture obviously declined along the aridity gradient, thereby reducing ammonia-oxidizing bacterial abundance and creating the nitrification step gradient.

In summary, my findings suggest that substrate quantity and/or quality strongly affect the degradation and N mineralization steps by changing fungal and prokaryotic community

compositions. Although soil moisture or pH may contribute to changes in the fungal and prokaryotic community, it is difficult to explain the changes observed in these communities without substantial effects of their substrate quality and quantity. However, in the nitrification step, soil moisture appeared to be more important than substrate for the ammonia-oxidizing bacteria, although ammonia-oxidizing archaeal population was resilient to changes in soil moisture. Hence, as rainfall patterns change, N degradation and mineralization will proceed well unless the SOM content changes, but the nitrification rate will slow as moisture decreases. Substrate and soil moisture reportedly determine N mineralization and nitrification rates (Ford et al., 2007; Marcos et al., 2016; Saetre and Stark, 2005), but substrate has a greater effect on N mineralization than does moisture, which has a greater effect on nitrification (Hu et al., 2015; Yahdjian and Sala, 2010), as demonstrated in this study.

Furthermore, the vulnerability of nitrification to soil moisture depletion resulting from climate change may control whole-forest N cycling. A decrease in N availability will limit N uptake, NPP and litter production (Norby et al., 2010; Ren et al., 2017; Tateno et al., 2017), consequently reducing SOM content (Fig. 6). This reduction in SOM content imposes substrate limitation on the fungal community, which will limit the whole N dynamics sequence through mineralization (Fig. 6). Thus, precipitation decreases may limit whole-forest N cycling, beginning with nitrification limitation, via the plant–soil feedback process. In this study, I observed no

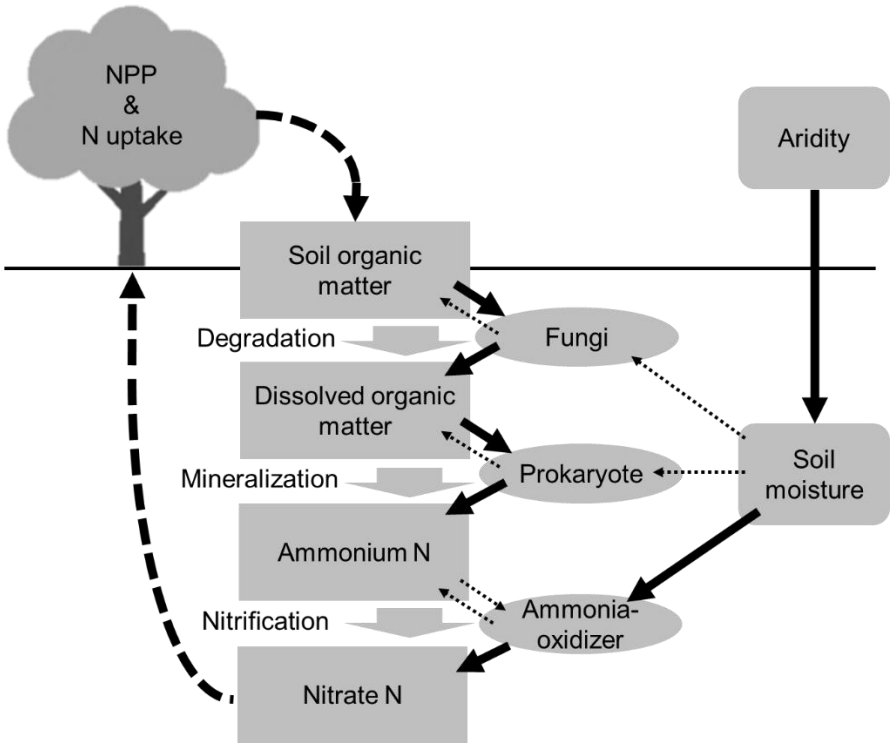

Fig. 6. Conceptual N cycling model based on results from this study. Black solid arrows and black thick dashed arrows refer to the effects observed in this study and the predicted effects, respectively. Aridity determines the nitrification step, which is controlled by soil moisture and ammonia oxidizers. The nitrification step would affect N uptake and net primary production (NPP), consequently controlling the degradation and mineralization steps by changing their substrates and drivers. Thin black dashed arrows indicate other possible effects that were not strongly supported in this study.

accumulation of specific N forms, perhaps because limitation in the nitrification step extended to the other steps; hence, the N cycling process was maintained in particular stable states, each suited to the precipitation at the different sites. Thus, I found balanced changes in extractable content of all N forms along the aridity gradient. The nitrification step is likely to become a temporary bottleneck step with increasing aridity. However, I expect the whole N cycling process to achieve a new stable state suited to the decreased precipitation pattern under future climate change.

3. Soil nitrogen cycling is determined by the competition between mycorrhiza and ammonia-oxidizing prokaryotes

3-1. Introduction

Mycorrhizal fungi play a critical role in soil C storage by controlling SOM decomposition through their ability to utilize soil N (Averill et al., 2014; Lindahl and Tunlid, 2015; Sterkenburg et al., 2018; Talbot et al., 2008; Zak et al., 2019). As described in Chapter 1, ECM fungi can produce many hydrolytic and oxidative extracellular enzymes (Chalot and Brun 1998, Courty et al. 2010, Kohler et al. 2015 but see Pelliter and Zak 2018), and obtain small organic N-bearing molecules from SOM, leaving behind a relatively C-rich substrate (Averill et al., 2014; Orwin et al., 2011). As a result, ECM fungi are thought to limit the amount of N available for free-living microbes, and to slow free-living microbial SOM decomposition (Averill and Hawkes, 2016; Fernandez and Kennedy, 2016; Gadgil and Gadgil, 1971). On the other hand, AM fungi lack saprotrophic ability, and are believed to use inorganic rather than organic N (Hodge and Storer, 2014; Read and Perez-Moreno, 2003; Smith and Read, 2008; Smith and Smith, 2011). As a result, AM fungi do not strongly limit the amount of N available for free-living microbes, and therefore do not prevent SOM decomposition by saprotrophic microbes.

The N competition between ECM fungi and free-living microbes is supported by models and observed global patterns of soil C storage (Averill et al., 2014; Orwin et al., 2011). Nonetheless, ECM fungi do not always appear to slow SOM decomposition (Brzostek et al., 2015; Drake et al., 2011; Phillips et al., 2014), and direct tests for the N competition are still lacking (Averill and Hawkes, 2016; Fernandez and Kennedy, 2016). In particular, it is unknown which form of N is competed for and which microbial groups compete for the N with ECM fungi. N transformation process comprises three main steps, and primarily driven by three type of microbes as described in Chapter 1. If the specific microbial functional abundance and the microbial N product are lower in ECM forests (forests dominated by ECM-symbiotic trees) than in AM forests (forests dominated by AM-symbiotic trees), that provides an evidence for the limitation during the step by the ECM fungi. That is, N competition is likely to occur during the mineralization step in ECM forests than in AM forests, if the prokaryotic decompositional ability, and the ammonium N pool, are lower in the ECM forests.

Furthermore, simple comparisons between ECM forests and AM forests of their N transformation steps and microbial communities cannot clarify the existence of mycorrhizal-mediated N competition. Many environmental factors, as well as mycorrhizal abundance, differ simultaneously between the forest types. The ratio of ECM trees to AM trees in forests is

negatively correlated with soil pH, probably related to the litter quality (Cheeke et al., 2016; Midgley and Phillips, 2016; Phillips et al., 2013). The ratio can be positively correlated with soil moisture, because ECM-dominated forests have a thicker litter layer that prevents evaporation, due to the poor chemical quality of the litter from ECM trees (Cornelissen et al., 2001; Phillips et al., 2013). These co-changing physicochemical properties are highly important factors for soil microbes, and thus for soil N cycling, as low soil pH limits the proliferation of soil ammonia-oxidizers and therefore limits the nitrification rate (Nicol et al., 2008; Stempfhuber et al., 2015), and high soil moisture reduces ammonium N content by enhancing plant N uptake (Homyak et al., 2017; Reichmann et al., 2013). Therefore, although soil inorganic N content declines with an increasing ratio of ECM trees to AM trees (Cheeke et al., 2016; Midgley and Phillips, 2016; Phillips et al., 2013), it is possible that the difference is more or less caused by soil physicochemical properties.

To understand mycorrhizal effects separately from the effects of soil moisture and pH, I propose to use an aridity gradient in dryland forests. Dryland soils impose many environmental stresses, including low soil moisture and high pH (alkalinity), which increase along the aridity gradient based on Chapter 2 and another study (Jiao et al., 2016). Using the aridity gradient is also useful for separating the effects of SOM quality and quantity from the direct mycorrhizal effect, because the aridity gradient provides SOM variation partly through changing the tree biomass based on Chapter 2 and another study (Feral et al., 2003), regardless of mycorrhizal effects. In addition, SOM quality and quantity in the deeper soil layer will change from those of the surface soils with relatively mild changes in pH and moisture (Fierer et al., 2003; Thoms et al., 2010).

Here, to evaluate N competition between ECM fungi and free-living microbes, I studied mycorrhizal effects on soil N transformation steps and its microbial drivers, and the effects of soil physicochemical properties, along environmental gradients (particularly a dryland aridity gradient). To this end, I examined AM-symbiotic black locust forests (Yang et al., 2014) and ECM-symbiotic oak forests (Zhang et al., 2013) on the Loess Plateau in northeastern China. To study N transformation steps, I focused on microbial-driver abundance for each step (saprotrophic and ECM fungi for degradation, N-degrading prokaryotes for mineralization, and ammonia-oxidizing prokaryotes for nitrification) and their N product contents (extractable organic N (EON), ammonium N, and nitrate N, respectively). I expected one of the N transformation steps to be clearly limited in the ECM forests, regardless of the aridity gradient. I hypothesized that, in ECM forests, microbial driver abundance and the N products in the step are consistently lower than in AM forests.

3-2. Materials and Methods

3－2－1． Study site

This study was conducted in three AM-symbiotic black locust forests and three ECM-symbiotic oak forests from the southern to central part of the Loess Plateau of China. Black locust forests were largely planted in the 1960s, and oak forests are native climax forests. These forests are very common in this region (Du et al., 2011). The three black locust forests were BL-Wet site, BL-Med site, and BL-Dry site in Chapter 2. Three oak forests were located in a mountainous area near Fuxian Prefecture (eastern Fuxian, 36°05′N, 109°33′E, and western Fuxian, N36°04′N, 109°09′E) and Mt. Gonglushan in Shaanxi Province. Because these two sites in Fuxian had almost the same mean annual rainfall, I defined these two sites as Oak-Wet site. We called Mt. Gonglushan the Oak-Med site. It was difficult to find a Oak-Dry site because this region was categorized as a forest-steppe transitional zone, i.e., the drought boundary of the forest, and most of the natural oak forests were already cut down because of human activity (Lü et al., 2003). Mean annual rainfall and mean annual air temperature were 577 mm and 9.0 ℃ in the Oak-Wet site (Li and Shao, 2006), 514 mm and 10.2 ℃ in the Oak-Med site, (Otsuki et al., 2005), which is neighboring in the BL-Med site.

In each forest, four plots (20 m × 20 m), which were > 30 m away from other plots, were established. All the plots were located on a flat or gentle slope near a ridge. The forest canopy was closed, and more than 90% of the canopy of all these plots were occupied by dominant species, in the oak forests as well as in the black locust forests as described in Chapter 2. All forests had understory cover of shrubs and herbaceous species. There were no ECM trees (i.e., *Pinaceae*, *Salicaceae*, *Myrtaceae*, *Fagaceae,* and *Betulaceae*) in the black locust forests (Wang and Qiu, 2006). Tree density, mean tree size (DBH and height), and the amount of organic layer in the oak forests are shown in Table 1 and 8.

Table 8. Tree density, total basal area, average and maximum DBH, height of canopy trees of black locust or oak, and amount of organic layer. All canopy trees more than DBH >5 cm in 100–400 m^2 plot were measured to show calculate tree density, total basal area, average and maximum DBH, and height. Average tree DBH and height shows means ± SDs. Litter in 30–50 cm square (4 replicates) was collected in 4 plots of each site to be used for the calculation for each site.

	Oak-Wet site		Oak-Med site
	Eastern	Western	
Tree density (trees ha^{-1})	1500	2000	575
Total basal area (m^2 ha^{-1})	40.8	43.2	13.9
Average tree DBH (cm)	16.8 ± 8.4	15.5 ± 6.1	16.6 ± 5.7
Maximum tree DBH (cm)	31.7	29.3	26.3
Average tree height (m)	7.9 ± 1.9	8.5 ± 1.5	8.5 ± 2.1
Maximum tree height (m)	10.0	13.0	10.8
Amount of organic layer (g m^{-2})	2903 ± 266	3278 ± 393	2222 ± 235

3－2－2． Soil sampling and measurement of soil physicochemical properties

Soil samples (0–10 cm in depth) were taken in the same way as in Chapter 2. Sampling was conducted in several occasions as follows: September 2015, April, June, and September 2016, August 2017, and June and October 2018 for the BL-Wet and BL-Dry sites. It was September 2015, April, June, and September 2016, June, August and October 2017, and June and October 2018 for the BL-Med and Oak-Med sites. It was October 2017 and June and October 2018 for the Oak-Wet site. The data for BL-Wet, Med, and Dry sites from September 2015 to September 2016 were shared with Chapter 2. To separate effects of SOM quantity and quality from other environmental factors, such as soil pH and subsurface soil samples (20–30 cm in depth) were taken at October 2018, at the same places as the 0-10 cm depth soil samples were taken.

Soil water content, pH, and the contents of total C, total N, EOC, EON, ammonium N and nitrate N, and net N mineralization and nitrification rate were measured in the same way as in Chapter 2. These transformation rates were measured by laboratory incubation released from alive plants.

3－2－3． Soil DNA extraction, quantification by real-time quantitative PCR, and sequencing analysis

DNA was extracted from 0.25 g soil samples using the MoBio Powersoil DNA Isolation Kit (MoBio, Carlsbad, CA, USA). The extracts were stored in a freezer until further analysis. Real-time quantitative polymerase chain reaction (qPCR) and sequencing analysis were performed in the same way as in Chapter 2. Sequence data were deposited in the Sequence Read Archive at NCBI under accession number DRA008550.

To analyze the fungal and prokaryotic community structure, the read number were equalized to 5645 and 2100 reads, respectively, using random pick-up based on the minimum read number. To search fungal trophic mode from fungal taxonomy and to predict functional gene abundance based on 16S rRNA gene, FUNGuild database (Nguyen et al., 2016), and PICRUSt pipeline (Langille et al., 2013), were used, respectively. FUNGuild program output saprotroph, symbiotroph, or pathotroph in the trophic modes. I picked up ECM guild from symbiotrophs. Unlike the previous version used in Chapter 2 (ver 1.0), the new FUNGuild program (ver 1.1) very often output multiple trophic modes and guilds for single OTUs (e.g., saprotroph – symbiotroph), and such OTUs were counted in both groups, i.e., OTUs of saprotroph - symbiotroph were counted both as saprotroph and as symbiotroph. To perform PICRUSt, sequences were re-picked and normalized in the same way as in Chapter 2, generating a table of Kyoto Encyclopedia of Gene and Genomes (KEGG) Orthologs (KOs, Kanehisa & Goto, 2000). I selected the same N-degrading gene sets as in Chapter 2. Because the total ITS gene number was significantly different between

the AM and ECM forests (Table 10), the total saprotrophic and ECM fungal gene abundances, as well as relative abundances, were calculated by multiplying the FUNGuild-based relative abundance and the qPCR-based fungal ITS gene abundance.

3－2－4． Statistical analyses

For all tests, the level of significance was set at 5%. A linear mixed-effect model (LMM) for forest type (black locust forests = -1, oak forests = 1) and aridity was conducted to test the effects of forest type and the aridity gradient on soil parameters. I used the minus mean annual rainfall to indicate the aridity level. The dataset from all the surface soil samples (0–10 cm) collected in several occasions were used for the LMM for forest type and aridity. The sampling occasion and plot (the Oak-Wet site has 8 plots and the other sites have 4 plots) were introduced as a random variable in these models. An LMM for forest type, aridity, and depth was also conducted to test the effects of forest type, the aridity gradient, and the soil depth (0–10 cm depth = -1, 20–30 cm depth = 1) on soil parameters. The dataset from the soil samples collected in October 2018 was used for the LMM for forest type, aridity, and depth. The sampling plot was introduced as a random variable in these models. The objective variables were standardized in the linear mixed-effect models. The standardization was conducted using mean and SDs. The lme4 and lmerTest package (Bates et al., 2014; Kuznetsova et al., 2017) in R (version 3.1.2, R Development Core Team, 2014) were used for conducting the linear mixed-effect models.

I conducted path analysis to identify how soil N transformation steps were determined by forest type and aridity (minus mean annual rainfall). The dataset from all the surface soil samples (0–10 cm) collected in several occasions were used. I also conducted path analysis to identify how soil N transformation steps are determined by forest type, aridity, and soil depth. The dataset from all the soil samples collected in October 2018 was used. I expected that the forest type and aridity (and depth) would affect soil water content, pH, total N content, the C: N ratio, and relative abundance of ECM fungi, and these factors would affect EON, ammonium N, and nitrate N content. Standardized values were used, and path diagrams were illustrated after nonsignificant relationships were eliminated. Path analysis was conducted using the sem package of R (Byrnes et al., 2016). PerMANOVA were used to test the significance of the effects of forest type and aridity on the community via the adonis function of the R vegan package (Oksanen et al., 2016).

3-3. Results

3－3－1． Soil physicochemical properties

Soil water content and pH significantly differed along the aridity gradient (Table 9). Soil total C

and N content and the C: N ratio were significantly affected by the interaction, as well as by forest type. Water content decreased and pH increased with the aridity gradient. Total C and N content and the C: N ratio were higher in the oak forests.

Table 9 Soil water content, pH, total carbon (C) and nitrogen (N) contents, C: N ratio, EOC content, and EOC: EON ratio of surface soils (0–10 cm). Values represent means ± standard deviations (SDs). Right side shows standardized coefficients and *P*-values (**P* < 0.05, ***P* < 0.01, ****P* < 0.001) based on linear mixed-effect model for forest type (black locust forests = -1, oak forests = 1, Type), standardized minus mean annual rainfall (Aridity) and their interactions (Type × Aridity). The models were performed with sampling occasion and plot as random variables.

	Black locust (BL) forest			Oak forest		Type		Aridity		Type ×Aridity	
	Wet site	Med site	Dry site	Wet site	Med site						
Water content (%)	21.7 ± 4.5	11.5 ± 4.4	10.0 ± 3.0	19.8 ± 4.8	15.0 ± 5.4	0.03		**-0.74**	***	0.12	
pH	7.07 ± 0.66	7.92 ± 0.33	8.20 ± 0.22	7.63 ± 0.22	7.55 ± 0.36	-0.05		**0.54**	*	-0.42	
C content (g kg^{-1})	30.3 ± 7.5	25.3 ± 5.0	12.9 ± 5.4	40.2 ± 10.1	41.0 ± 8.1	**0.66**	***	-0.25		**0.34**	*
N content (g kg^{-1})	2.79 ± 0.58	2.31 ± 0.43	1.16 ± 0.45	3.01 ± 0.66	3.23 ± 0.57	**0.52**	***	-0.27		**0.51**	**
C: N ratio	10.7 ± 0.7	10.9 ± 0.4	11.0 ± 0.8	13.3 ± 0.9	12.6 ± 0.7	**0.81**	***	-0.2		**-0.28**	*
EOC content (mg kg^{-1})	233 ± 268	241 ± 42	180 ± 28	488 ± 165	452 ± 159	**0.62**	***	-0.19		-0.08	
EOC: EON ratio	12.0 ± 6.6	15.8 ± 6.3	33.6 ± 44.7	11.3 ± 4.7	12.5 ± 4.0	**-0.16**	*	**0.32**	*	-0.11	
Net N mineralization rate (mg kg^{-1} day^{-1})	0.92 ± 1.48	0.55 ± 0.71	0.58 ± 0.60	-0.31 ± 0.77	0.80 ± 1.16	-0.10		0.29		**0.45**	***
Net nitrification rate (mg kg^{-1} day^{-1})	1.98 ± 1.71	0.75 ± 0.42	0.74 ± 0.35	0.62 ± 0.40	1.05 ± 0.69	-0.17		-0.09		**0.50**	**

3－3－2． Soil extractable N dynamics

Soil extractable N composition was highly changed by the forest type (Fig. 7). The EON content was significantly higher in the oak forests, and nitrate N content was significantly higher in the black locust forests. EON content was also affected by the aridity gradient, and the EON content declined along the gradient. Ammonium N content was not significantly affected by the forest type, aridity gradient, or their interaction. Net N mineralization and net nitrification rate were not

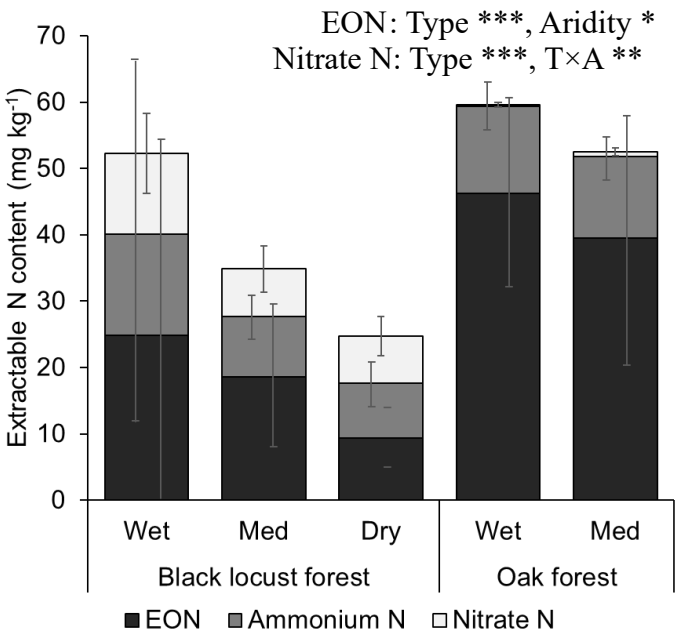

Fig. 7 Soil EON, ammonium N, and nitrate N content (mean ± SD) of surface soils (0–10 cm). The significant results of the linear mixed-effect model for forest type (Type), standardized minus mean annual rainfall (Aridity) and their interactions (T × A) were shown in the upper right. The models were performed with sampling occasion and plot as random variables. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

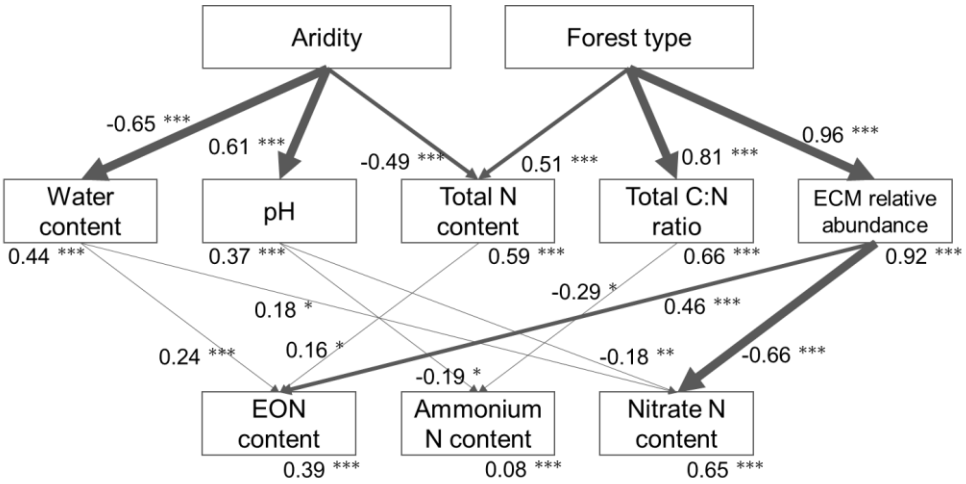

Fig. 8 The path diagram of the relationships among the experimental design (forest type and aridity (minus mean annual rainfall)), the mediating variables (water content, pH, total N content, total C: N ratio, and ECM relative abundance) and extractable N contents of surface soils (0–10 cm). Standardized values were used. Values and asterisks next to arrows indicate path coefficients and P values (**P* < 0.05, ***P* < 0.01, ****P* < 0.001), respectively. Value under the box represents coefficient of determination. The significant relationships are illustrated. Forest type was read as ordinal categorical data: black locust forests = -1; oak forests = 1.

affected by forest type or the aridity gradient but were significantly affected by their interaction (Table 9). The path analysis showed that aridity affected soil EON, ammonium N, and nitrate N content through modifying soil moisture, pH, and total N content (Fig. 8). The forest type also affected soil EON, ammonium N, and nitrate N content through modifying total N content, the C: N ratio, and the ECM relative abundance. Among these effects, the effects of ECM relative

abundance was the strongest on soil EON content and especially on nitrate N content.

3－3－3． Soil fungal community

Fungal ITS gene abundance based on qPCR analysis was significantly affected by the forest type, aridity gradient, and their interaction (Table 10). The fungal abundance was higher in the oak forests, and the abundance decreased along the aridity gradient (Table 10). Fungal community structure was significantly affected by forest type (PerMANOVA, $F = 35.3$, $R^2 = 0.17$, $P < 0.001$), the aridity (PerMANOVA, $F = 16.9$, $R^2 = 0.08$, $P < 0.001$), and their interaction (PerMANOVA, $F = 6.6$, $R^2 = 0.03$, $P < 0.001$). The NMDS separated samples depending on forest type and the aridity gradient (Fig. 9a).

Table 10. Log_{10} number of fungal ITS, bacterial and archaeal 16S rRNA gene abundance, and the predicted abundances of genes associated with N-degradation of surface soils (0–10 cm). Values represent means ± SDs. The predicted abundances of genes were calculated in 1000 reads per samples enumerated using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) pipeline procedures. Right side shows standardized coefficients and P-values (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) based on linear mixed-effect model for forest type (black locust forests = -1, oak forests = 1, Type), standardized minus mean annual rainfall (Aridity) and their interactions (Type × Aridity). The models were performed with sampling occasion as a random variable.

	Black locust (BL) forest			Oak forest		Type		Aridity		Type ×Aridity	
	Wet site	Med site	Dry site	Wet site	Med site						
Fungal ITS	7.69 ± 0.48	7.46 ± 0.44	7.57 ± 0.50	8.37 ± 0.53	7.80 ± 0.60	**0.33**	***	**-0.49**	***	**-0.46**	***
Bacterial 16S rRNA	9.15 ± 0.49	9.06 ± 0.49	8.99 ± 0.60	9.38 ± 0.45	9.05 ± 0.56	0.02		**-0.19**	*	-0.12	
Archaeal 16S rRNA	6.85 ± 0.42	6.85 ± 0.39	6.81 ± 0.37	6.71 ± 0.33	6.42 ± 0.54	**-0.44**	***	0.07		0.18	
NAG	755 ± 90	667 ± 44	692 ± 43	744 ± 61	762 ± 58	**0.34**	**	-0.22		0.22	
Chitinase	178 ± 31	188 ± 14	177 ± 18	184 ± 24	202 ± 26	**0.31**	*	0.28		0.27	
LAP	455 ± 39	470 ± 33	486 ± 27	548 ± 33	547 ± 35	**0.81**	***	**0.22**	*	-0.06	
Arginase	226 ± 19	217 ± 13	206 ± 16	265 ± 22	269 ± 21	**0.77**	***	-0.02		**0.27**	*
Urease	434 ± 59	439 ± 38	436 ± 54	533 ± 55	505 ± 54	**0.55**	***	0		-0.02	

The relative and total abundances of saprotrophs and ECM were significantly affected by forest type (Fig. 10). The relative abundance of saprotrophs was higher in the black locust forests, and that of ECM was higher in the oak forests (Fig. 10a). However, because the fungal total abundance based on qPCR was significantly higher in the oak forest, the total abundance of saprotroph was higher in the oak forest when multiplying the qPCR-based fungal ITS gene

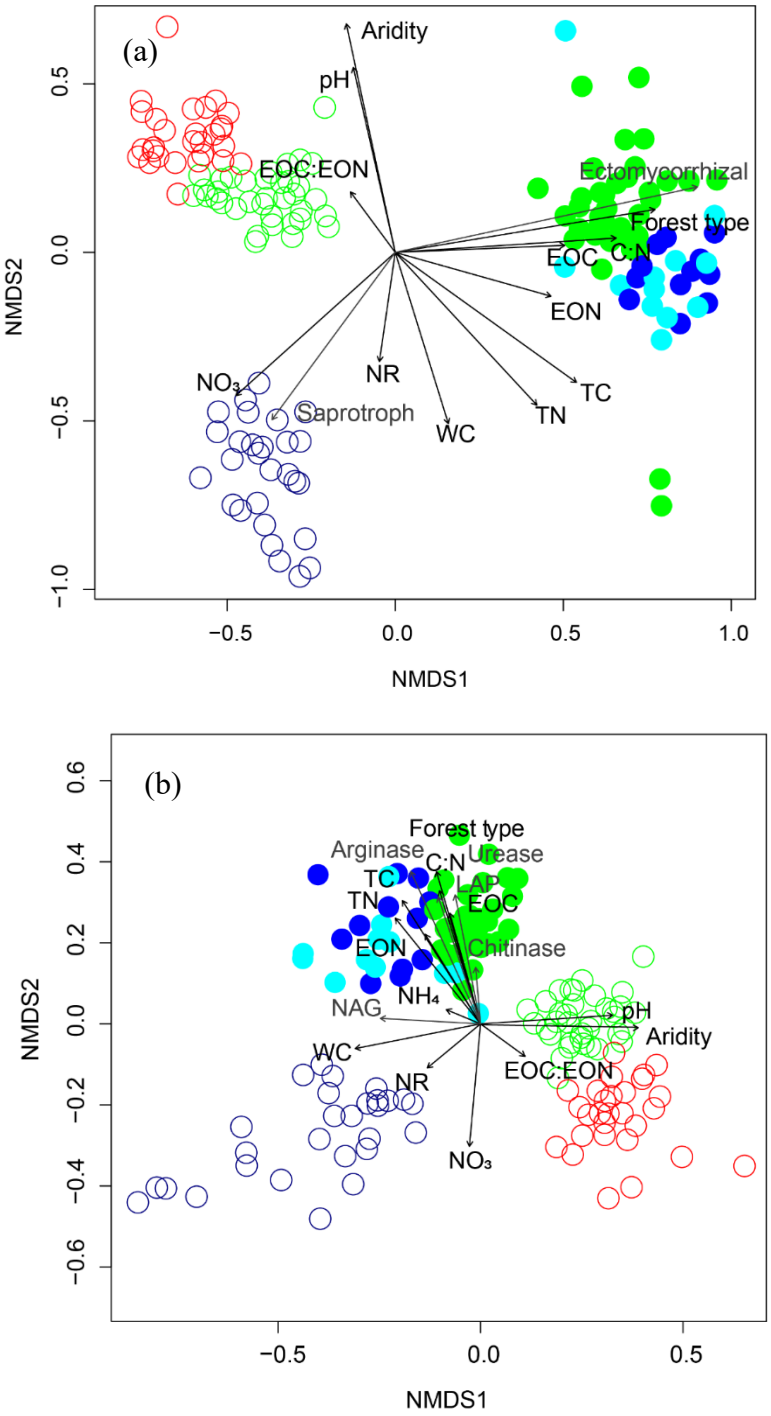

Fig. 9 The NMDS based on Bray-Curtis dissimilarities of (a) fungal community structures and (b) prokaryotic community structure of surface soils. The fill and color represent forest type and site, respectively. Open and filled symbols indicate samples from black locust forests and oak forests, respectively. Navy, green, and red symbols refer to BL-Wet, BL-Med, and BL-Dry site, respectively. Blue, cyan, and green refer to eastern Fuxian (Oak-Wet site), western Fuxian (Oak-Wet site), and Oak-Med site, respectively. Only significant vectors are shown on the ordination. Black vectors represent experimental design and soil properties. Gray vectors represent relative abundance of trophic group or C and N-degrading genes. The experimental design is forest type (black locust forests = -1, oak forests = 1, Forest type) and standardized minus mean annual rainfall (Aridity). WC, water content; TC, total C content; TN, total N content; C: N, total C: N ratio; NO_3, nitrate N content; NH_4, ammonium N content; EOC, extractable organic C content; EON, EON content; EOC: EON, extractable organic C: N ratio; MR, net N mineralization rate; NR, net nitrification rate. NMDS analysis was performted in the same way as in Chapater 2. For NMDS of fungal community, soil physicochemical properties, organic matter quality and quantity, extractable N content, and fungal trophic groups and guilds were tested by envfit. For NMDS of prokaryotic community, the same soil and N properties as a fungal community and predicted abundance of N-degrading genes were tested.

abundance and the FUNGuild-based relative abundances (Fig. 10b). The ECM total abundance was also higher in the oak forest, when multiplying the qPCR-based fungal ITS gene abundance and the FUNGuild-based relative abundances (Fig. 10b). The relative abundance of ECM, total abundances of saprotroph and ECM were significantly affected by the aridity gradient, and all the

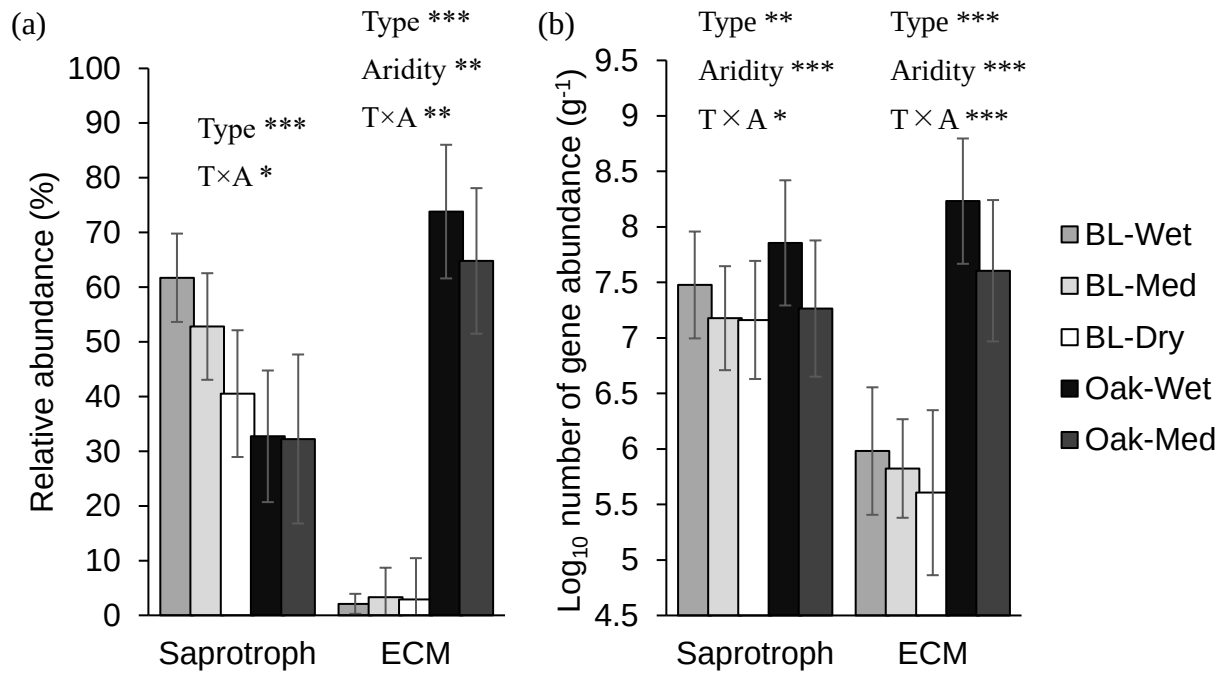

Fig. 10 (a) Relative abundance and (b) total abundance of saprotrophic and ECM fungal trophic groups (mean ± SD) of surface soils (0–10 cm), based on trophic mode (saprotroph) and guild (ECM) of FUNGuild. The total abundances were calculated by multiplying the qPCR-based fungal ITS gene abundance and the FUNGuild-based relative abundances. The significant results of the linear mixed-effect model for forest type (Type), standardized minus mean annual rainfall (Aridity), and their interactions (T × A) are shown below. The models were performed with sampling occasion and plot as random variables. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

abundances were significantly affected by the interaction. Those of saprotrophs and ECM decreased along the aridity gradient. Dominant ECM taxon was Sebacinaceae, Thelephoraceae, and Cortinarius.

3－3－4． Soil prokaryotic community

Total bacterial abundance based on qPCR analysis was not affected by forest type but was affected by the aridity gradient (Table 10). Total archaeal abundance based on qPCR analysis was affected by the forest type and was higher in black locust forests, and most of the archaea were ammonia-oxidizers (Table 10; Fig. 11). The prokaryotic community structure was determined by the forest type (PerMANOVA, $F = 25.3$, $R^2 = 0.13$, $P < 0.001$), the aridity (PerMANOVA, $F = 18.2$, $R^2 = 0.09$, $P < 0.001$), and their interaction (PerMANOVA, $F = 3.9$, $R^2 = 0.02$, $P < 0.001$). The NMDS separated samples based on the forest type and the aridity gradient (Fig. 9b). Predicted abundances

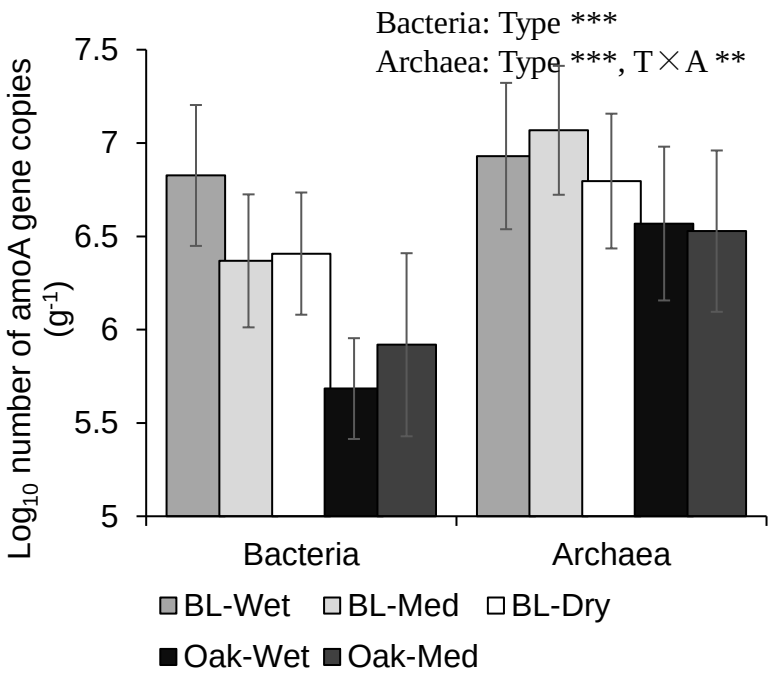

Fig. 11 Log_{10} number of soil ammonia-oxidizing bacterial and archaeal *amoA* gene abundance (mean ± SD) of surface soils (0–10 cm). The significant results of linear mixed-effect model for forest type (Type), standardized minus mean annual rainfall (Aridity), and their interactions (T × A) are shown in the upper right. The models were performed with sampling occasion and plot as random variables. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

of all the N-degrading genes were significantly affected by the forest type, and higher in the oak forest (Table 10). Those of LAP and arginase were also significantly affected by the aridity and the interaction, respectively.

Soil ammonia-oxidizing bacterial and archaeal abundances based on qPCR analysis were significantly affected by the forest type and the abundances were higher in the oak forests (Fig. 11). The ammonia-oxidizing archaeal abundance was also affected by the interaction.

3－3－5． Soil N transformation steps and microbial communities in subsurface soils

Total C and N contents and the C: N ratio significantly differed by soil depth, as the C and N contents and ratio were largely lower in subsurface soils at the same sampling occasion (Table 11). The quantity and quality of SOM varied more greatly than other factors such as moisture and pH did in subsurface soils (Table 11), as we expected and other studies showed (Fierer et al., 2003; Thoms et al., 2010). All the extractable N content was also significantly affected by soil depth, as the contents were lower in the subsurface soils (Fig. 12). Nitrate N content was still higher in the black locust forests and the content was almost 0 in the oak forests even in subsurface soils. The path analysis for samples from October 2018, wherein soil depth was an added factor affecting soil physicochemical properties, as well as the aridity and forest type, showed that the soil C: N ratio strongly affected EON content and ammonium N content, but ECM relative abundance, which was not affected by soil depth, was still strongly affected nitrate N content (Fig. 13). PerMANOVA showed the fungal community was more strongly affected by forest type (PerMANOVA, $F = 9.0$,

Table 11 Water content, pH, total carbon (C) and nitrogen (N) contents, the C: N ratio, extractable organic C content, extractable organic C: N ratio, net N mineralization rate, and net nitrification rate at 0–10 cm and 20–30 cm soil depth in black locust forests and oak forests of October 2018. Values represent means ± SDs. Right side shows standardized coefficients and *P*-values (**P* < 0.05, ***P* < 0.01, ****P* < 0.001) based on the linear mixed-effect model for forest type (black locust forests = -1, oak forests = 1, Type), standardized minus mean annual rainfall (Aridity), and their interactions (Type × Aridity). The models were performed with sampling plots as a random variable.

		Black locust (BL) forest			Oak forest		Type		Aridity		Depth	
		Wet site	Med site	Dry site	Wet site	Med site						
Water content (%)	0-10	24.9 ± 3.0	6.8 ± 0.6	13.9 ± 2.2	19.8 ± 2.0	11.0 ± 3.4	0.11		**-0.93**	***	**-0.46**	***
	20-30	17.0 ± 1.1	6.1 ± 0.3	6.4 ± 1.2	13.2 ± 1.2	6.0 ± 0.6						
pH	0-10	7.06 ± 0.66	7.84 ± 0.05	8.10 ± 0.15	7.41 ± 0.15	7.40 ± 0.25	-0.14		**0.68**	***	**0.64**	***
	20-30	7.48 ± 0.81	8.52 ± 0.04	8.65 ± 0.12	8.44 ± 0.14	8.44 ± 0.01						
Total C content (g kg^{-1})	0-10	33.1 ± 5.88	29.1 ± 3.72	14.1 ± 5.75	38.2 ± 3.66	38.1 ± 10.92	**0.18**	**	**-0.32**	***	**-0.85**	***
	20-30	9.4 ± 1.74	4.8 ± 0.37	3.2 ± 1.38	8.7 ± 2.11	6.4 ± 0.58						
Total N content (g kg^{-1})	0-10	2.98 ± 0.47	2.65 ± 0.35	1.24 ± 0.44	2.77 ± 0.24	2.93 ± 0.65	0.06		**-0.4**	***	**-0.86**	***
	20-30	1.00 ± 0.18	0.54 ± 0.04	0.35 ± 0.15	0.84 ± 0.18	0.63 ± 0.08						
Total C: N ratio	0-10	11.08 ± 0.29	11.0 ± 0.34	11.17 ± 0.96	13.81 ± 0.41	12.84 ± 0.91	**0.50**	***	-0.09		**-0.77**	***
	20-30	9.33 ± 0.23	8.73 ± 0.15	8.96 ± 0.07	10.28 ± 0.50	10.18 ± 0.31						
EOC content (mg kg^{-1})	0-10	191 ± 30	198 ± 15	157 ± 24	398 ± 73	284 ± 76	**0.39**	***	-0.2		**-0.70**	***
	20-30	119 ± 9	95 ± 7	101 ± 17	130 ± 11	112 ± 13						
EOC: EON ratio	0-10	8.8 ± 0.8	10.1 ± 0.6	12.6 ± 2.0	9.9 ± 0.7	10.8 ± 1.4	-0.07		-0.21		**0.66**	***
	20-30	19.9 ± 4.6	14.3 ± 0.8	13.0 ± 2.3	16.7 ± 3.5	13.5 ± 5.1						
Net N mineralization rate (mg kg^{-1} day^{-1})	0-10	0.02 ± 0.74	-0.51 ± 0.31	-0.14 ± 0.47	-0.66 ± 0.36	-0.28 ± 0.35	-0.26		0		**-0.37**	**
	20-30	-0.61 ± 0.20	-0.57 ± 0.17	-0.75 ± 0.22	-0.92 ± 0.74	-0.69 ± 0.13						
Net nitrification rate (mg kg^{-1} day^{-1})	0-10	1.15 ± 0.26	0.00 ± 0.31	0.68 ± 0.20	0.41 ± 0.21	0.23 ± 0.28	**-0.36**	**	**-0.43**	*	**-0.53**	***
	20-30	0.26 ± 0.08	0.12 ± 0.06	0.09 ± 0.09	0.04 ± 0.04	0.02 ± 0.01						

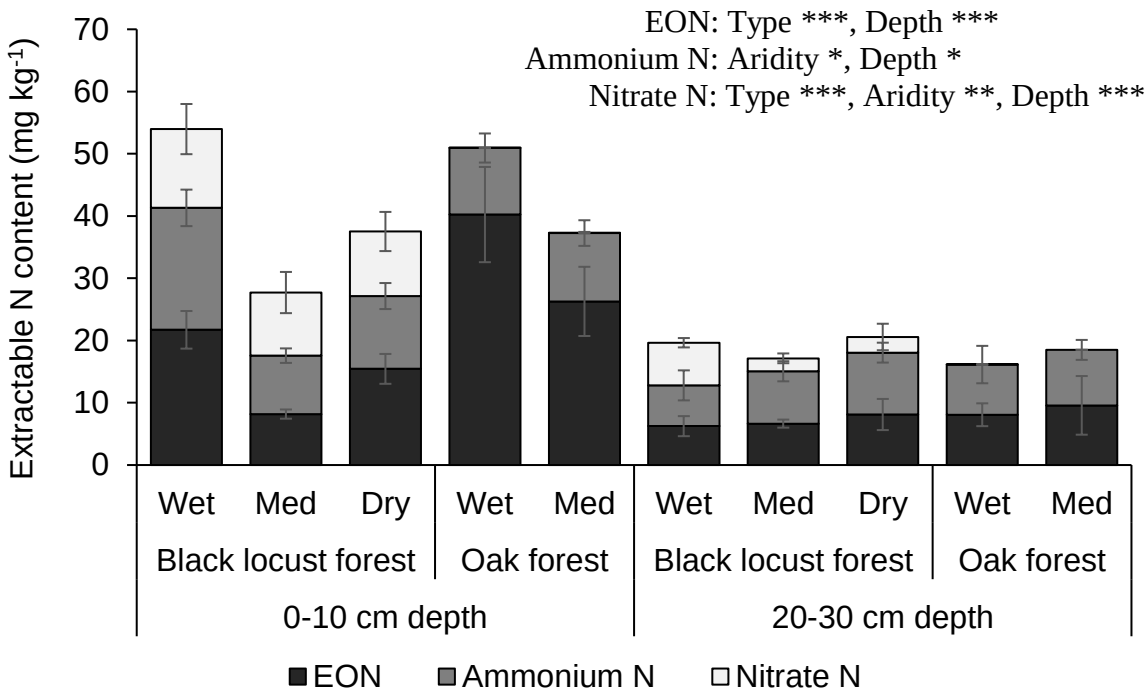

Fig. 12 The EON, ammonium N, and nitrate N content (mean ± SD) at 0–10 cm and 20–30 cm soil depth in black locust forests and oak forests of October 2018. The significant results of linear mixed-effect model for forest type (black locust forests = -1, oak forests = 1, Type), minus standardized mean annual rainfall (Aridity) and soil depth (surface (0–10 cm depth) = -1, subsurface (20–30 cm depth) = 1, Depth) are shown in the upper right. The models were performed with plot as a random variable. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

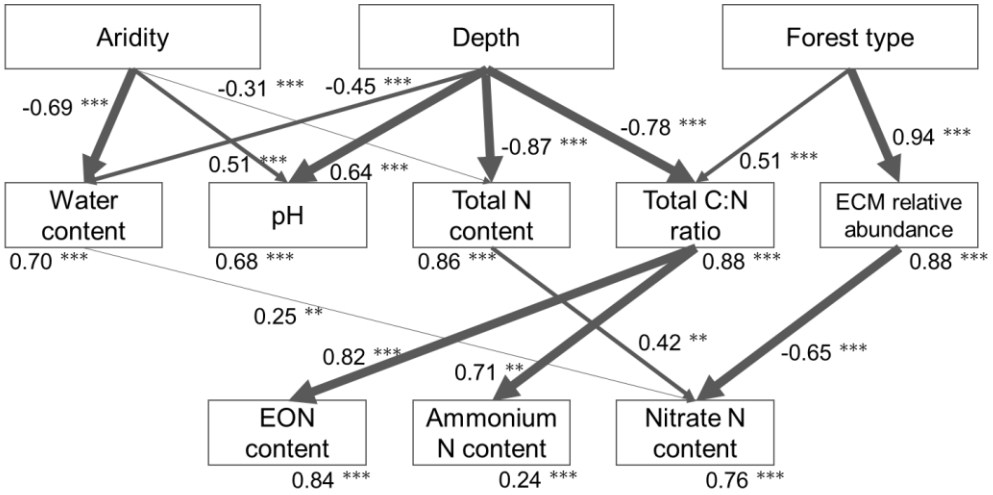

Fig. 13 The path diagram of the relationships among the experimental design (forest type, aridity (minus mean annual rainfall) and soil depth), the mediating variables (water content, pH, total N content, total C: N ratio, and ECM relative abundance) and extractable N contents for both 0–10 cm and 20–30 cm depth soils of October 2018. Standardized values were used. Values and asterisks next to arrows indicate path coefficients and P values (*P < 0.05, **P < 0.01, ***P < 0.001), respectively. Values under the box represent coefficient of determination. The significant relationships are illustrated. Forest type and soil depth were read as ordinal categorical data: black locust forests and surface soils = -1; oak forests and subsurface soils = 1.

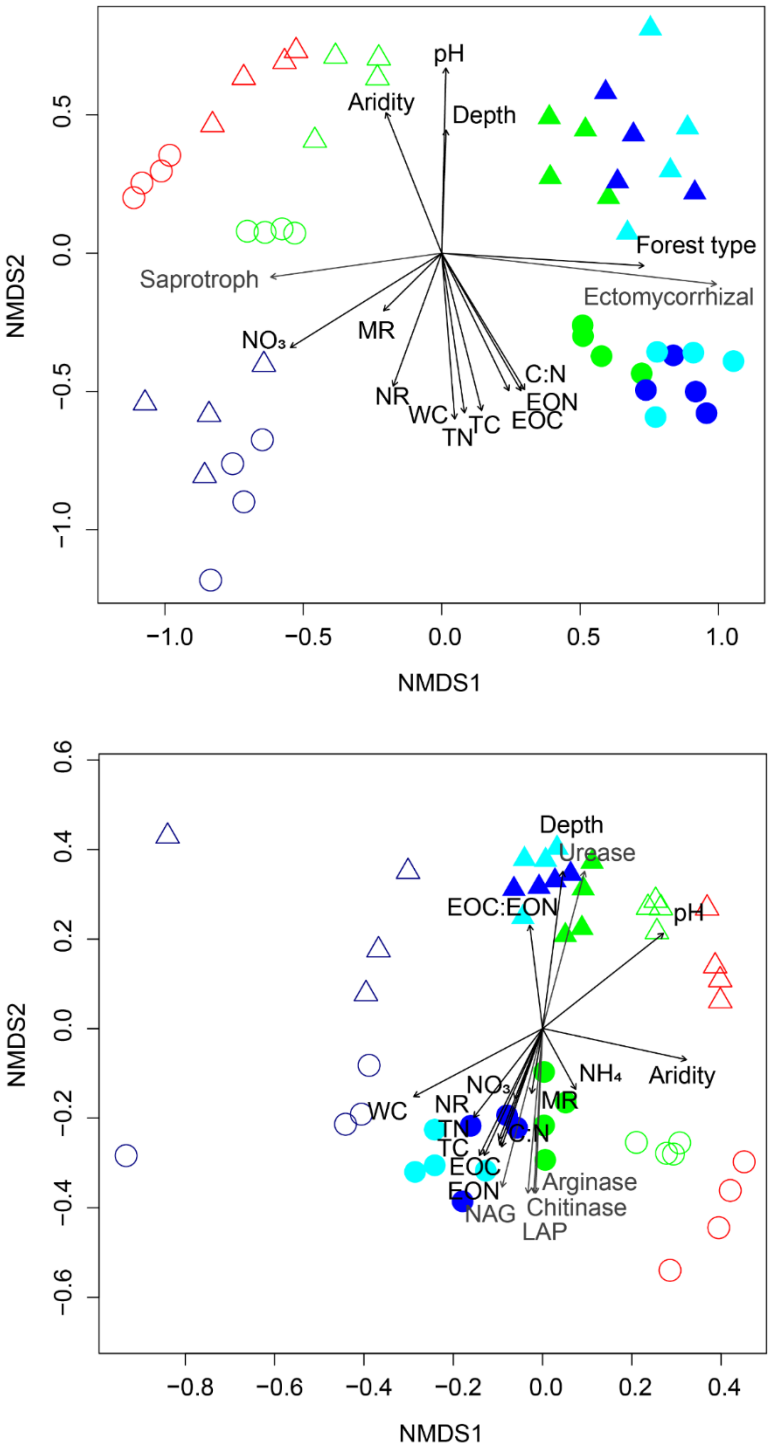

Fig. 14 The NMDS based on Bray-Curtis dissimilarities of (a) fungal community structures and (b) prokaryotic community structure at 0–10 cm and 20–30 cm soil depth in black locust forests and oak forests of October 2018. The fill, color, and shape represent forest type, site, and depth, respectively. Open and filled symbols indicate samples from black locust forests and oak forests, respectively. Navy, green, and red symbols refer to BL-Wet, BL-Med, and BL-Dry site, respectively. Blue, cyan, and green refer to eastern Fuxian (Oak-Wet site), western Fuxian (Oak-Wet site), and Oak-Med site, respectively. Circle and triangle symbols refer to 0–10 cm depth soils and 20–30 cm depth soils, respectively. Only significant vectors are shown on the ordination. Black vectors represent experimental design and soil properties. Gray vectors represent relative abundance of the trophic group or C and N-degrading genes. The experimental design is forest type (black locust forests = -1, oak forests = 1, Forest type), standardized minus mean annual rainfall (Aridity), soil depth (surface soil = -1, subsurface soil = 1, Depth). WC, water content; TC, total C content; TN, total N content; C: N, total C: N ratio; NO_3, nitrate N content; NH4, ammonium N content; EOC, extractable organic C content; EON, EON content; EOC: EON, dissolved organic C: N ratio; MR, net N mineralization rate; NR, net nitrification rate. NMDS analysis was performted in the same way as in Fig. 9.

$R^2 = 0.15$, $P < 0.001$) than by mean annual rainfall (PerMANOVA, $F = 3.6$, $R^2 = 0.06$, $P < 0.001$), or by soil depth (PerMANOVA, $F = 3.5$, $R^2 = 0.06$, $P < 0.001$), but the prokaryotic community was more strongly affected by soil depth (PerMANOVA, $F = 10.5$, $R^2 = 0.16$, $P < 0.001$) than by

Table 12 Log_{10} number of soil fungal ITS gene, bacterial and archaeal 16S rRNA gene abundance, and ammonia-oxidizing bacterial and archaeal *amoA* gene abundance at 0–10 cm and 20–30 cm soil depth in black locust forests and oak forests of October 2018. T Values represent means ± SDs. Right side shows standardized coefficients and *P*-values (**P* < 0.05, ***P* < 0.01, ****P* < 0.001) based on the linear mixed-effect model for forest type (black locust forests = -1, oak forests = 1, Type), standardized minus mean annual rainfall (Aridity) and soil depth (surface (0–10 cm depth) = -1, subsurface (20–30 cm depth) = 1, Depth). The models were performed with sampling plots as a random variable.

		Black locust (BL) forest			Oak forest		Type		Aridity		Depth	
		Wet site	Med site	Dry site	Wet site	Med site						
Fungal ITS	0-10	8.50 ± 0.18	8.26 ± 0.17	8.50 ± 0.04	9.02 ± 0.11	8.88 ± 0.13	**0.44**	***	0.12		**-0.67**	***
	20-30	7.90 ± 0.20	7.85 ± 0.26	7.46 ± 0.34	8.10 ± 0.12	7.92 ± 0.08						
Bactetrial 16S rRNA	0-10	9.58 ± 0.26	9.73 ± 0.20	9.86 ± 0.09	9.85 ± 0.07	9.73 ± 0.14	0.21		0.01		**-0.68**	***
	20-30	9.48 ± 0.27	9.38 ± 0.05	9.22 ± 0.21	9.58 ± 0.10	9.51 ± 0.11						
Archaeal 16S rRNA	0-10	7.29 ± 0.19	7.22 ± 0.32	7.26 ± 0.13	6.84 ± 0.07	7.04 ± 0.11	-0.08		**-0.30**	*	**0.71**	***
	20-30	7.67 ± 0.65	7.66 ± 0.09	7.51 ± 0.16	7.81 ± 0.12	7.74 ± 0.13						
Bacterial *amoA*	0-10	6.75 ± 0.09	6.04 ± 0.29	6.22 ± 0.10	5.58 ± 0.09	5.93 ± 0.17	**-0.38**	***	**-0.37**	**	**0.65**	***
	20-30	7.24 ± 0.67	7.20 ± 0.09	7.04 ± 0.24	6.96 ± 0.13	7.01 ± 0.09						
Archaeal *amoA*	0-10	7.31 ± 0.35	7.24 ± 0.37	7.17 ± 0.12	6.45 ± 0.19	6.97 ± 0.12	**-0.51**	**	-0.34		**0.42**	***
	20-30	7.52 ± 0.52	7.54 ± 0.07	7.28 ± 0.24	7.26 ± 0.17	7.29 ± 0.20						

Table 13 The relative abundance of the fungal trophic groups (saprotroph and ECM), and the predicted abundances of prokaryotic genes associated with N-degradation at 0–10 cm and 20–30 cm soil depth in black locust forests and oak forests of October 2018. Values are means ± SDs. Right side shows standardized coefficients and *P*-values (**P* < 0.05, ***P* < 0.01, ****P* < 0.001) based on linear mixed-effect model for forest type (black locust forests = -1, oak forests = 1, Type), standardized minus mean annual rainfall (Aridity) and soil depth (surface (0–10 cm depth) = -1, subsurface (20–30 cm depth) = 1, Depth). The models were performed with sampling plots as a random variable.

		Black locust (BL) forest			Oak forest		Type		Aridity		Depth	
		Wet site	Med site	Dry site	Wet site	Med site						
Saprotroph	0-10	60.1 ± 5.3	63.6 ± 5.5	49.7 ± 9.8	35.0 ± 17.2	41.4 ± 29.1	**-0.64**	***	-0.06		0.07	
	20-30	58 ± 12.4	63.1 ± 9.6	55.1 ± 17.9	29.3 ± 19.0	33.4 ± 6.1						
ECM	0-10	4.2 ± 3.9	1.4 ± 0.5	6.1 ± 11.0	72.8 ± 8.4	68.0 ± 15.6	**0.91**	***	-0.06		**0.07**	*
	20-30	4.8 ± 3.1	3.0 ± 1.0	1.3 ± 1.1	66.1 ± 18.5	53.8 ± 22.4						
NAG	0-10	680 ± 94	634 ± 11	664 ± 25	758 ± 68	727 ± 63	**0.44**	**	-0.27		-0.13	
	20-30	705 ± 57	648 ± 32	669 ± 23	708 ± 16	692 ± 13						
Chitinase	0-10	158 ± 44	182 ± 15	159 ± 13	182 ± 32	197 ± 43	**0.43**	**	0.13		**0.49**	***
	20-30	179 ± 18	207 ± 20	193 ± 26	229 ± 15	220 ± 21						
LAP	0-10	475 ± 49	512 ± 8	494 ± 25	568 ± 11	565 ± 26	**0.63**	***	**0.51**	***	**-0.48**	***
	20-30	419 ± 32	495 ± 15	508 ± 17	486 ± 16	518 ± 5						
Arginase	0-10	221 ± 20	228 ± 10	198 ± 3	265 ± 18	284 ± 21	**0.35**	***	0.05		**-0.70**	***
	20-30	187 ± 18	202 ± 10	200 ± 13	186 ± 11	197 ± 18						
Urease	0-10	501 ± 67	458 ± 44	418 ± 28	522 ± 45	566 ± 36	0.07		0.03		**-0.62**	
	20-30	399 ± 55	467 ± 48	429 ± 30	352 ± 28	419 ± 30						

forest type (PerMANOVA, $F = 6.8$, $R^2 = 0.10$, $P < 0.001$), or by the aridity (PerMANOVA, $F = 5.0$, $R^2 = 0.07$,$P < 0.001$), as shown in the NMDS (Fig. 14). The abundances of total fungi, saprotrophic fungi, ECM fungi, ammonia-oxidizing bacteria, and archaea were affected by soil depth, but they were also significantly affected by the forest type (Table 12). And even in subsurface soils, total and ECM fungal abundances were still higher in the oak forests, and ammonia-oxidizing bacterial and archaeal abundances were still higher in the black locust forests. The predicted abundances of the prokaryotic potential to produce N-degrading enzymes were also affected by soil depth, but they were also significantly affected by the forest type (Table 13). And even in subsurface soils, the prokaryotic potentials to produce N-degrading enzymes were still higher in the oak forests.

3-4. Discussion

Consistent with my hypothesis, soil extractable N composition differed by forest type, regardless of the aridity gradient (Fig 7). I found that EON content was higher and nitrate N content was substantially lower in the ECM forests than in the AM forests. This is consistent with the pattern of extractable N composition previously reported (Fitzhugh et al., 2003; Midgley and Phillips, 2016; Phillips et al., 2013). However, my findings suggest that the extractable N composition is more likely caused by mycorrhizal type than by soil physicochemical properties (Fig. 8). On the other hand, ammonium N content did not differ between the ECM forests and AM forests. The trend of ammonium N content between the two forests was different depending on the previous studies (Fitzhugh et al., 2003; Midgley and Phillips, 2016; Phillips et al., 2013). Furthermore, the soil microbial community corresponded to the soil extractable N composition: the abundance of nitrate N producers, ammonia-oxidizing bacteria and archaea, was consistently lower, with lower nitrate N content, in the ECM forests than in the AM forests (Fig. 11). In contrast, the abundances of free-living EON producers and ammonium N producers, saprotrophic fungi and prokaryotes, were not consistently lower in the ECM forests: their EON content was higher than in the AM forests, and they had similar ammonium N content (Fig. 10, Table 10).

Because ammonia-oxidizing prokaryotes are highly sensitive to soil moisture and pH (Chen et al., 2013; Gleeson et al., 2010; Marcos et al., 2016), it was expected that ammonia-oxidizing prokaryotic abundance would not differ by forest type, and would differ more along the soil moisture and pH gradients. Nevertheless, ammonia-oxidizing prokaryotic abundance was consistently higher in the AM forest, regardless of the aridity gradient (Fig. 10), which suggests that ECM fungi affected the ammonia-oxidizers. However, the lower abundance of free-living ammonia-oxidizers in the ECM forests seemed not to result from the lack of N substrate for ammonia-oxidizing prokaryotes, because this substrate (ammonium N), was sufficient in the ECM

forests (Fig. 7). I propose two potential reasons for this: ECM fungi might directly limit the growth of ammonia-oxidizers by secreting inhibitory compounds in order to monopolize the available N, because ECM fungi are known to produce antimicrobial substances and can alter bacterial function and activity (Frey-klett et al., 2005; Olsson et al., 1996). The laboratory incubation experiment revealed that net nitrification rate did not differ clearly between the forest types (Table 9), implying that the inhibitory effects of ECM on ammonia oxidizers may have been removed by cutting and removing the roots before incubation. The second possibility is that ammonium N was not accessible to the ammonia-oxidizing prokaryotes, even though the content was high, because drought reduced substrate diffusion and microbial mobility (Nguyen et al., 2018; van Meeteren et al., 2008). If ECM fungi obtain ammonium N before ammonia-oxidizing prokaryotes can access it, ECM fungi may limit the growth of ammonia-oxidizing prokaryotes by limiting substrate availability, even when ammonium N content appears to be high.

In contrast to the nitrification step, the mineralization step and the degradation steps appear not to be limited in the ECM forest. The fact that soil depth had a notable effect on soil fungal abundance and prokaryotic community structure (Fig. 14, Table 12) suggests that substrate quantity and quality are important for the fungal and prokaryotic community structure. Substrate quality and quantity have been reported to be the primary factors affecting the soil fungal and prokaryotic community structure in Chapter 2 and another study (Tian et al., 2017). Interestingly, my findings do not strongly support previous findings that ECM fungi limit saprotrophic growth (Gadgil and Gadgil 1971; Fernandez and Kennedy 2016). Nevertheless, I found that soil C content and C: N ratio were higher in the ECM forest than in the AM forest; previous authors have attributed this pattern to the actions of ECM fungi (Averill et al. 2014, Orwin et al. 2011).

The EON content was somewhat higher in the ECM forests than in the AM forests (Fig. 7). Since the abundance of the expected primary producers of EON, saprotrophic fungi, was not higher in the ECM forest (Fig. 10), other groups are likely to contribute to the high EON content in the ECM forest. Dominant ECM fungal taxa, especially the genus Cortinarius (the third most abundant ECM taxa in this study), have strong abilities to decay SOM (Bödeker et al., 2014). ECM fungal presence has been reported to raise the gross N-depolymelization rate (Averill and Hawks 2016). It has also been reported and suggested that ECM fungi do not transfer to the host plant all of the N that they mobilize (Näsholm et al., 2013; Pellitier and Zak, 2018). Hence, it is possible that ECM fungi increase the input to the soil EON pool. In addition, EON content declined strongly with increasing soil depth, through a reduction in soil C: N ratio (Fig. 12), suggesting that the SOM quality was also likely to be important in determining EON content. For further clarification, it would be useful to calculate the process rate (EON production rate) per unit abundance of the microbial driver (ECM and saprotrophic fungi). This will require more detailed data, such as the

gross transformation rate.

It remains unclear whether N competition is responsible for slowing SOM decomposition in the ECM forests we studied, because total saprotrophic fungal abundance and prokaryotic potential to produce N-degrading enzymes were not low in the ECM forests (Fig. 10; Table 10). I propose an alternative hypothesis, that N competition also causes high soil C storage (Fig. 15). The extremely low soil nitrate N content in the ECM forests is likely to make plants more dependent on ECM fungi for N acquisition (Hobbie and Hobbie, 2008). In fact, I observed lower nitrate N content and higher ECM fungal abundance in wetter than in dryer ECM forest soils (Figs 7 and 10). The increase in the plant dependence on ECM fungi can increase soil C in the form of microbial biomass, because ECM fungi have high microbial biomass (Högberg et al., 2002); such dependence can also increase plant C investment in ECM fungi in soils (Hobbie and Hobbie, 2008; Näsholm et al., 2013). This is likely to contribute to high soil C storage, as previously suggested (Averill et al., 2014; Orwin et al., 2011). Although the relative contributions to soil C storage of these two functions of ECM fungi (slowing SOM decomposition via competition with free-living microbes; and increasing ECM dependence via competition with ammonia-oxidizing prokaryotes) may vary depending on the ecosystem, ECM fungi are likely to increase soil C storage more than AM fungi, via either or both of these mechanisms.

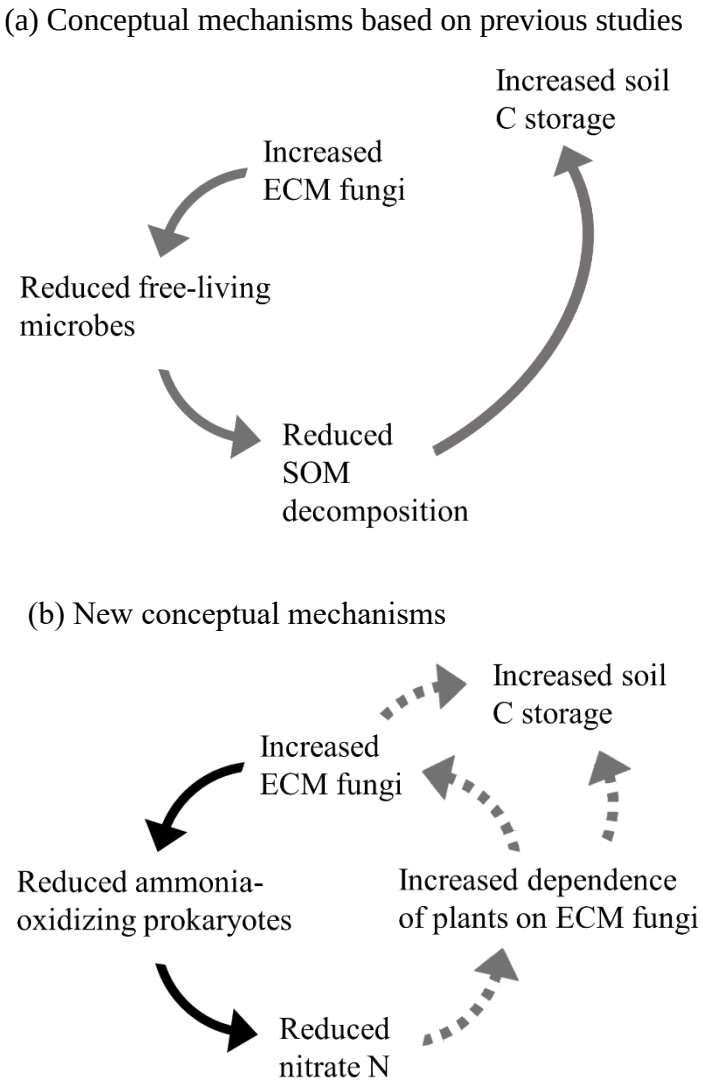

Fig. 15 Conceptual feedback mechanisms of ECM fungal function for soil C storage (a) based on previous literature (Orwin et al. 2011, Averill et al. 2014) and (b) results from this study. In (b), the black arrows and the gray dashed arrows refer to the observed effects and the predicted effects, respectively.

４. Root mycorrhizal community composition and nitrogen uptake patterns of understory trees differ between different mycorrhizal forests

４-１. Introduction

Soil N is an essential nutrient for plants that influences the productivity, composition, and functioning of forests (LeBauer and Treseder, 2008; Tanner et al., 1998; Wright et al., 2011). Plants primarily use soil inorganic N, such as ammonium and nitrate, but plants can also use organic N as a significant resource in soils with low N availability (Jones and Kielland, 2002; Näsholm et al., 1998; Nordin et al., 2001). In addition, plants can access various soluble organic compounds such as amino acids and peptides by associating with mycorrhizal fungi (Pellitier and Zak, 2018; Read and Perez-Moreno, 2003; Smith and Read, 2008). Root mycorrhizal community composition is also important, because it reportedly had significant effects on the N acquisition of host plants (Mensah et al., 2015; Veresoglou et al., 2011). Thus, plants have many strategies to take up soil N and change their form of N uptake in response to soil N availability, although each plant species has its own typical form of N that it takes up (Andersen et al., 2017; Daryanto et al., 2019; Uscola et al., 2017; Wei et al., 2015).

Many environmental factors affect soil N availability, root mycorrhizal community, and then plant N utilization. For example, increases in soil temperature lead to greater soil inorganic N content, resulting in increased inorganic N uptake rates by plants (Kuster et al., 2016; Warren, 2009) and the temperature increases also control root mycorrhizal colonization and community structure (Soudzilovskaia et al., 2015; Zhang et al., 2019). The soil N availability and root mycorrhizal community are sometimes correlated, as the colonization decrease and community composition shift with increasing N availability (Kjøller et al., 2012; Lilleskov et al., 2002). Furthermore, the N utilization of plants is affected by co-existing vegetation. When plants compete for a particular source of N, competitively inferior plants alter their N utilization such as slowing their rate of uptake and changing the N source that they take up, in response to the N environment which competitively superior plants created (Ashton et al., 2010; McKane et al., 2002). Root mycorrhizal community are reported to change with presence of other plant (Duponnois et al., 2011; Stinson et al., 2006). On the other hand, even when the environment such as soil N availability changes, some plants do not change root mycorrhizal community (Gehring et al., 2017; Martínez-García and Pugnaire, 2011) and the primary source of N that they take up (Ashton et al., 2010; Wang and Macko, 2011).

Natural abundance of stable isotopes of N (δ^{15}N) can reveal plant N use processes (Koba et al., 2003; Templer et al., 2007). The δ^{15}N in plant leaves and roots including mycorrhizal fungi

can indicate plant dependence for N acquisition on mycorrhizal fungi, because mycorrhizal fungi transfer N with lower δ^{15}N values to host plants; i.e. mycorrhizal-dependent plants show leaf δ^{15}N < root δ^{15}N (Emmerton et al., 2001; Hobbie and Colpaert, 2003; Kohzu et al., 2000). In addition, metagenomics analysis of fungi in plant roots reveal which mycorrhizal taxa are associated with plants (Koorem et al., 2017). The δ^{15}N signature of leaves can indicate the primary source of N that they take up, because leaf δ^{15}N is thought to have similar values to the δ^{15}N of primary source of N that they take up in soil (Liu et al., 2018; Takebayashi et al., 2010). For example, the δ^{15}N of leaves tended to be low if plants depend on soil nitrate (Falkengren-grerup et al., 2004; Kahmen et al., 2008), because δ^{15}N of soil nitrate is usually much lower than that of ammonium (Choi et al., 2005; Takebayashi et al., 2010; but see Houlton et al., 2007). In addition, the measurement of δ^{18}O of soil nitrate can indicate which process the nitrate N is derived from, nitrification (δ^{18}O < 10‰) or atmospheric deposition (Fang et al., 2012; Kendall et al., 2007). The δ^{15}N values in the soils can also explain soil N transformation process, because net mineralization and nitrification rate are typically positively correlated with the soil δ^{15}N values (Templer et al., 2007). Thus, by using a combination of isotopic and mycorrhizal analyses, we can more deeply understand how soil N is derived from and plants utilize the N.

Canopy tree species can have a critical impact on the N utilization of co-existing understory plants through changing the environment, including N availability and mycorrhizal community in soil. The mycorrhizal type of the dominant tree species is getting more attention as a potentially large factor controlling soil N availability (Averill et al., 2014; Averill and Hawkes, 2016; Phillips et al., 2013). ECM fungi and AM fungi have different effects on soil N cycling as described in Chapter 3. Accordingly, soil N availability, especially nitrate N content, are reported to be higher in the AM forest than in the ECM forest, as Chapter 3 and other studies showed (Phillips et al. 2013; Midgley and Phillips 2016). Furthermore, different canopy trees would provide different mycorrhizal inoculum source to the co-existing understory trees. In addition, I proposed in Chapter 3 that low nitrate N content in the ECM forests accelerate plant dependence on mycorrhizal fungi for N acquisition, which result in high plant C investment in roots, which led greater C storage in soils. I can strengthen the new hypothesis in Chapter 3 if plants in the ECM forest highly depend on mycorrhizal fungi for N acquisition. In contrast, plants in the AM forest would take up highly available nitrate N, without strongly depending on mycorrhizal fungi.

Here, I examined root mycorrhizal community, δ^{15}N and N concentrations of leaves and fine roots in co-existing understory plants, as well as the δ^{15}N and contents of the soil N source that plants can take up in a neighboring AM and ECM dominated forest. I hypothesized that forest mycorrhizal type controls N utilization of understory plants (i.e. mycorrhizal association and N source). Specifically, I expected (1) root mycorrhizal community of understory trees differ between

the two forests in response to soil N availability and root mycorrhizal community of canopy trees. In addition, I expected (2) the understory trees in the AM forest do not depend upon mycorrhizal fungi for N acquisition (i.e. leaf δ^{15}N ≪ root δ^{15}N), alternatively access highly available nitrate N (i.e. leaf δ^{15}N ≒ soil nitrate δ^{15}N), which is derived from the nitrification (i.e. soil nitrate δ^{18}O < 10‰). On the other hand, I expected (3) the understory trees in the ECM forest highly depend upon mycorrhizal fungi (especially, specific taxonomy) for N acquisition (i.e. leaf δ^{15}N < root δ^{15}N). However, it might be possible that the species of the understory tree be a primary factor for its N utilization, resulting in forest mycorrhizal type as a secondary and obscure factor.

4-2. Materials and Methods

4－2－1. Study site

This study was conducted in BL-Med site and Oak-Med site. Six understory tree species appeared commonly and frequently in both forests; *Rosa multiflora* (Rosaceae), *Cotoneaster multiflorus* (Rosaceae), *Platycladus orientalis* (Cupressaceae), *Syringa pekinensis* (Oleaceae), *Acer ginnala* (Sapindaceae) and *Acer stenolobum* (Sapindaceae). B. Wang & Qiu (2006) reported that the genus *Rosa* and *Cotoneaseter* are AM trees, and *Platycladus* and *Syringa* are unknown, and *Acer* is an AM or AM + ECM tree, although *Acer* is usually thought as AM tree (Phillips et al., 2013). Other reports showed *Platycladus* and *Syringa* are symbiotic with AM fungi (Kapoor et al., 2008; Wu et al., 2019). The relative photosynthetic photon flux density (rPPFD) of the black locust forest and the oak forest were 7.6 ± 2.7 and 9.7 ± 3.2, respectively. Black locust was known as a N-fixing tree, but the black locust trees in my site seemed not to actively fix N, because nodules were never found in their roots and N-fixing bacteria in soil was rather lower in the black locust forest than in the oak forest (Table 14). To investigate whether the black locust forest fix N, six black locust individuals (ID 1 - 6) were randomly selected, and one of their roots growing in 0-10cm depth soil was sampled in the black locust forest. The number of nodules, root tips were visually counted, and the length of the thickest and longest main roots were measured. I also predicted the prokaryotic N-fixing gene in soils from the sequencing of 16S rRNA gene in Chapter 3. The activities of N_2 fixers are limited under drought stress (Boring and Swank, 1984; Polania et al., 2016; but see Wurzburger and Ford Miniat, 2014), and the N accumulation in soil of my site was much reduced in comparison with that in soils of black locust forests receiving more precipitation (Tateno et al., 2007).

4－2－2. Sample collection

Table 14 (a) The number of nodules, root tips, and main root length of black locust trees. (b) Predicted abundance of N-fixing gene (nitrogenase, EC1.18.6.1) in 1000 reads per samples enumerated using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) pipeline procedures. The method of soil sampling, DNA extraction and sequencing analysis were descried in Chapter 3. Values are means ± SDs. The significant effects of the forest (F) was shown as the result of one-way ANOVA of the forest type. $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

(a)

ID	Nodule	Root tips	Root length (cm)
1	0	114	11.5
2	0	90	15.7
3	0	249	17.1
4	0	91	12.9
5	0	346	18.7
6	0	272	17.8

(b)

	Black locust forest	Oak forest	*F* value
0 - 10 cm soil	258.6 ± 30.4	302.6 ± 34.6	**41.2** ***
20 - 30 cm soil	323.3 ± 32.2	355.8 ± 27.4	2.4

I examined five mature individuals (each 3-5 m height) for each understory tree species in each forest. I also examined five mature individual canopy trees in each forest. I sampled healthy leaves and fine roots from each individual and together they made a pair. Approximately 10 grams of root samples (mostly fine roots but including some coarse roots) were collected from surface soils (0-30 cm depth) where the majority of roots are located. Root samples were separated into subsamples for isotopic analysis and those for DNA analysis. Approximately 10 grams of leave samples were collected randomly from the whole crown and the petiole was included. I also collected soil from 0-10 cm depth around each tree individual to detect whether extractable N content are specifically determined by the near tree species (Fig. S1). The effects of forest type on soil extractable N content was much larger than the effects of tree species, so we measured the δ^{15}N only for each forest representative soils. Collection of leaf, root and the soils around trees was conducted in mid-September 2015.

For measurement of δ^{15}N of the representative soil extractable N in each forest, soil samples (0–10 cm depth) were collected from five locations of each forest. Both forest soil has organic and mineral horizon within 0-10 cm depth. The sampling locations were approximately 5 m from each other. These samples were collected in June and September of 2016. Each soil sample was divided into a subsample for water content analysis, and that for N extraction. Soil inorganic

N was captured by ionic resin capsules each containing about 1 g of mixed ion exchange resins (IER; PST-2, Unibest, Bozeman, MT). Seven resin capsules were placed at a depth of 10 cm in soil in each forest from June 2016. The set locations were *ca*. 10 m distant from one another. The resin capsules were collected in September 2016 (three months later). The collected resin capsules were washed well with distilled water to remove adhering soils.

For additional measurements of stable isotopes in bulk soil, soil samples (0-10 cm depth and 20-30 cm depth) were collected at four locations (*ca*. >20 m distant from one another) in each forest. Each sample were generated to composite and mix four soil samples collected in four points (*ca*. 5 m distant from one another). The soil samples for 0-10 cm depth were collected in September of 2016 and October 2017, and the soil samples for 20-30 cm depth were collected in August 2017. Each soil sample was divided into a subsample for water content analysis, and then for isotopic analysis of N. Soil water content was determined after samples were dried at 105°C for >3 days.

4－2－3． DNA extraction from roots and metagenomics analysis

Root samples were carefully washed three times, and 300 root tips were picked up from each root sample and crushed in each 1.5mL microtube with BioMasher (Nippi. Inc., Tokyo, Japan). To the crushed tissue, 350 μL of CTAB buffer (100 mM Tris; 1.4 M NaCl; 20 mM EDTA; 2% acetyl

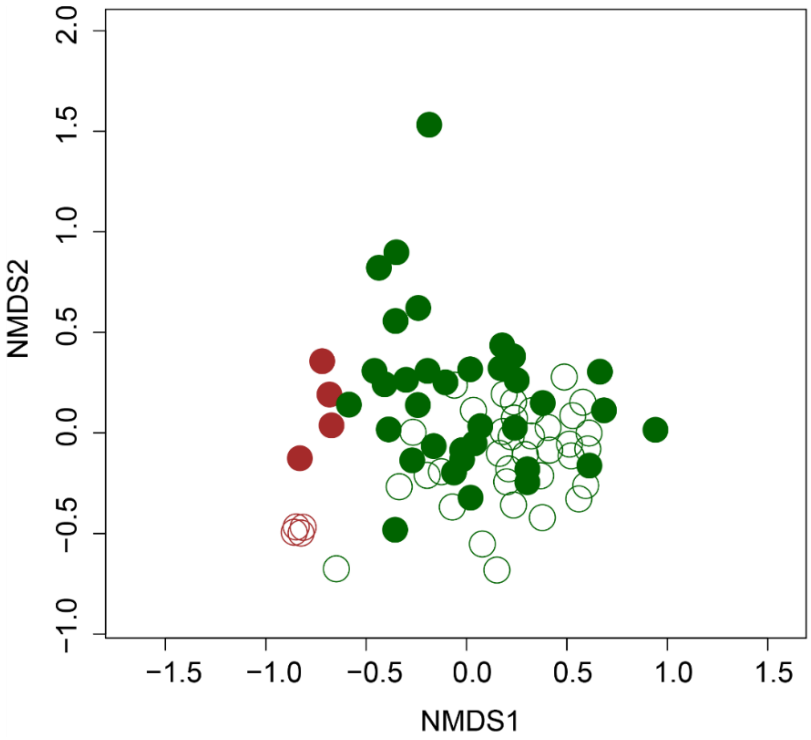

Fig. 16 The NMDS based on Bray-Curtis dissimilarities of fungal community structures in roots and soils. The fill and color represent the forest and sample type (root or soil), respectively. Open and filled symbols indicate samples from black locust forests and oak forests, respectively. Green and brown symbols refer to root and soil, respectively. The NMDS analysis of community structure dissimilarity based on the Bray-Curtis index was performed using the metaMDS function in the vegan package (Oksanen et al., 2016) of R.

trimethyl ammonium bromide) was added, and the mixture was incubated for 30 min at 65 ℃ (Sato and Murakami, 2008). After incubation, an equal volume of chloroform was added and

centrifuged for 15 min at 12,000 rpm. The supernatant was then transferred to a new microtube. An equal volume of isopropanol and a one-tenth volume of sodium acetate (pH 5) were added to the supernatant and then the mixture was centrifuged for five minutes at 12,000 rpm. After being washed with 70% ethanol, the precipitated DNA was dissolved in 200 μL of TE buffer (10 mM Tris; 1 mM EDTA, pH 8.0). The DNA extracts were kept in a -20 ^{O}C freezer until sequencing analysis.

Because all the understory trees are primarily thought as AM trees, AM fungal community was measured. I amplified the small subunit of the nuclear ribosomal RNA (SSU rDNA) in the DNA extracts using the primer sets AMV4.5NF / AMDGR (Lee et al., 2008) to reveal AM fungal community in the tree roots. The reaction solution ratio was 20:2:2:3:13 Q5 High-Fidelity DNA Polymerase (New England Biolabs Inc., MA, USA) / 10 mM forward primer / 10 mM reverse primer / 10-fold-diluted DNA sample / sterilized distilled water. Cycling conditions were as follows: initial denaturation at 98 °C for 30 sec, followed by 35 cycles of 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 30 sec, followed by a 2-min final extension at 72 °C. Amplification was performed using two replicates from each sample, after which amplification was checked by agarose gel electrophoresis and the replicates were composited to make one solution for purification. Purification was conducted using Agencourt AMPure XP (Beckman Coulter Inc., Brea, CA, USA) following the manufacturer's protocol. After purification, appropriate amounts of samples were combined into one tube and mixed to equalize the DNA quantity in each sample, which was computed using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). The combined samples were separated by the agarose gel electrophoresis, and the gels containing SSU rDNA genes were extracted by QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) following manufacture's instruction. Based on the size and quality of DNA in the gel extracts, which were checked using the Agilent High Sensitivity DNA Kit and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), a dilution library of approximately 25 pM was prepared. The subsequent step in the analysis has been described in Chapter 2. The SSU rDNA sequences shorter than 250 bp in length were removed; Maarj*AM* was used to extract AM fungal sequences (Öpik et al., 2010). Sequence data were deposited in the Sequence Read Archive at NCBI under accession number DRA009209.

In my analyses of the AM fungal community, I standardized the read number to 2790 using random pick-up based on the minimum read number. OTUs whose relative abundance were <1% were removed. The OTU tables was converted into 1 or 0 binary OTU tables, as >0 and 0 were defined "presence (1)" and "not presence (0)", respectively. AM taxonomy was defined based on the family. For the groups whose family was not identified but order was identified, I refed the order ("Other Glomerales" and "Other Archaeosporales"). The relative frequency was calculated

as how many individuals had the taxonomy (0 ~ 5 for one tree species in one forest). I carefully washed the surface of plant roots to avoid the contamination of soils, so that the root fungal community was clearly different from the soil fungal community (Fig. 16).

4－2－4． Measurement of the $\delta^{15}N$ or $\delta^{18}O$ and IER-captured nitrate N content

Leaf and root samples were washed with distilled water. The leaf, root and bulk soil samples were oven-dried at 60℃ for over 24 h and ground into powder. Bulk soil samples were hydrolyzed using HCl to remove carbonate. Leaf and root samples were loaded into tin capsules for isotope analysis, and total C and N concentration and stable isotope ratios were measured using an isotope ratio mass spectrometer (DELTA V Advantage, Thermo Fisher Scientific Inc., Waltham, USA) with an elemental analyzer (Flash 2000, Thermo Fisher Scientific Inc., Waltham, USA). The N stable isotope ratios of bulk soil samples were analyzed with an Elemental Analyser (Eurovector) coupled to an isotope Ratio Mass Spectrometer (Delta plus xp, Thermo Fisher Scientific, USA). The precision of the on-line procedure was better than ± 0.2‰ for $\delta^{15}N$.

For extractable N analysis, soil samples were extracted with 2M KCl solution (soil: KCl = 1: 10, w/w). IERs were shaken with 20 ml of 2M KCL for 20 min three times, making a total extract of 60 ml per one resin. Before preparing 2M KCl solution, the KCl was muffled at 450℃ for 4 h. The extracts were filtered using a precombusted glass-fiber filter (GF/F; Whatman Int. Ltd., Maidstone, UK, muffled at 450℃ for 4 h). The KCl extracts were frozen until the analyses of N concentration and isotopic composition. I measured the concentration of ammonium and nitrate N in soil and resign extracts, and that of total extractable N (TEN) in soil extracts, by using microplate reader (Synergy™ HTX, BioTek, USA). For determining the concentration of ammonium and nitrate N, the indophenol-blue method and a modified acidic Griess reaction (Miranda et al., 2001) were used, respectively. TEN was oxidized to nitrate N, using persulfate oxidization and measured as nitrate N (Miyajima et al., 2005). The extractable organic N (EON) is then calculated as [EON] = [TEN] – [Nitrate] – [Ammonium]. When [Nitrate] was under the detection limit, we assigned [Nitrate] as zero.

The $\delta^{15}N$ and $\delta^{18}O$ of nitrate were measured using the denitrifier method (Casciotti et al., 2002; Sigman et al., 2001; Thuan et al., 2018). We converted the nitrate into nitrous oxide (N_2O) using a denitrifier (*Pseudomonas aureofacien*) that lacked the enzyme to convert N_2O to N_2. The N_2O produced was introduced into the isotope ratio mass spectrometer (Sercon 20/22 equipped with Cryoprep and GC; Sercon Ltd., UK). The $\delta^{15}N$ of TEN was determined using persulfate oxidation followed by the denitrifier method (Houlton et al., 2007; Koba et al., 2012). The ammonium N in the KCl extract was recovered with the diffusion method (Holmes et al., 1998)

and the ammonium N was captured onto a glass fiber filter (muffled at 450℃ for 4 h, GF/D; Whatman Int. Ltd., Maidstone, UK) during shaking for over 48 h and drying for over 24 h. The captured ammonium N was converted into nitrate N by the persulfate oxidization then its N isotope ratio was determined by the denitrifier method (Thuan et al., 2018).

Natural abundance of ^{15}N and ^{18}O was expressed in per mil (‰) deviation from international standards: $\delta^{15}N$ or $\delta^{18}O = [R_{sample}/R_{standard}-1]\times1000$, where R is ^{15}N / ^{14}N or ^{18}O / ^{16}O, respectively. Atmospheric N and Vienna standard mean ocean water were used as the international standards for N and O, respectively. Calibrations for these isotopic analyses were carried out using several international and inhouse standards (USGS32, 34, 35 and IAEA NO_3^- for nitrate N, USGS25, 26 and IAEA N-2 for ammonium N and calibrated alanine, glycine, and histidine for TEN (Takebayashi et al., 2010)). The natural abundance $\delta^{15}N$ values of soil nitrate N was measured for nine and one samples in the black locust forest and in the oak forest, respectively, due to very low nitrate N concentration in other ten samples. For these ten samples, $\delta^{15}N_{EON}$ was calculated assigning [Nitrate] as zero. The $\delta^{15}N$ of EON was calculated using the following mass and isotopic balance equation: $\delta^{15}N_{EON} = \{\delta^{15}N_{TEN} \times [TEN] - (\delta^{15}N_{nitrate} \times [Nitrate] + \delta^{15}N_{ammonium} \times [Ammonium])\}/[EON]$. The difference in the $\delta^{15}N$ between leaves and roots of the same individual tree was defined as: $\delta^{15}N_{root\text{-}leaf} = \delta^{15}N_{root} - \delta^{15}N_{leaf}$.

4－2－5． Statistical analysis

The NMDS analysis of AM fungal community structure dissimilarity based on the Bray–Curtis index was performed using the metaMDS function in the vegan package (Oksanen et al., 2016) of R software. The envfit function in the vegan package was used to identify significant correlations between the NMDS values of points and the forest type, the species of the trees (one tree species = 1, other tree species = 0), and the presence of fungal taxonomies, to illustrate the vectors on the NMDS ordination plot. To know the effects of soil environment, the extractable N content, moisture and pH in soil around each tree individual were illustrated on the ordination. To identify the specific AM fungi which highly contribute to the N acquisition of the host tree, the vectors of $\delta^{15}N_{root\text{-}leaf}$ (the indicator of mycorrhizal dependence) and leaf N concentration of the host trees were illustrated on the ordination. PerMANOVA was performed to test the significance of the effect of forest type and the tree species on the microbial community, using the adonis function in the R vegan package. The 1 or 0 binary OTU tables were used to illustrate NMDS and conduct PerMANOVA.

I used two-way ANOVA to test the significant differences between the forest type (black locust (AM) forest or oak (ECM) forest) and plant species for the following: the presence (1 or 0) of AM taxonomy (Family level) and the total number of AM fungal OTUs in roots, and the content, $\delta^{15}N$ and/or $\delta^{18}O$ of N in soils and that captured in IER. I set the difference of sampling occasion (timing) as a random effect. Tukey's multiple comparisons was used to examine potential differences among $\delta^{15}N$ of leaves, roots and soils. I used a paired *T*-test to examine potential differences in $\delta^{15}N$ between the leaf and root of the same individual plants. Student's *T*-test was used to test for significant differences in leaf N concentration between the same plant species across the two forests. Prior to these tests, I tested for normality and homogeneity of variance in the data using the Kolmogorov–Smirnov and median Levene's tests, respectively. The car package in R software (version 3.1.2; R Development Core Team, 2014) was used to perform Levene's test

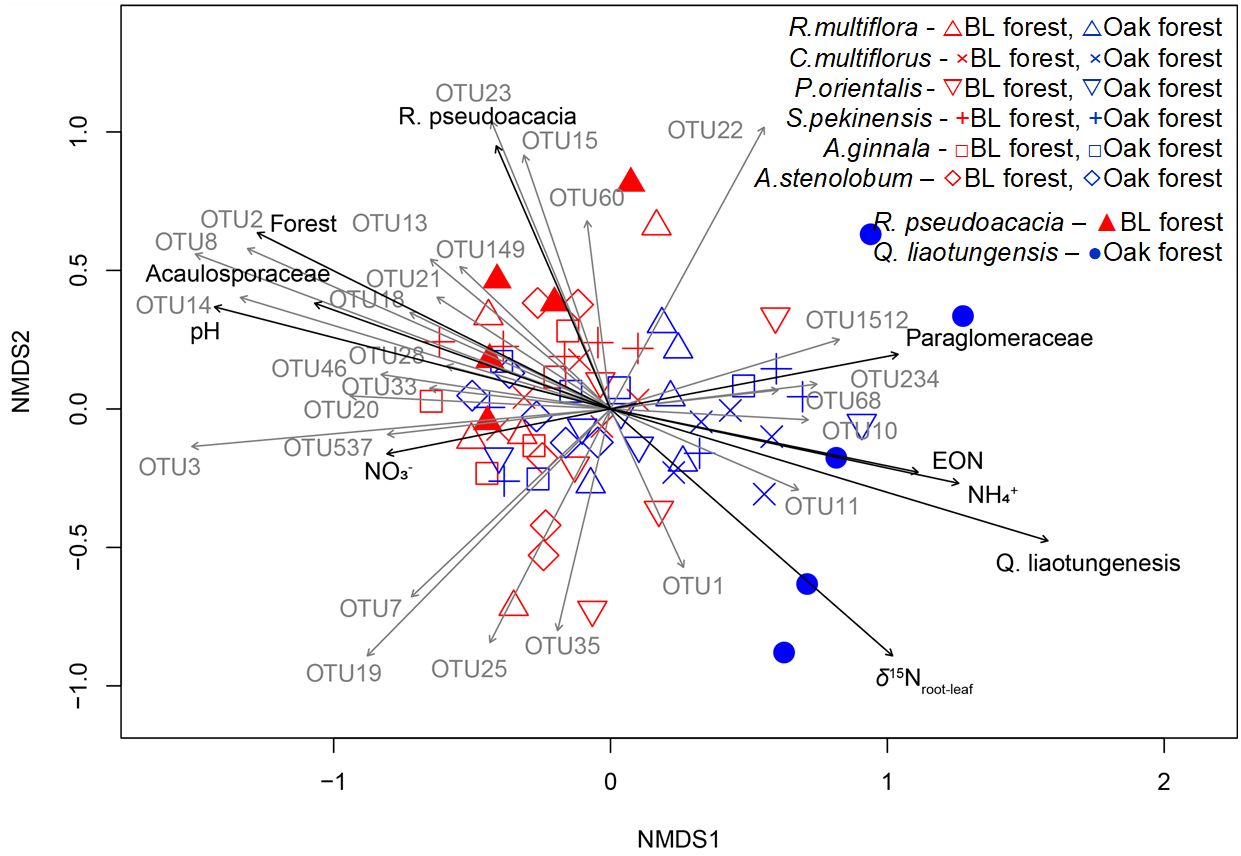

Fig. 17 The NMDS based on root AM fungal community dissimilarities. Red and blue symbols represent the plants in the black locust forest and in the oak forest, respectively. The symbols indicate as follows: △; *R.multiflora*, ×; *C.multiflorus*,▽; *P.orientalis*, +; *S.pekinensis*, □; *A.ginnala*, ◇; *A.stenolobum,* ▲; *R.multiflora,* ●*; Q.liaotungenesis*. Only significant vectors are plotted on the ordination. Black vectors represent the experimental design (Forest (black locust forest =1, and oak forest =0) and Species), the presence of fungal taxonomy, the soil extractable N content (EON, NH_4^+; ammonium, NO_3^-; nitrate) and $\delta^{15}N_{root\text{-}leaf}$ (the indicator of mycorrhizal dependence). Dark gray vectors represent the presence of the frequent OTUs.

(Fox et al., 2014). When the *P* values of Kolmogorov–Smirnov or Levene's test prior to ANOVA was <0.05, the data were square-root or reciprocally transformed. When the *P* values of Kolmogorov–Smirnov or Levene's test prior to *T* test was <0.05, Welch's *T* test was alternatively used. When the *P* values of Kolmogorov–Smirnov test was >0.05 but the *P* values of Levene's test was <0.05 prior to the multiple comparison, Games-Howell's test was alternatively used. I chose a significance level of $P < 0.05$ for all tests.

4 - 3 . Results

4－3－1． AM fungal community in the tree roots

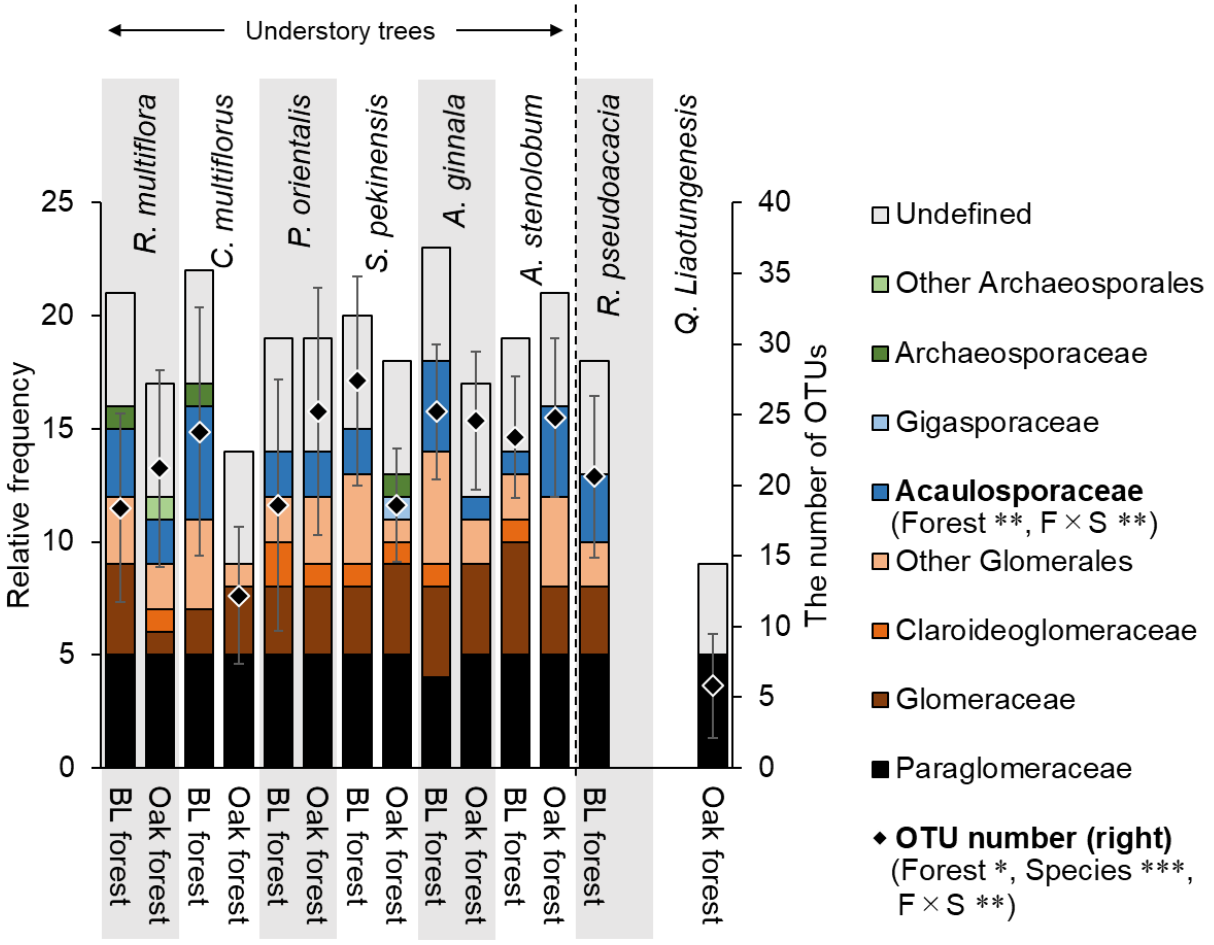

Fig. 18 Relative frequency of AM fungal Family and the averaged total number of AM fungal OTUs in roots of roots living in the black locust (BL) forest and the oak forest. The blanket below the legends indicates the significant effects of forest type (Forest), tree species (Species) and the interaction (Forest × Species; F × S) based on the two-way ANOVA with the forest type and species. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Black, red, blue and green groups belong to the Order Paraglomerales, Glomerales, Diversisporales and Archaeosporales.

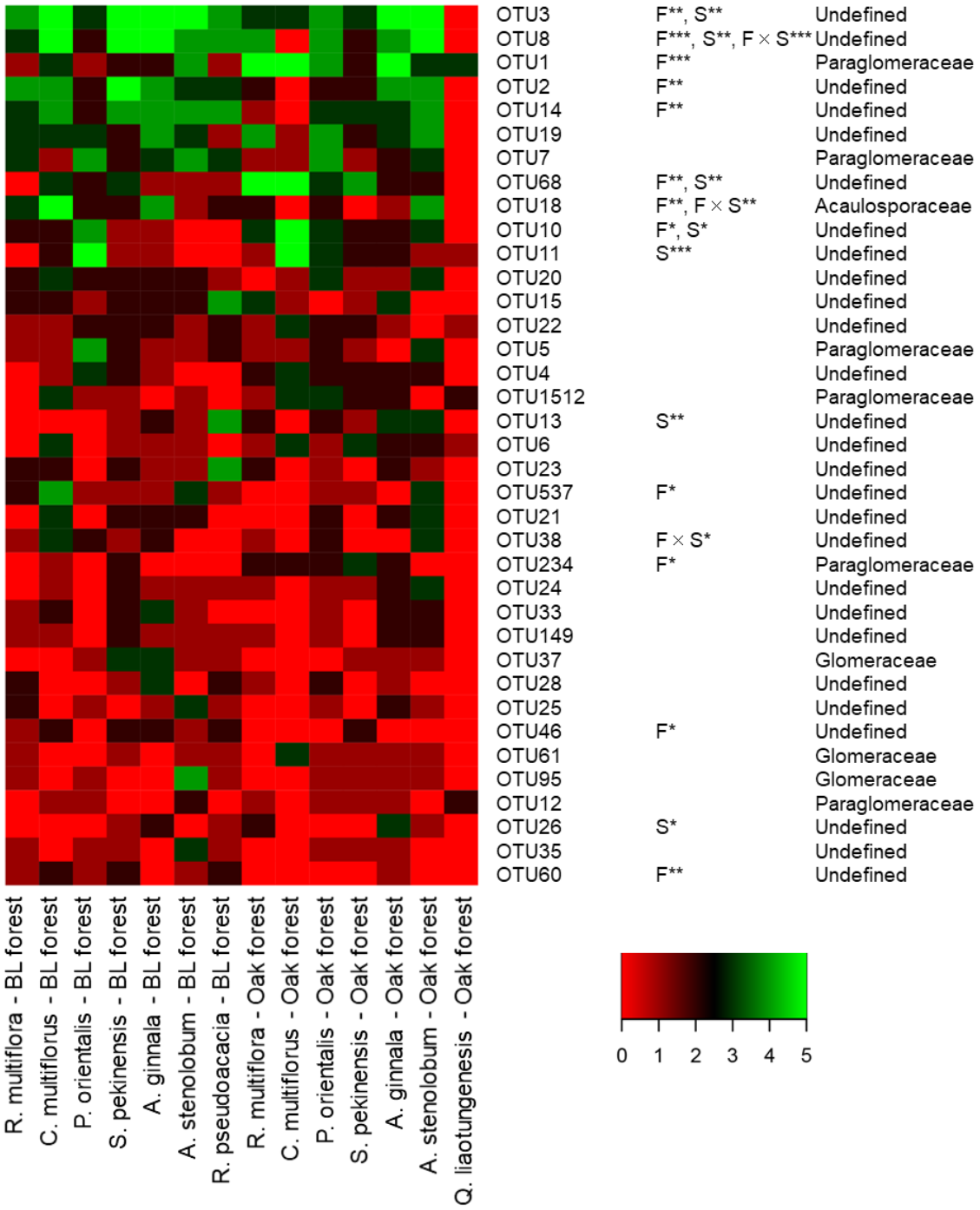

Fig. 19 Relative frequency of AM fungal OTUs in roots of the co-existing understory trees in the black locust (BL) forest and in the oak forest. The right side shows OTU ID, the result of two-way ANOVA, and the taxonomy (The Family). The significant effects of the forest (F), plant species (S) and the interaction (Forest × Species; F × S) was shown as the result of two way ANOVA of the forest and the species. $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. AM taxonomy was based on the family. OTUs which appeared in >9 individuals were showed. OTUs was aligned from top to bottom along the total frequency in roots of 70 tree individuals.

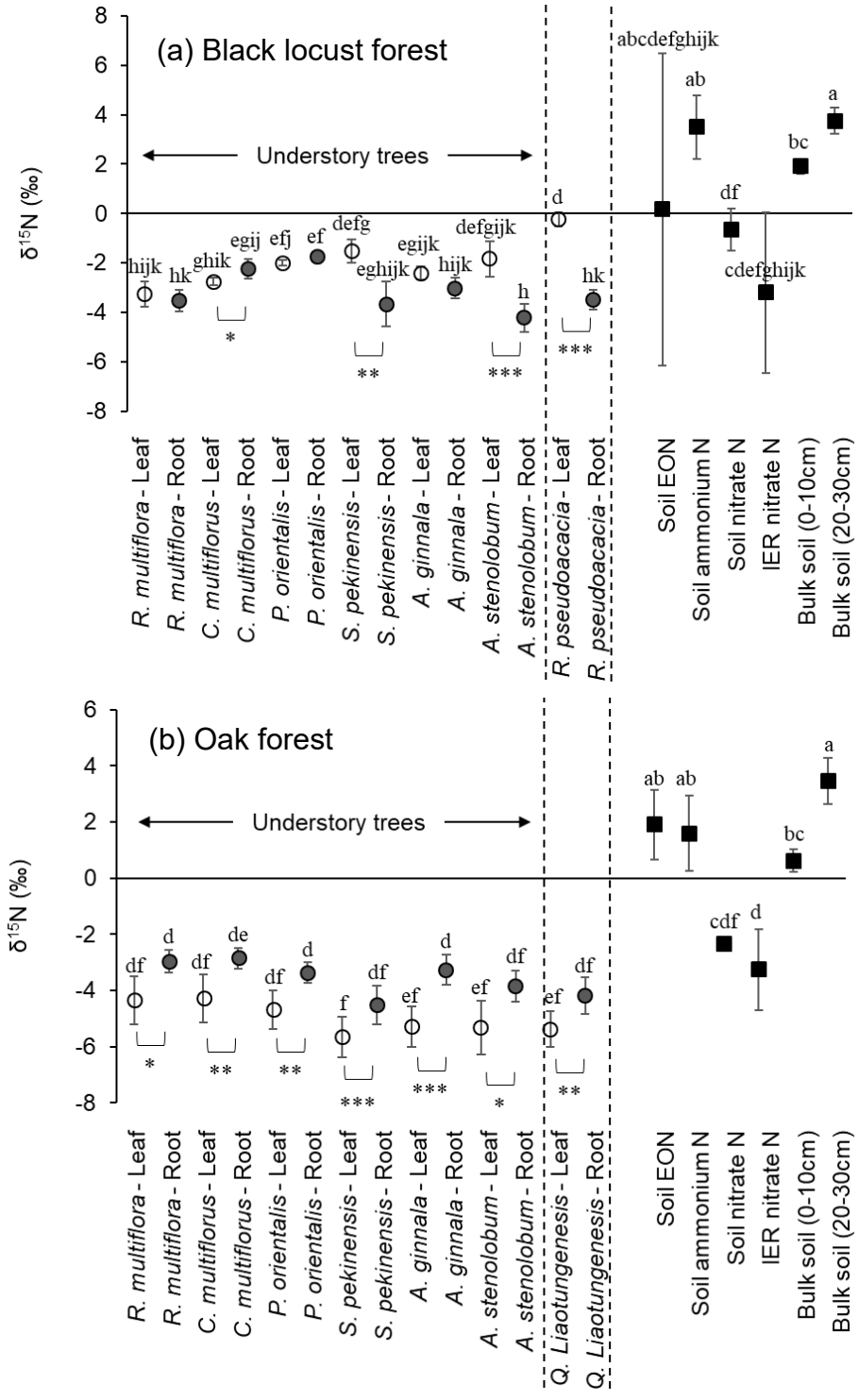

Fig. 20 The δ^{15}N of leaves and roots of the six understory trees and the canopy trees, and the δ^{15}N of soil N sources (soil extractable N and bulk soil) in (a) the black locust forest and (b) the oak forest (mean ± SD). The results of paired *T* test between δ^{15}N of leaves and roots from the same individual trees were shown below of the plots. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Different lowercase letters above the plots indicate significant pairwise differences (Tukey–Kramer or Games-Howell test).

AM fungal community structure was the most strongly affected by forest type ($F = 4.34$, $R^2 = 0.05$, $P < 0.001$), and then by the host plant species ($F = 1.86$, $R^2 = 0.16$, $P < 0.001$), but not significantly affected by the interaction between forest type and plant species (based on PerMANOVA). The NMDS also showed the large difference of the community structure between the two forests. The forest type, extractable N content and pH in soil around the trees, and the canopy tree species was significantly correlated with the plots (Fig. 17). The community structure of the canopy tree was more similar to that of the understory trees in the same forest than in the different forest (Fig. 18). The presence of Acaulosporaceae and the number of OTUs were significantly affected by the forest (Fig. 18). The presence of Acaulosporaceae was also affected by the interaction, and the number of OTUs was also affected by the plant species and the interaction. The Family Acaulosporaceae more frequently appeared in the black locust forest, except for in the roots of *P. orientalis* and *A. stenolobum* (Fig. 2). The number of OTUs in the roots of *C. multiflorus*, *S. pekinensis* and *A. ginnala*. was higher in the black locust forest than in the oak forest (Fig. 18). Acaulosporaceae the most frequently appeared in *C. multiflorus* of the black locust forest (it appeared in every individuals). Most of AM fungal OTUs more frequently appeared in the AM forest than in the ECM forest (Fig. 19). However, OTU1 and 234 (Family Paraglomeraceae), and 10 and 68 (the Family and Order was not identified) were significantly more frequent in the ECM forest than in

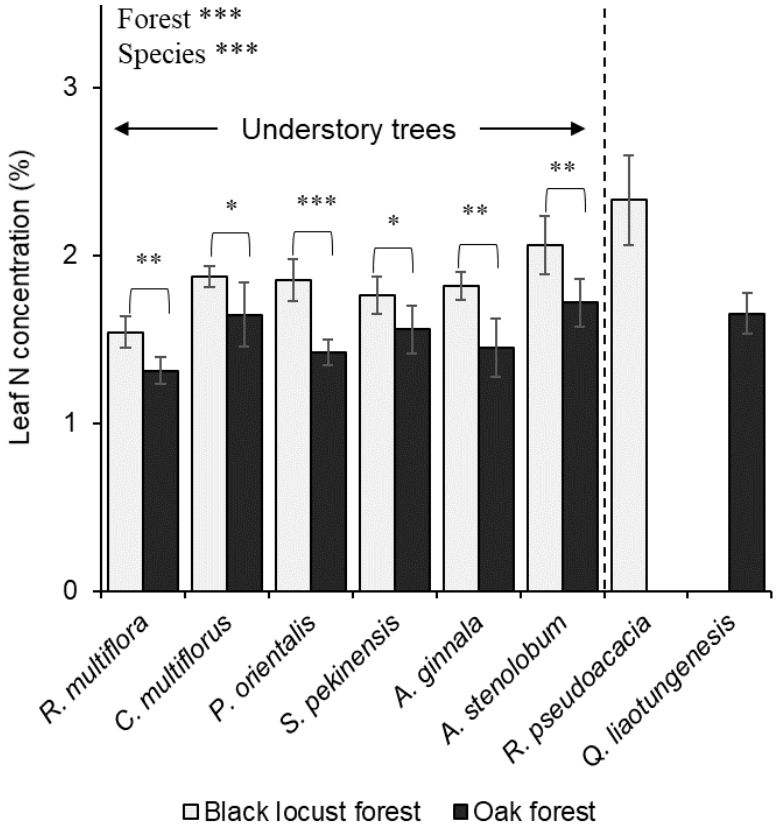

Fig. 21 The leaf N concentrations of the six understory trees and the canopy trees in the black locust forests and in the oak forests. Values are means ± S.D. Significant differences between forests in each species (identified by Student's or Welch's *T* test) are indicated on the bar. The results of two-way ANOVA with the forest type (Forest) and the plant species (Species) were shown in upper side. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

the AM forest (Fig. 19). Further, OTU1 was highly correlated with $\delta^{15}N_{root\text{-}leaf}$ (Fig. 17).

4－3－2． The chemical properties of the understory trees and soils

In the oak forest, leaf $\delta^{15}N$ was consistently lower than root $\delta^{15}N$ in all the species (Fig. 20b). On the other hand, in the black locust forest, the relationship between leaf and root $\delta^{15}N$ was different depending on the plant species (Fig. 20a). Leaf $\delta^{15}N$ of *S. pekinensis* and *A. stenolobum* was significantly higher than their root $\delta^{15}N$, although leaf $\delta^{15}N$ of *C. multiflorus* was lower than its root $\delta^{15}N$ in the black locust forest (Fig. 20a). Leaf N concentration was significantly affected by

Table 15 The content and $\delta^{15}N$ of soil extractable N in soil (0-10cm depth), nitrate N in ion exchanged resin (IER) and bulk soil (0-10cm depth and 20-30cm depth), and the $\delta^{18}O$ of nitrate N in soil (0-10cm depth) and captured in IER (mean ± SD). (n) shows the number of samples. Right side shows *F*-values and *P*-values (*P < 0.05, **P < 0.01, ***P < 0.001) based on one-way ANOVA with the forest (the black locust forest or the oak forest). The models were performed with sampling occasion as a random variable. The data of soil extractable N content and total N content was from Chapter 3.

		Black locust forest	(n)	Oak forest	(n)	*F* value	
Content (mg kg^{-1}) or (mg cap^{-1})	Soil EON	18.57 ± 10.76	(36)	39.50 ± 18.94	(36)	**59.3**	***
	Soil ammonium N	9.08 ± 3.27	(36)	12.29 ± 3.30	(36)	**66.5**	***
	Soil nitrate N	7.18 ± 3.45	(36)	0.74 ± 0.56	(36)	**238.4**	***
	IER nitrate N	0.239 ± 0.180	(7)	0.030 ± 0.022	(7)	**18.1**	**
	Soil total N (0-10cm depth)	2312 ± 434	(36)	3234 ± 571	(36)	**68.4**	***
	Soil total N (20-30cm depth)	544 ± 44	(4)	629 ± 76	(4)	3.8	
$\delta^{15}N$ (‰)	Soil EON	0.17 ± 6.33	(10)	1.90 ± 1.24	(10)	0.7	
	Soil ammonium N	3.50 ± 1.27	(10)	1.59 ± 1.33	(10)	**10.1**	**
	Soil nitrate N	-0.65 ± 0.86	(9)	-2.35	(1)	3.8	
	IER nitrate N	-3.20 ± 3.24	(7)	-3.25 ± 1.44	(7)	0.0	
	Soil total N (0-10cm depth)	1.91 ± 0.31	(8)	0.62 ± 0.39	(8)	**54.4**	***
	Soil total N (20-30cm depth)	3.76 ± 0.52	(4)	3.46 ± 0.81	(4)	0.4	
$\delta^{18}O$ (‰)	Soil nitrate N	1.51 ± 2.33	(9)	-3.10	(1)	**9.8**	*
	IER nitrate N	4.10 ± 2.29	(7)	38.7 ± 16.17	(7)	**31.6**	***

the forest type and plant species, and consistently higher in the black locust than in the oak forest in all understory tree species (Fig. 21).

In the black locust forest, the $\delta^{15}N$ of understory trees were not significantly different form the $\delta^{15}N$ of soil EON, IER-captured nitrate N and sometimes soil nitrate N, but consistently lower than that of soil ammonium N (Fig. 20a). The $\delta^{18}O$ of nitrate N in soils and captured in IER of the black locust forest was lower than 10‰ (Table 15). In the oak forest, the $\delta^{15}N$ of the understory trees were significantly lower than $\delta^{15}N$ of EON and ammonium N (Fig. 20b). The $\delta^{15}N$ of soil nitrate N of the oak forest was measured only in one sample (Table 15). The $\delta^{15}N$ of IER-captured nitrate N was not significantly different with the $\delta^{15}N$ of some understory trees (Fig. 20b), but the $\delta^{18}O$ of nitrate N captured in IER was much higher than 10‰ in the oak forest (Table 15).

In the surface soil (0-10 cm depth), EON and ammonium N content were higher in the oak forest than in the black locust forest, but the nitrate N content was 10 times higher in the black locust forest than in the oak forest (Table 15). The content of nitrate N captured in IER was also higher in the black locust forest than in the oak forest (Table 15). The $\delta^{15}N$ of bulk soil and soil ammonium N was significantly higher in the black locust forest than in the oak forest (Table 15).

4-4. Discussion

As I expected in my hypothesis (1), mycorrhizal community composition associated with roots were significantly different between the two forests. Soil N availability should be an important factor for the difference in the community structure, because the structure significantly correlated with extractable N contents (Fig. 17), although soil pH, which co-change with forest mycorrhizal type (Cheeke et al., 2016; Phillips et al., 2013), may also be important for the community structure (Fig. 17; An et al., 2008; Moora et al., 2014) . The Family Acaulosporaceae was significantly more frequent in the AM (black locust) than ECM (oak) forest (Fig. 18), and some OTUs in the Family Paraglomeraceae were significantly more frequent in the ECM than AM forest (Fig. 19). Consistent to my result, the Order Acaulospora and Paraglomus are reported to increase in soils with high-nitrate N content (Van Diepen et al., 2011), and low-nutrient soils (Kouadio et al., 2017; Rodríguez-Echeverría et al., 2017), respectively. The canopy trees may provide the fungal inoculum source to the understory trees, because black locust roots or forest soils often had Acaulospora (He et al., 2016; Yang et al., 2015). Thus, root mycorrhizal community of understory trees differ between the AM and ECM forest, in response to soil N availability and root mycorrhizal community of canopy trees.

Regarding hypothesis (2), in the AM forest, the understory trees did not strongly depend upon mycorrhizal fungi, because leaf $\delta^{15}N$ was not significantly lower than root $\delta^{15}N$ except for *C. multiflorus* (Fig. 20a). The understory trees likely to use nitrate N, as indicated by $\delta^{15}N$ of soil

nitrate N, especially IER-captured nitrate N, being similar to δ^{15}N of the leaves of the understory trees (Fig. 20a). The fact that δ^{15}N of ammonium N was higher than δ^{15}N of the understory trees suggests that they likely not to use ammonium N (Fig. 20a). The δ^{15}N of soil EON was also not significantly different from δ^{15}N of the understory trees. It needs further research to reveal if the trees actively use the EON, but EON might not be major N source because most of the EON is high-molecular-weight and recalcitrant (Jones et al., 2005), and only small part of EON is possible N source for plants (Takebayashi et al., 2010). Supporting that the understory trees take up nitrate N, the δ^{15}N of roots was higher than that of leaves, especially of *S.pekinensis* and *A.stenolobum*. Nitrate N assimilation occurs both in roots and shoots, with lighter nitrate N typically assimilated in roots, and then the heavier nitrate N is transferred to shoots and leaves, leading to greater leaf δ^{15}N than root δ^{15}N (Evans, 2001; Evans et al., 1996; Yoneyama and Kaneko, 1989). Further, the nitrate N in the AM forest comes primarily from nitrification, because δ^{18}O of soil and IER-captured nitrate N was < 10 ‰, although the IER-captured nitrate N in the ECM forest should be mostly derived from atmosphere (Table 15; Fang et al., 2012; Kendall et al., 2007). In addition, the greater δ^{15}N values in the AM than ECM forest soils support the nitrification rate is higher in the AM forest, since they are typically positively correlated (Templer et al., 2007). Thus, consistent with my hypothesis (2), the understory trees in the AM forest likely take up rich nitrate N pool which is derived from active nitrification.

In terms of hypothesis (3), all plant species in the ECM forest likely uptake N depending on mycorrhizal fungi, as indicated by the fact that leaf δ^{15}N was lower than root δ^{15}N (Fig. 20b), which suggests mycorrhizal fungi in roots transferred lighter N to leaves (Emmerton et al., 2001; Hobbie and Colpaert, 2003; Kohzu et al., 2000). Although the transfer of N from AM fungi to host plants has not yet been demonstrated (Aguilar et al., 1998; Wheeler et al., 2000), AM fungi thought to transfer ^{15}N depleted N to host plants, as shown by AM plants being more depleted compared to non-mycorrhizal plants (Craine et al., 2015). In addition, AM trees have shown lower leaf δ^{15}N than root δ^{15}N, although it is no information the trees actually associated with the AM fungi (Pardo et al., 2013; Templer et al., 2007), and reports are very limited. The OTU1 (Paraglomeraceae), which was significantly more frequent in the ECM forest and correlated with $\delta^{15}N_{root\text{-}leaf}$ (Fig. 17, 19), should have an important role in N acquisition of the understory trees in the ECM forest. Species in Paraglomeraceae were reported to increase growth of host plants (Lu and Wu, 2017; Wu et al., 2011). In the agreement with my hypothesis (3), the understory trees in the ECM forest depend upon mycorrhizal fungi for N acquisition. The results can further support my suggestion in Chapter 3 that ECM forests accelerate plant dependence on mycorrhizal fungi for N acquisition, which may result in high plant C investment to soils.

I also found the patterns of N utilization of the understory trees were affected by the host

tree species. For example, *C. multiflorus* had significantly lower δ^{15}N in leaves than roots in the AM forests, while other tree species had higher or similar δ^{15}N in leaves than roots (Fig. 20a). The Genus *Cotoneaster* was reported to show smaller growth when fertilized by nitrate N than by ammonium N (Kraus et al., 2002). AM fungal family Acaulosporaceae appeared frequently in the roots of *C. multiflorus* in the AM forest (Fig. 18, 19), and Acaulospora was reported to increase in soils with high-nitrate N content (Van Diepen et al., 2011). *C. multiflorus* may not actively access the rich nitrate N pool and may depend for nitrate N uptake on Acaulosporaceae in the AM forest.

I conclude that while the tree species is an important factor for describing patterns of the N utilization, the effect of forest type is even stronger. I suggest that canopy trees with different mycorrhizal type can alter the root mycorrhizal community and the N utilization patterns of co-existing understory trees, even if the two forests are very close in proximity (neighboring to each other). Furthermore, the understory trees in the AM forest had higher leaf N concentrations than in the ECM forest (Fig. 21). The higher N concentrations of understory trees may contribute to further accelerate the N cycling as a feedback, because high N concentration of litter increase decomposition rate (Cotrufo et al., 1995; Sun et al., 2018), although more researches such as about the amount and N concentration of litterfall of understory trees are needed.

5 . General discussion

5 - 1 . Discussion

In Chapter 2, I found the aridity level had a big impact on soil N cycling, as increasing aridity reduced all the extractable N contents. However, the microbial drivers of each step responded differently to the aridity gradient. My study suggested the nitrification step is the most sensitive to rainfall pattern change because the abundance of the driver, ammonia-oxidizers, was determined by soil moisture, although other two steps were likely to be controlled by substrate quantity and quality, which should more stable under rainfall pattern change as described in Chapter 2 (Fig. 22). In Chapter 3, I found the difference in mycorrhizal type between the black locust forest and the oak forest also has a substantial impact on soil N cycling. The presence of ECM fungi strongly limited the nitrification step, as the nitrate N content and the abundance of ammonia-oxidizers consistently lower in the oak forest than in the black locust forest regardless of other soil physicochemical properties. The other two steps were likely to be more largely controlled by SOM than by forest type. Thus, my study suggests climate change (rainfall pattern change) and land-use change (exotic plantation) in this region strongly alter soil N cycling, especially the nitrification step (Fig. 22). The result is consistent with other study, suggesting nitrification is more sensitive to environmental change than mineralization (Chen et al., 2019; Hu et al., 2016). Once the ammonia-oxidizers was reduced by environmental factors (moisture and ECM presence in this

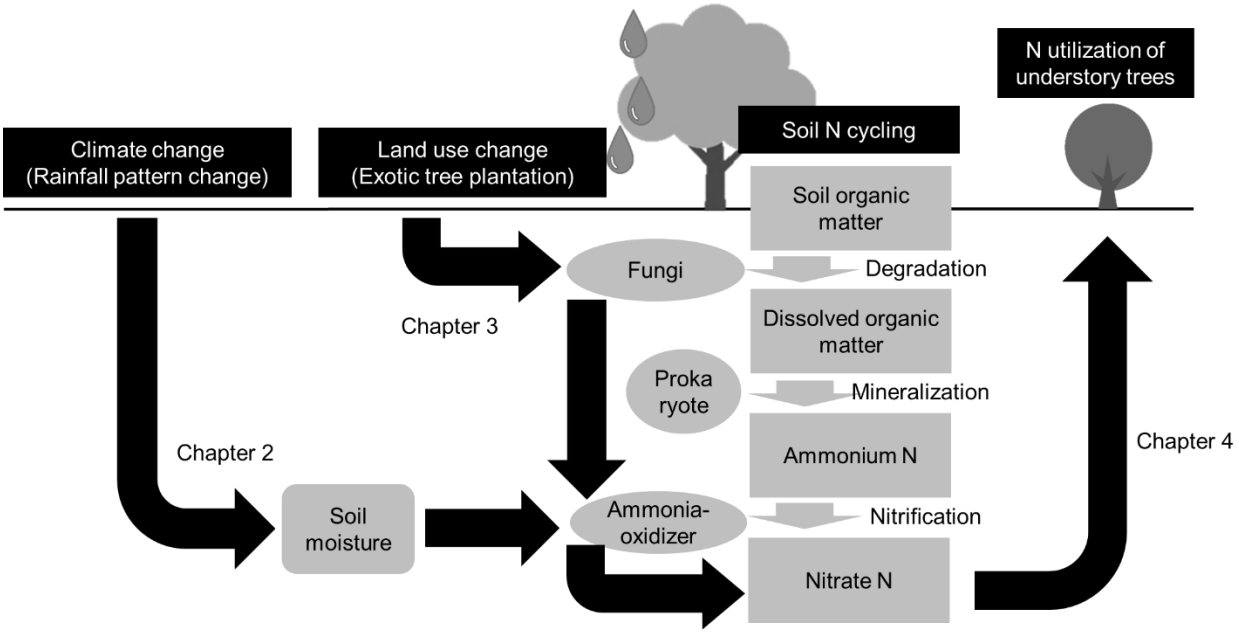

Fig. 22 Summary of the findings in this study. Black bolded arrows indicate the strongest effects observed in each chapter.

case), they could not be replaced by the same functional microbes because of their less diversity than ammonifying microbes (Isobe et al., 2019). Accordingly, Chapter 4 suggested the difference in soil N availability between the black locust forest and the oak forest controlled the N utilization of understory trees. The understory trees likely used rich nitrate N pool in the black locust forest, but they depend upon mycorrhizal fungi for N acquisition under low soil N availability in the oak forest. Thus, my study suggested the response of nitrification to environmental change is a key factor controlling the whole forest N cycling in this region.

The change in soil N cycling along the aridity gradient may be milder in the oak forests than in the black locust forest, although the difference of the compared aridity level was smaller between the oak forests than between the black locust forests. For example, soil inorganic N content was 27 mg/kg and 16 mg/kg in the BL-Wet and BL-Med sites, respectively, although it was 13 mg/kg in both of the Oak-Wet and Oak-Med sites on average. For supporting the milder effects of aridity level on soil N cycling in the oak than black locust forests, I also found the soil N cycling was more sensitive to soil moisture in the black locust forest than in the oak forest according to artificial rainfall experiment (Tatsumi et al. unpublished). In this experiment, increasing soil water content increased nitrate N content in the black locust forest, but not in the oak forest. These results are consistent with the suggestion that exotic tree plantations respond more sensitively to climate change than the native forests (Afreen et al., 2011), although more research is needed to conclude because I could not separate mycorrhizal type and exoticness in this study. In the oak forests, ECM fungi primarily controlled the soil N cycling, so the soil N cycling would be stable unless ECM fungi are affected by the aridity gradient. Otherwise, if ECM fungi are suppressed by the soil drought (Gehring et al., 2006; Nilsen et al., 1998; Sims et al., 2007), an alternative increase in the free-living microbes due to the release from the N competition may drive the N transformations. It was also reported the soil N availability did not change in beech (ECM species) forests along a rainfall gradient of Central Europe (Meier and Leuschner, 2014). My study suggested the future increase in the aridity level reduce the N cycling rate and further reduce NPP in the black locust forest rather than in the oak forests.

Chapter 4 supported my expectation in Chapter 2 that the reduction in nitrate N production along the aridity gradient further reduces N uptake of plants growing in the black locust forests. Chapter 4 showed plants in the black locust forest primarily used nitrate N, so the soil moisture change would strongly affect the N uptake of the plants living in the black locust forest. On the other hand, plants in the oak forest were likely to uptake N through mycorrhizal association rather than using nitrate N probably because of the consistently low nitrate N content. AM fungi are relatively tolerant to soil drought (Klironomos et al., 2001; Morte et al., 2000). Hence, plants in the oak forest depended for N acquisition on the less sensitive way to soil moisture (i.e. AM

association) than direct nitrate N uptake by plant roots without mycorrhizal association. The N utilization in the oak forest may further make NPP less sensitive to rainfall pattern change in the oak forest than in the black locust forest, because I suggested NPP decrease in response to increasing aridity through the reduction in nitrate N uptake in Chapter 2, but further research is needed to conclude this.

In Chapter 4, the leaf N concentration of understory trees, as well as their N utilization, differed between the forests. The leaf N concentration is important for the plant photosynthetic rate (Sims and Pearcy, 1989; Sinclair and Hone, 1989), so the understory growing competition in the black locust forest should be also different from in the oak forest to some extent. For example, plant species, which is more inflexible in their N utilization than the selected six common species, may not get enough N to meet their necessary demand for growth competition in the black locust forest. It is possible that the change in the growth competition may contribute to the lower tree biodiversity in the black locust forest than in the oak forest (Otsuki et al., 2005), although further research is needed to test the possibility. For supporting this, it was reported that soil N enrichment reduced the plant biodiversity probably because of other resource competition (Barbara et al., 2008; Humbert et al., 2016). Thus, the effects of canopy trees on soil N availability likely cause further effects on the ecosystem function.

In Chapter 4, only AM understory tree species were targeted, because the common species in black locust forests and oak forests were only AM species. However, it is important to know the N utilization of ECM understory plants (e.g. *Populus davidiana* in this region) between the two forests, because the succession from black locust (AM) forest to oak (ECM) forest have not been observed in the region. Since ECM trees receive more amount of N from mycorrhizal fungi than AM trees (Smith and Read, 2008), mycorrhizal inoculum provided by canopy trees would be more important for ECM trees than AM trees. I found oak seedlings planted with oak forest soils had higher ECM colonization, survival rate, and leaf N concentrations than the seedlings planted with black locust forest soils (Tatsumi et al. unpublished). ECM fungi should have important role for oak growth, such as improving their N uptake. In this region, natural succession to oak forests originally occurs from *Populus davidiana*, and Gneus *Populus* can be symbiotic with ECM fungi (Wang and Qiu, 2006), and ECM fungi were probably provided from *P. davidiana* to oak seedlings (Zhang et al., 2014). In addition, native ECM trees were reported to share ECM fungi with introduced exotic ECM trees (Bahram et al., 2013; Jairus et al., 2011). It may be more important whether the plated trees are ECM trees or not than whether they are exotic or not for the natural succession from the planted trees to the indigenous ECM trees. It is valuable to consider for use of the ECM trees for afforestation instead of the black locust, although further test is still necessary.

In addition, climate change (rainfall pattern change) and land-use change (exotic

plantation) likely affected forest C storage as well as N cycling. The black locust plantations had lower soil C storage than native oak forests, and the prolonged drought due to the rainfall pattern change would reduce the NPP in the black locust forests. Aged black locust plantations retained more C than the barren land (Zhang et al., 2018), although their soil C storage sometimes did not significantly change (Tian et al., 2017; Zhang et al., 2018). Hence, the conversion of barren areas to black locust plantation should be meaningful in terms of terrestrial C retention. However, the use of ECM trees rather than AM trees for the plantation should have more soil C storage based on my study. Pine trees (*Pinus tabulaeformis*) were also frequently used for afforestation in this region, and indigenous to the region (Yamanaka et al., 2014), and an ECM tree (Wang and Qiu, 2006). A study in Loess Plateau showed a higher C content in a pine forest than in a black locust forest (Cao et al., 2018). I also found similar soil extractable N composition to the oak forest (very low nitrate N content) in the pine forest nearby the Med-Oak site (Tastumi et al. unpublished). Planting of pine tree (ECM tree) may be useful to improve the natural succession as described above. Further research of pine forests in this region should be interesting to determine the tree species for afforestation if concerning the succession and terrestrial C retention.

5-2. Conclusion

My study suggested that climate change (rainfall pattern change) and land-use change (exotic plantation) strongly alter soil N cycling, especially the nitrification step, and the nitrate N availability is likely a key factor controlling the whole forest N cycling, through changing the N utilization of the co-existing trees. In addition, the change in N utilization of understory trees could be the reason of the slow succession from black locust forests to oak forests, if oak trees are not able to change the N utilization as other species do. Furthermore, the black locust forests would have lower C storage than the oak forests, because ECM fungi (symbiotic with oak) increase soil C storage than AM fungi (symbiotic with black locust). And, NPP would be more largely reduced by increasing aridity in the black locust forest than in the oak forest, because trees in the black locust forest depended on nitrate N, which would sensitively decrease with the increasing aridity. Thus, the altered N cycling by climate change and land-use change will further alter ecological function (forest species composition and terrestrial C storage). My study provided important knowledge to predict the future environment and consider the forest management of this region, from the viewpoint of the N cycling driven by soil microbial community.

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