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Comparative studies on microbial community structures and their function in boreal and subarctic ecosystems
(北方林およびツンドラ生態系における土壌微生物群集とそれらの機能性についての比較研究)

PhD Thesis

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Chapter 1 **General Introduction**

1.1. Boreal forest and tundra ecosystems

1.1.1. Characteristics of vegetation in boreal forest

The boreal forest is distributed in northern Eurasia, northern North American continent, and Scandinavia (Fig. 1.1). Only limited tree species, spruce, pine, fir, and birch, are able to grow due to high adaptation to the adverse climate under low temperature in such environments. These boreal forest area accounts for approximately one-third of the total forest on the earth and is an important global carbon pool (Lindroth et al., 1998). The low temperature in long winter and relatively short photosynthetically active season inhibit the activity of soil fauna and microbes leading slow litter decomposition (Hyvönen et al., 2007). Meteorological conditions (temperature and precipitation) and soil environmental factors (pH, moisture content, and presence or absence of permafrost) in boreal forests are varied, thus boreal tree species are different among the forest types and geotopological characteristics. Our study sites are located in Swedish Scandinavian mountains and Finnish northern Lapland, both of which involves tree line with birch trees (*Betula pubescense*). In vegetative succession, conifer forests of spruce (*Picea abies*) and pine (*Pinus sylvestris*) are distributed throughout the southward area.

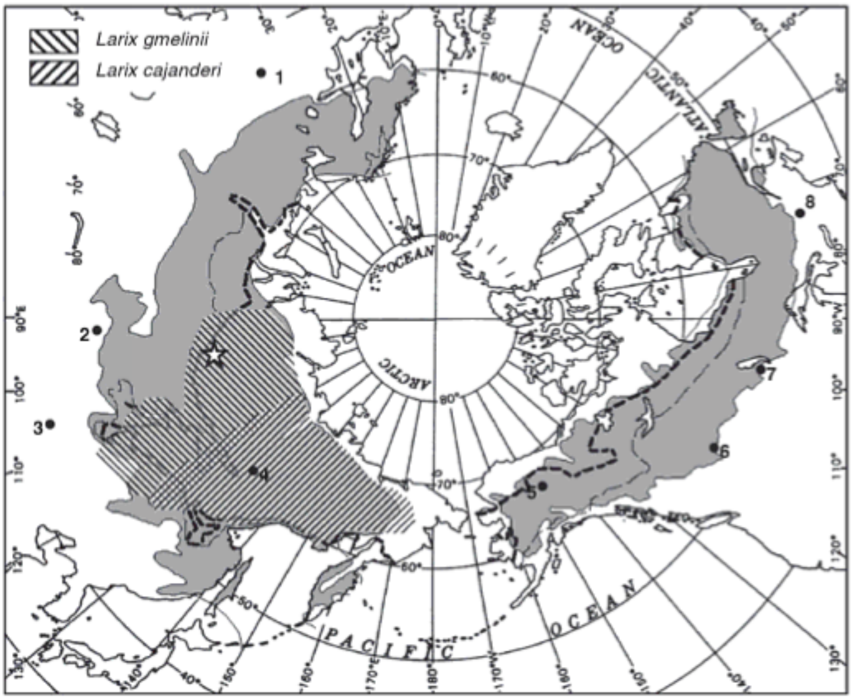


Figure 1.1 The distribution of boreal forest (Ozawa et al., 2010)

1.1.2. Characteristics of vegetation in subarctic tundra

The subarctic tundra is distributed in the northern Scandinavian mountains, where heath and meadow are found as patchy distribution. Heath vegetation is dominated by evergreen and deciduous dwarf shrub on relatively low nutrient dry condition, while meadow vegetation is dominated by forbs, graminoid and sedges on relatively high nutrient wet condition. Heath and meadow vegetation can be observed through forest till tundra in every elevation along the tree line. It has been pointed out that bacterial activity is higher in meadow soil than in heath soil, which probably means fungal activity, particularly ericoid mycorrhizal fungi, is relatively high in heath soil (Sundqvist et al., 2014).

1.2. Impact of global warming on subarctic ecosystems

Climate change is a critical issue throughout the global warming caused by human activity (IPCC 2007). It has long been estimated that the tree species comprised of boreal forest migrate northward the tree line, leading to the increase of photosynthesis and litter supply to the soil (Bonan, 2008; Xu et al., 2011). Global warming had a significant effect on forest ecosystems at high latitude of the forest limit and subarctic tundra, primarily because of acceleration of organic carbon decomposition in soil (Hyvönen et al., 2007). Although the trees of boreal forest provide a large quantity of organic carbon in the form of photosynthetic products allocated to the root and also in the form of leaf litter, the availability of this carbon for nitrogen-fixing bacteria is limited owing to the low temperatures of boreal forest soils, leading to ineffective litter decomposition in the soil (Kleczewski et al., 2012). Therefore, environmental changes exert a significant effect on the amount of available carbon in boreal forest bed soils, thereby impacting microbial processes in the soils (Hyvönen et al., 2007; García-Palacios et al., 2015).

1.3. Nitrogen cycle in terrestrial ecosystems

A large part of nitrogen exists in nature in the form of dinitrogen gas (N_2) in the atmosphere. However, most of the higher plants cannot utilize this inactive gas form of nitrogen. Conversely, inorganic nitrogen, such as nitrate (NO_3^-) and ammonium (NH_4^+), and organic nitrogen such as protein and nucleic acid are important available or storage forms of nitrogen necessary for green plants. Thus, nitrogen changes its form and state due to biological process in circulation in the global ecosystem. Generally, once the organic nitrogen such as protein and nucleic acid are supplied to the soil in the forest ecosystems, soil microbes and soil fauna in the forest floor start to decompose them into inorganic nitrogen, nitrate and ammonium. Also, biological nitrogen fixation supplies a part of available nitrogen to the soils.

Tree growth is limited by nitrogen. In northern region, low temperature retards litter decomposition, leading to less mineral nitrogen supply. Besides, nitrogen input and output in forest ecosystems show somewhat imbalance, with unknown nitrogen sources. Prokaryotic free-living nitrogen fixers can reduce nitrogen gas present in space between soil particles to ammonium, but several reports rather showed that the level of nitrogen-fixation in the boreal forest soil is too low to explain nitrogen imbalance (Vitousek & Howarth, 1991). Boreal forests may also deposit nitrogen via nitrate precipitation from fallen dusts and polluted air. Indeed, Haber-Bosch process contributes to supplying more than 55% of available nitrogen source in current global nitrogen cycle, but in the boreal forest ecosystem, those can be ignored because impacts of chemical fertilization and air pollution in boreal forests are obviously small (Gundale et al., 2014).

Previous studies have demonstrated that annual nitrogen mineralization cannot supply the total annual nitrogen demand of plants in boreal forest ecosystems due to the effect of low temperatures (Kielland, 1994; Schimel & Chapin, 1996). Kielland (1994) suggested that organic nitrogen, particularly in the form of dissolved amino acids, constitutes a large portion of the nitrogen budget for plants in boreal forest limit ecosystems. Some arbuscular and ericoid mycorrhizal fungi show high ability to assimilate glycine and glutamate, but many ectomycorrhizal fungi that mainly support dry boreal forest ecosystem were less responsible to such organic nitrogen sources (Grelet et al., 2009;

Whiteside et al., 2012). In relatively wet, boreal Scandinavian spruce forests, cyanobacteria that inhabit the surface of ubiquitous feather mosses covering the forest bed are thought to act as nitrogen suppliers (Deluca et al., 2002), but it is still mystery in the forests established on dry heath or wet sphagnum moss peatland. The measured value of nitrogen supply was sometimes below the estimated value (Binkley et al., 2000) means that not all sources of nitrogen supply are clearly understood.

1.3.1. Nitrogen cycle in the boreal forest

Boreal forests maintain a large amount of biomass. Nevertheless, neither inorganic nitrogen supply by decomposition of litters nor high nitrogen fixation from the soils and the roots were detectable (Binkley et al., 2000). Annual nitrogen mineralization cannot supply the total annual nitrogen demand of plant in the boreal forest ecosystems due to the effect of low temperatures. In relatively wet, boreal Scandinavian spruce forests, cyanobacteria that inhabit the surface of ubiquitous feather mosses covering the forest bed are thought to act as nitrogen suppliers (Deluca et al., 2002). Conversely, in the relatively dry heath forest where moss carpet is poor or absent, the source of nitrogen in such a boreal ecosystem remains a mystery.

The biological nitrogen fixation in forest soil and litter has been estimated approximately 0.5 kg N ha^{-1} yr^{-1}, which is 1/10 of nitrogen necessary for maintaining biomass productivity in similar types of forest (Rosen & Lindberg, 1980). In conifer forest in northern Europe, it has been reported that nitrogen input is due to symbiotic nitrogen fixation by cyanobacteria which is associated with ubiquitous feather moss covering 80% of forest bed (Deluca et al., 2002).

This report implied a solution of nitrogen imbalance in moss-rich forest, however nitrogen source in other ecosystems without ground-covering moss remains a mystery.

1.3.2. Nitrogen cycle in tundra

In the arctic tundra, the cyanobacteria-associated nitrogen fixation is a major nitrogen source, which supplies adequate nitrogen demand of surrounding vegetation, epiphytic cyanobacteria on organic layer, lichens, and bryophyte (Stewart et al., 2011). Conversely, ericaceous *Empetrum hermaphroditum* and *Vaccinium vitis-idaea* acquire nitrogen from ericoid mycorrhiza and ectomycorrhiza in subarctic tundra heath (Michelsen et al., 1996). In meadow, epiphytic cyanobacteria are absent in forbs and herbaceous species, and nitrogen source is partly unclear (Sundqvist et al., 2011).

1.3.3. Nitrogen fixation

Biological nitrogen fixation is performed by nitrogen-fixing bacteria to convert atmospheric dinitrogen gas to ammonia by nitrogenase, responsible for the reduction of one nitrogen (N_2) molecule to ammonium (NH_3) consuming 16 ATP to yield 2 molecules of ammonium. Nitrogenase complex consists of 2 enzymes, 2 cofactors, and reducing agents. One of the enzyme unit called dinitrogenase is heterotetrametric MoFe protein that is encoded by *nifD* and *nifK* genes, and another is dinitrogenase reductase, homodimetric Fe protein encoded by *nifH* gene. Dinitrogenase reductase encoding *nifH* gene highly conserved among nitrogen-fixing bacteria has been used for detection and classification of nitrogen-fixing bacteria (Ohkuma et al., 1996; Raymond et al., 2004).

1.3.4. Contribution of archaea to nitrogen cycle

Domain *Archaea* has long been classified into two phyla *Euryarcheota* and *Crenarchaeota*, which account for 80% and 20% of archaeal species respectively. The *Euryarcheota* includes methanogens, which produce methane, and haloarchaea, which survive in a high salinity environment. The *Creanarchaeota* includes thermophilic or hyper-hemophilic archaea and sulfur-dependent extremophiles. Phylum *Thaumarchaeota*, previously involved in *Creanarchaeota*, was currently proposed as a new archaeal phylum in 2008, which was found from moderate environments such as marine and soil as mesophilic archaea (Brochier-Armanet et al., 2008). Some of ammonia oxidizing archaea (AOA) have been reported successful isolation, but most of them are autotrophic or mixotrophic archaea difficult to culture (Könneke et al., 2005; Lehtovirta-Morley et al., 2011; Tourna et al., 2011). The first step of ammonia oxidation occurred by the ammonia monooxygenase (AMO) on cell membrane is oxygenation of NH_3 to produce NH_2OH. The AMO is encoded by an *amoA* gene.

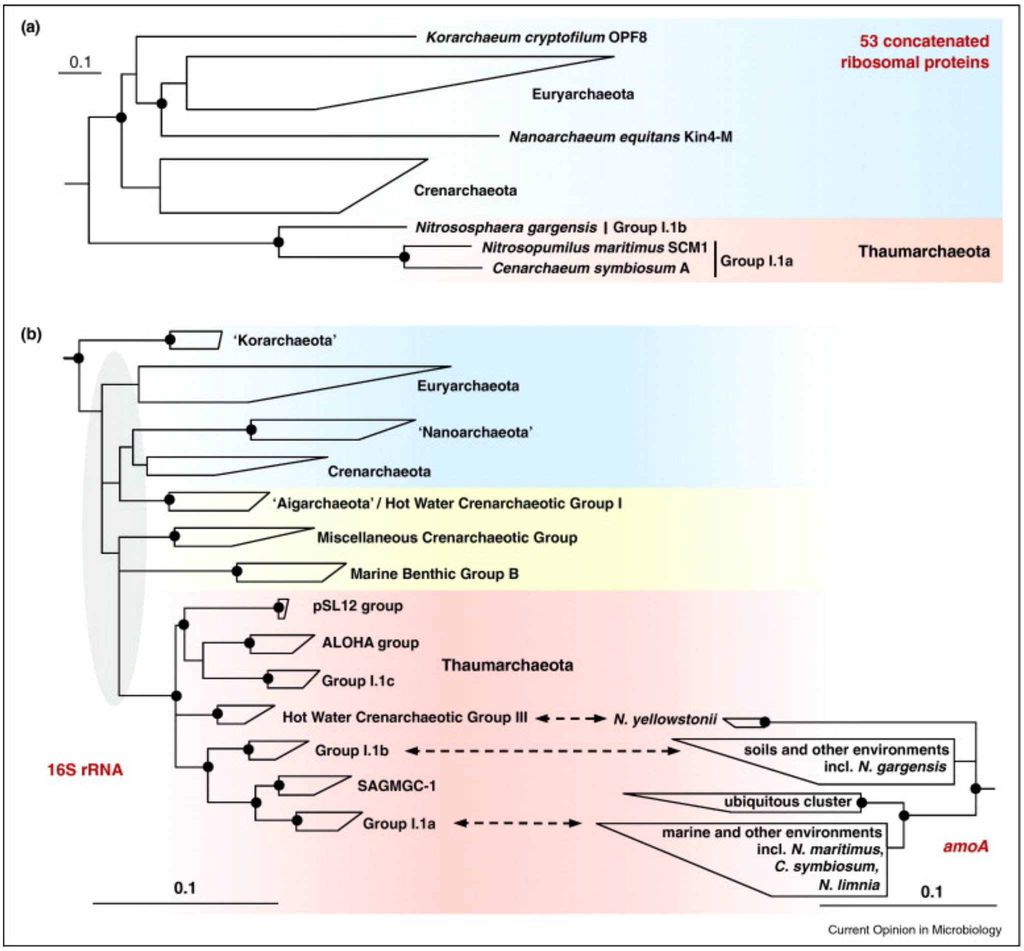


Figure 1.2 Phylogenetic tree of Archaea (Pester et al., 2011)

The archaeal *amoA* gene was more abundant than bacterial *amoA* gene in many studies: e.g. in waste water treatment plant, hot spring sediment, forest soil, and marine ecosystems (Lehtovirta-Morley et al., 2011; Mußmann et al., 2011; Tourna et al., 2011). The ammonium oxidization is rate-limiting process of nitrification throughout nitrogen cycling, and ammonium oxidizing bacteria (AOB) had long been believed to play a central role in soil and marine ecosystems, till archaeal *amoA* gene was detected in an order of magnitude larger than bacterial *amoA* (Alves et al., 2013). Although AOB abundance is lower than AOA, it showed higher growth rate, which still suggested its preferential ammonia oxidization under an environment with a high ammonium concentration.

On the other hand, AOAs are capable of utilizing low concentration of ammonium even in the acidic condition (Martens-Habbena et al., 2009). Hence, *Thaumarchaeota* might play a role in global nitrogen cycling as a part of nitrification. This ammonia-oxidization is speculated to be a motive forth for nitrogen cycling in carbon-poor mineral soil in boreal forest ecosystems. They may accelerate nitrogen circulation in terrestrial nitrogen cycle.

1.4. Podzolization in the boreal forest and relation with material circulation

1.4.1. Occurrence and time scale of podzols

The podzol covers approximately 485 million ha of world terrestrial area though boreal to tropic regions (Sauer et al., 2007; FAO-UNESCO, 2002) (Fig. 1.3). The boreal regions are, however, main podzolized region, where Scandinavia, Russia, and Canada are widely involved. Thus, low temperature and relatively wet environment caused podzolization. In addition, podozolic regions are also distributed in the high-mountain regions, subarctic tundra, polar desert (Blume et al., 1996).

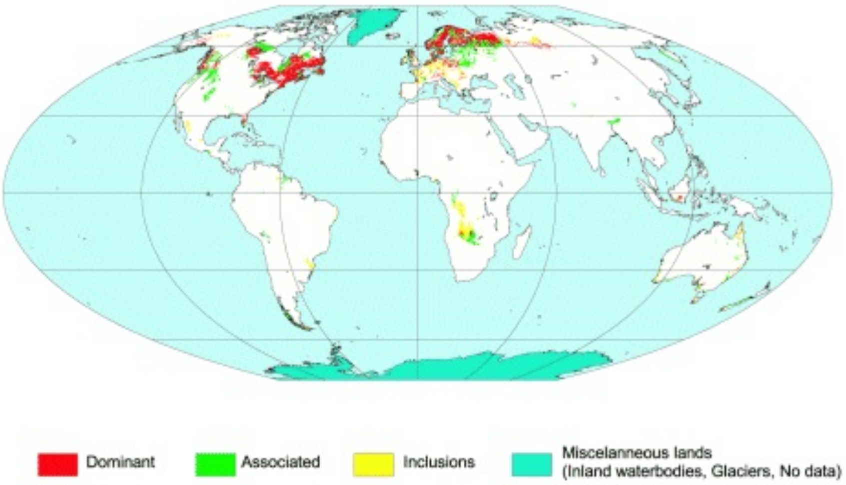


Figure 1.3 World distribution of podzols (FAO-UNESCO, 2002)

The time spans for podzol formation depend on the climatic condition and soil properties. There have been reported the time spans of the podzol formation in various regions. In Scandinavia, the primitive podzol was observed with in 100-500 years (Mokma et al., 2004). It usually takes 1000-6000 years to mature (Sauer et al., 2008). In some cases, the mature podzols were observed within 220-300 years in low temperature and wet climate (Koutaniemi et al., 1988; Evans, 1999). In North America, initial podsolization was observed within 170-1000 years, while in the most studies, the mature podzols did within 2300 years to 10000 years (Barrett & Schaetzl, 1992). In any cases, 100-year-horizons need for podsolization.

1.4.2. Podzolization processes and influences on material circulation

Several theories have been proposed for the mechanisms of podzolization. The podzolization is a complex process of mobilization and immobilization of organic matter and minerals. The initial process is to leach organic matter and minerals from the topsoil to the subsoil and eventually forms an eluvial horizon. The podzolization caused low nutrient and acidic condition leading to low microbial activity in slow litter decomposition as constant release of organic acids. Besides, low pH enhances destruction of primary silicates and minerals which leads to release of Al, Fe and Si. These elements and organic matter are supplied from the topsoil and accumulate in the subsoils. It is suggested that the ions of Fe, Al and Si leached together with organic acids form water-soluble complexes with organic matter for the mobilization process. The organic matters and minerals are then accumulating in the subsoil. The process of immobilization is hypothesized as follows: polymerization of organic matters, oxidation of the complex, mineral ions addition to the complex during the translocation, and bacterial degradation (Lundström et al., 2000). Due to Fe deficiency, nitrogen fixation would be suppressed because of unavailable Fe which needs for the active center of nitrogenase.

1.5. Research aims

So far as reported, nitrogen imbalance in the boreal forest has yet been a mystery. Hence, the soil microbial community structures in subarctic ecosystems including boreal forest, tree line, and tundra were investigated to reveal their roles in the ecosystems, particularly in the relation with their nitrogen cycling capacity. This study aimed to reveal contribution of microbial communities to the nitrogen cycling especially in nitrogen fixation and nitrification. Besides, relationship among the microbial community, vegetation, and soil environment were investigated.

The specific queries to be solved in this study are as follows: (1) what is the major interaction between response of soil microbial communities to water-soluble organic carbon and their nitrogen fixing potentials, (2) what is the major member and roles of thaumarchaeal communities detected from boreal forest soils, (3) what is the relationship between vegetation, soil properties, microbial community structure and nitrogen fixing potentials in tundra ecosystems, and (4) what is the contribution of soil microbial communities to podzolization in boreal forest.

Chapter 2

A Comparative Study on Soil Microbial Community linked to Water Soluble Organic Carbon (WSOC)

2.1. Background

Climate change is a critical issue throughout the world. The boreal tree species migrate to north, leading to alteration of not only vegetation diversity but also carbon cycle in the ecosystems. In addition, nitrogen availability is one of the most important factors influencing the primary production of terrestrial plants (Vitousek & Howarth, 1991; DeLuca & Zackrisson, 2007), especially at high latitudes, where organic layer of the soil is slowly decomposed due to annually low temperatures. Several reports on the effect of global warming on subarctic ecosystems are available. A heating treatment on bulk soils in birch and spruce tree line areas resulted in the incensement of soil organic carbon (Rinnan et al., 2008; Hagedorn et al., 2014). Besides, in subarctic heath along the birch tree line, a combination of long-term heating and litter addition increased nitrogen supply to the ecosystem, while moss-associated nitrogen fixation by *Nostoc* cyanobacteria was decreased (Sorensen & Michelsen, 2011). Another study suggested that along with soil respiration by heterotrophs, increased soil temperature affects balance between microbial community structure and metabolism of inorganic nitrogen (Xu et al., 2011).

Thus, many of previous studies have demonstrated that annual nitrogen mineralization cannot supply the total nitrogen demand of plants in the boreal forest ecosystems due to slow litter decomposition under low temperature conditions (Kielland, 1994; Schimel & Chapin, 1996; Binkley et al., 2000). As described in Chapter 1, understanding nitrogen cycle in boreal forest ecosystems in the aspect of soil microbial ecology is insufficient, particularly in heathy forests poor in carpet mosses (Lett & Michelsen, 2014).

The hypothesis of this study is that accelerated litter decomposition in boreal forest due to the climate change activates nitrogen cycling particularly in the subsoil horizon relatively shallow. Hence, I compared microbial community structure and nitrogen-fixing potentials in the soils of following 2 sites: a spruce forest edge in high latitude and altitude in Pallas, Finnish Lapland with a poor litter supply and its slow decomposition, and a planted larch forest in cold-temperate zone in Tomakomai, Japan with a large amount of litter supply and its rapid decomposition. The eubacterial and archaeal community structures in the soils were investigated using PCR-DGGE and high-throughput sequencing system. Nitrogen-fixing potentials of the soil microbial communities were evaluated using a culturing method to examine their response to different concentrations of a WSOC-mimicking carbon source mixture.

2.2. Materials and Methods

2.2.1. Site description and soil sampling

Samples were collected from a spruce forest in northern Finland and a planted larch forest in Hokkaido, Japan (Fig. 2.1). The spruce forest is located inside Pallas National Park in Pallas, Lapland, in northern Finland in the high-latitude boreal forest limit area (68°02′N, 24°04′E, alt 710 m). The organic layer (O-horizon), spodzol (E-horizon), and mineral soil (B1- and B2-horizons) were sampled from triplicate soil pits in mid-September, 2009. Each of the soil pits was a size of 20×20 cm^2 square to a depth of 30 cm. The planted larch forest is located inside Tsutamori Forest Park in Tomakomai, Hokkaido, Japan (48°39′N, 141°47′E, alt 25 m), established on volcanic lapilli/sand bed. The organic layer (O-horizon) and mineral soils (A- and B-horizons) were sampled in early June 2011. Official permission was obtained from the Park and Wildlife Finland, Metsähallitus, and permission to import the banned items (21Y637) to Japan was obtained from Minister of Agriculture, Forestry and Fisheries via the Sapporo Branch of Yokohama Plant Protection Station. The permission to obtain soil samples from the Tutamori Forest Park was obtained from Tomatoh Enterprise that was commissioned from Tomakomai City for the managements of vegetation and soils in the park.

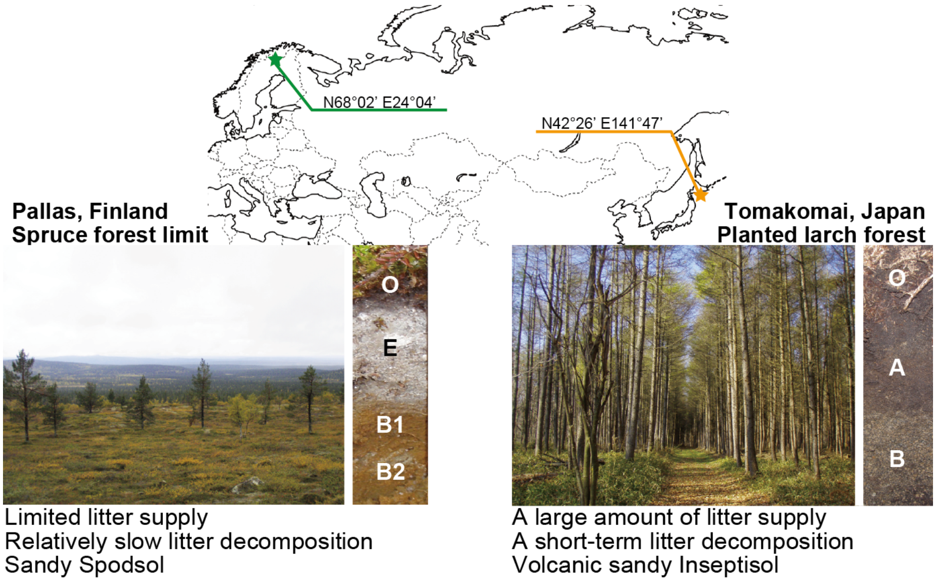


Figure 2.1 Sampling site and soil profiles of Pallas and Tomkomai

2.2.2. Measurement of water-soluble organic carbon in soil and soil pH

WSOC was measured using a 2 g portion of each soil sample mixed with 20 mL of MilliQ water and shaken for 1 h at 130 rpm. Extracted soil solution thus obtained was filtered through a 0.45 μm PTFE membrane (Millipore, Merck , Germany) and analyzed organic carbon using a TOC-5000A total organic carbon analyzer (Shimadzu, Kyoto, Japan), according to the method shown by Toma et al. (2010). Soil pH was measured using a 3 g portion of each soil sample mixed with 15 mL of deionized water and shaken by hand for 30 min, and the pH of the resulting soil suspension was subsequently measured using a Navi-H pH F-52 portable pH meter (Horiba, Kyoto, Japan).

2.2.3. Soil cultured medium and acetylene reduction assay

The nitrogen fixing potentials were evaluated by acetylene reduction assay (Hardy et al., 1968) using 300 mg portion of the soil that was inoculated to the medium and incubated at 15°C for 7 days. Winogradsky's nitrogen-poor mineral medium (less than 5 mg-N L^{-1}) was used for this acetylene reduction assay (Hara et al., 2009). In this medium, a mixed carbon source mimicking root exudate, containing D-fructose, D-glucose, sucrose, D-mannitol, succinic acid, and DL-malic acid at a molar ratio of 2:2:2:2:1:1, was used (Bürgmann et al., 2005). The medium was adjusted to pH 5.0 with a solution of 2 M H_2SO_4 close to approximate soil pH value (in water), and was solidified with 3 g L^{-1} gellan gum (Hashidoko et al., 2002). A set of soils from one soil cross-section were randomly selected among three of each site and cultured at different concentrations (0.02, 0.04, 0.075, 0.10, 0.25, 0.50, and 2.0%) of the mixed carbon source.

A 300 mg of soil from B1- and B2-horizons at Pallas, and A- and B-horizons at Tomakomai was each inoculated to a 10 mL of medium in a 30 mL gas chromatography vial with a headspace volume of 22.5 mL in triplicate. After a 7-day pre-incubation, a 2.3 mL volume of acetylene gas was injected into the headspace. After an additional 3-day incubation, a 50 μL of headspace gas was analyzed by a Hitachi G-5000 capillary gas chromatograph (Hitachi, Ltd., Tokyo, Japan) connected to a CP-PoraPLOT U 10-m glass capillary column (0.32 mm internal diameter; Chromapack, Middelburg, Netherlands). The analytical conditions of the gas chromatography were as follows: injection at 150°C, detection at 250°C using a flame ionization detector, column head pressure of 60 kPa, and column temperature of 50°C. Peak retention times of C_2H_4 and C_2H_2 generally observed were 0.90 min and 1.30 min, respectively.

2.2.4. DNA extraction from soil and cultured soil gel medium

DNA was extracted from the soil samples using ISOIL Large for Beads ver.2 (Nippon Gene, Toyama, Japan), with a 10-fold reduction in the scale of the standard protocol. A 0.5 g portion of each soil sample was subjected to the DNA extraction following the instruction of the DNA extraction kit. Total DNA in the cultured media was extracted using ISOIL for Beads Beating (Nippon Gene) using a 900 μl of cultured media. The resulting DNA samples from the soil samples and the cultured medium were quantified at 280 and 260 nm using a Gene Quant *Pro* RNA/DNA Calculator (Pharmacia, Uppsala, Sweden).

2.2.5. PCR-DGGE analysis for soil bacterial community cultured

Denaturing gradient gel electrophoresis (DGGE) was perform to determine the composition of the eubacterial and archaeal communities in soil samples and in the cultured medium. To characterize the eubacterial community in soil and culture inoculated with the soil, 16S ribosomal RNA (rRNA) V3-5 region was amplified using *341*-forward primer with a GC clump (*341_{gc}F*) (Yan et al., 2007) and *907*-reverse (*907R*) primer (Muyzer et al., 1993) by AmpliTaq GOLD 360

Master Mix (Applied Biosystems, Waltham, MA, USA). The cycling condition was as follows: 95°C for 10 min, 30 cycle of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, then one cycle of 72°C for 7 min. The resulting amplicons (570 bp) were subjected to DGGE analysis.

To characterize the archaeal community in soil and culture inoculated with the soil, 16S rRNA V4-5 region was amplified using nested PCR: 1st PCR with a primer pair of *arch23f* (5'-TGC GAY CTG GTY GAT YCT GCC-3′) (Burggraf et al., 1991) and *1492r* (5'-TAC GGY TAC CTT GTT ACG ACT T-3') (Lane, 1991), 2nd PCR with *arch519f* (5'-CAG CAG CCG CGG TAA TAC-3') (Øvreås et al., 1997) and *arch915R* primer with a GC clamp (*arch915gcR*) (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3') (Coolen et al., 2004). The cycling condition for 1st PCR was as follows: 95°C for 10 min, 30 cycle of 95°C for 30 sec, 60°C for 30 sec, 72°C for 1.5 min, then one cycle of 72°C for 7 min. The resulting amplicons (1.5 kbp) purified were subjected to 2nd PCR under the cycling condition as follows: 95°C for 10 min, 30 cycle of 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, then one cycle of 72°C for 7 min. Resulting amplicons of the 2nd PCR were subjected to DGGE analysis.

The DGGE analyses for the eubacteria and archaea were performed in 30-70% denaturing gradient gel using a DCode system (Bio-Rad, Hercules, CA, USA) at 60°C, 100V for 16 h (Kowalchuk et al., 1997; Tzeneva et al., 2008). The gel plate completed electrophoresis was dyed with SYBR Green I for 30 min and scanned by an image analyzer Typhoon 9400 (GE healthcare, Little Chalfont, UK).

2.2.6. Sequence analysis for DGGE bands

The major DGGE bands were excised with 200-μL pipette tip under a blue

LED light and then dissolved in 10 µL of sterile water. Each DNA band obtained from the DGGE gels for nucleotide sequencing was subjected to PCR with AmpliTaq GOLD 360 (Applied Biosystems) using the eubacterial universal primer pair *314F/901R* or the archaeal universal primer pair *arch519F/arch 915R* (5'-GTG CTC CCC CGC CAA TTC CT-3') eubacterial and archaeal 16S rRNA gene respectively at 25 cycles under the same reaction condition described above. The resulting amplicons were purified and then sequenced using a ABI Prism 310 genetic analyzer (Applied Biosystems) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). In case the initial sequencing step was unsuccessful to give multiple fragments, the PCR products were cloned in *E. coli* DH5α cells (Promega) using pGEM T-vector (Promega, WI, USA). Colonies possessing the target DNA-inserted plasmid were selected by blue/white colony assay on LB/ampicillin/X-Gal plates. Colony PCR was performed with a *T7* and *SP6* (5'-TAA TAC GAC TCA CTA TAG GG-3'/5'-TAT TTA GGT GAC ACT ATA G-3') primer pair under the following condition: 95°C for 10 min, 30 cycle of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, then one cycle of 72°C for 7 min. The resulting DNA sequence data were subjected to BLASTn searches against the DNA Database of Japan (DDBJ) and the National Center for Biotechnology Information (NCBI) database GenBank.

2.2.7. High-throughput sequencing for soil bacterial and archaeal community

Metagenomics analyses of the soil eubacterial and archaeal community structures were performed using a next-generation sequencing system, Ion Torrent Personal Genome Machine (Life Technologies, CA, USA). Bacterial 16S rRNA gene region was amplified using an Ion 16S Metagenomics Kit (Life Technologies) by primer sets V2-4-8 and V3-6, 7-9, covering the variable regions. PCR amplification was performed under the following condition: 95°C for 10 min, 25 cycle of 95°C for 30 sec, 55°C for 30 sec, 72°C for 20 sec, and then one cycle of 72°C for 7 min. The resulting amplicons were mixed in equal amount and the resulting mixture was purified by Agencourt AMPure XP (Beckman Coulter, CA, USA) according to the manufacturer's instruction. Archaeal 16S rRNA gene region was amplified by nested PCR: 1st PCR with the primer pair of *arch23F/1492r*, 2nd PCR with a primer pair of *519F/915R*. The PCR condition was same with the library preparation for DGGE. The resulting amplicon with *519F/915R* was purified by Agencourt AMPure XP (Beckman Coulter). All the samples were quantified using a Qubit dsDNA HS assay kit (Life Technologies).

The bacterial and archaeal 16S rRNA gene libraries were prepared using the Ion Plus Fragment Library Kit (Life Technologies) and barcoded with the IonXpress Barcode Adapters 1-16 Kit (Life Technologies) to distinguish four soil samples (B1 and B2 from Pallas and A and B from Tomakomai for bacteria and archaea respectively). The library and template were prepared according to the manufacturer's instructions. The adaptor ligated and nick-repaired libraries were amplified using following condition: 95°C for 5 min, 6 cycle of 95°C for 15 sec, 58°C for 15 sec, and 70°C for 1 min. The amplified libraries were qualified and quantified using an Agilent 2100 Bioanalyzer with High Sensitivity DNA Analysis Kits (Agilent Technologies, CA, USA). Each library was diluted to 26 pM and subjected to emulsion PCR to amplify sequencing templates onto Ion Sphere Particles (ISPs), using the Ion OneTouch 2 system (Life Technologies), and enriched with the Ion OneTouch ES (Life Technologies) using the Ion PGM

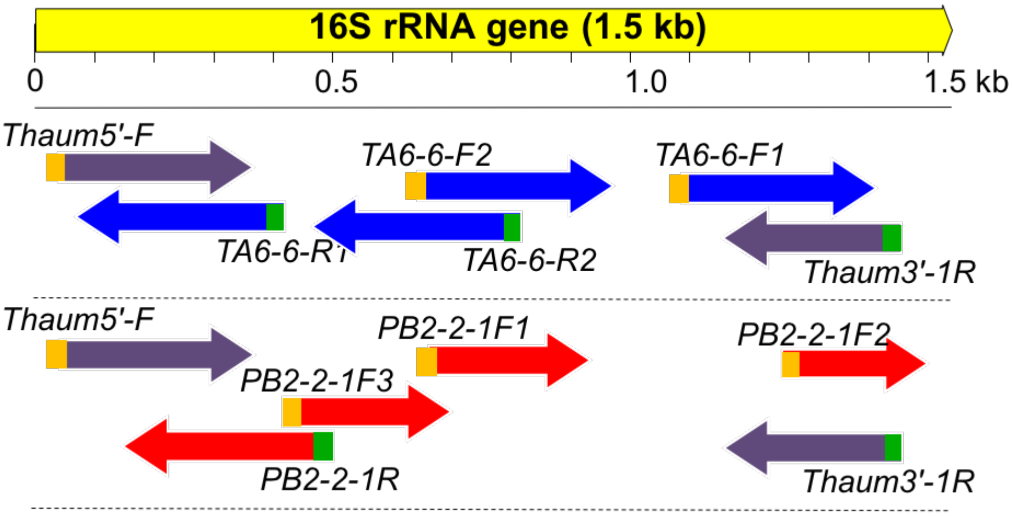
Template OT2 400 Kit (Life Technologies). The enriched ISPs were subjected to single-end sequencing using the Ion Torrent PGM system (Life Technologies) using the Ion PGM Sequencing 400 Kit (Life Technologies) and Ion 316 Chip v2 (Life Technologies) for 850 flows.

The bacterial sequences were processed for data analysis using the Ion Torrent platform-specific pipeline software, Ion Reporter 5.0 16S Metagenomics Workflow based on the reference of MicroSEQ 16S Reference Library v2013.1 (Life Technologies) and Greengenes v13.5 (The Greengene Database Consortium, http://greengene.secondgenome.com/). The sequences less than 10 copies were discarded. The archaeal sequences were processed for data analysis using Mothur software package v1.38.1 (Schloss et al., 2011). Each sequence was subjected to quality control and remove the low-quality sequences (sequence of <200 bp with average quality score of <25, ambiguous character, and homoprimer >8) and integrated resulting 12987 sequences (Huse et al., 2007).

The merged sequences were converged as 370~430 bp in length. The sequences were merged to a unique sequence resulting in total 10822 sequences, in which 7482 unique sequences were involved. These sequences were aligned with SILVA archaeal reference v119 (https://www.arb-silva.de/documentation/release-119/) (Pruesse et al., 2007). Non-specific sequences in the library were removed, then the overhangs at the both ends were trimmed to obtain 10290 sequences with 6775 unique sequences. The resulting sequences were subsequently subjected to pre-clustering in 3 differences in the sequence to converge the as 2477 unique sequences. These reads were then subjected to chimera-checking using the UCHIME algorithm (Edgar et al., 2011) to remove chimeric sequences. The resulting 1894 unique sequences in the total of 9424 sequences were classified using the Bayesian method with the SILVA reference v128 (https://www.arb-silva.de/documentation/release-128/). These unique sequences were pairwise aligned and the resultant distance matrix was clustered using the nearest-neighbor algorithm at 97% similarity (Schloss et al., 2011). Randomly selected 31 sequences were subjected to phylogenetic analysis using neighbor-joining method combined with the sequences obtained from DGGE analysis using MEGA with a bootstrap test of 1000 replications.

2.2.8. Phylogenetic analysis of archaeal community in soil and cultured soil

The DNA sequences obtained from DGGE were aligned using MEGA v7.0.16. Without gap removal, the phylogenetic analysis of the archaeal DGGE bands (positions from 527 to 760) was conducted using ClustalW with 1,000 bootstrap replicates. A novel universal primer pair, *Thaum5′-F* (5'-CGG ACC YGA CTG CTA TCR GA-3) and *Thaum3′-1R* (5'-GGT TCC CCT ACG ACT ACC TTG-3') (positions 22-41 and 1442-1462 from 16S rRNA gene of *Methanocaldococcus fervens* AG86^{T}), newly designed in this study for archaea of phylum *Thaumarchaeota* using Primer3 Input version 0.4.0 (Untergasser et al., 2012). To identify the major archaeon in the mineral soils of Pallas and Tomakomai, the major 16S rRNA genes in DNA recovered from the soil and the cultures were further amplified using combination of the thaumarchaeal primer pair (Fig. 2.2). The resulting amplicon was cloned in the same way described in 2.2.5.



primer	sequence	position
Thaum5'-F	5'-CGGACCYGACTGCTATCRGA-3'	22-41
Thaum3'-1R	5'-GGTTCCCCTACGACTACCTTG-3'	1422-1462
TB6-6-R1	5'-TTAGCGGAAATCACTCAGAGT-3'	385-405
TB6-6-F1	5'-CTAGTTGCTACTGCTGTTCTCA-3'	1064-1085
TB6-6-R2	5'-CCTGCGGCACTGGGCTGGCACAAG-3'	791-811
TB6-6-F2	5'-AGGGGTAAAATCCTTTGATCCATT-3'	623-655
PB2-2-1-F1	5'-TGGTGGTCCTCTATGGAT-3'	614-631
PB2-2-1-R	5'-TGGGCTGCCGGAGATACTACAA-3'	470-491
PB2-2-1-F2	5'-AGAGGCAATCCCTAAATGCTA-3'	1223-1243
PB2-2-1-F3	5'-GGTAATACCAGCACCTCAGA-3'	383-402

Figure 2.2 Primers used for archaeal 16S ribosomal RNA gene-targeted PCR-DGGE analysis and for full sequence determination of the region.

2.2.9. Phylogenetic analysis of archaeal *amoA* gene and amino acid sequences

Archaeal *amoA* gene-targeted PCR-DGGE was performed with the amplicons of a primer pair of *CrenamoA23*$_{gc}$*f* (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAT GGT CTG GCT WAG ACG-3') and *CrenamoA616r* (5'-GCC ATC CAT CTG TAT GTC CA-3') using DNA recovered from soil and cultured soil under the condition used from the 16S rRNA genes. The major DGGE bands were excised and dissolved in 10 µL sterile water.

Each DNA band obtained from the DGGE gels was subjected to PCR using a primer pair of *CrenamoA23f* (5'-ATG GTC TGG CTW AGA CG-3') and *CrenamoA616r* (5'-ATG GTC TGG CTW AGA CG-3') under the condition previously described and then sequenced. The nucleotide sequences obtained were converted to amino acid sequences for ammonia monooxygenase subunit A. Using the *amoA* gene and its amino acid sequence from *Nitrososphaera vienensis* EN76 genomic DNA as a positional standard (Tourna et al., 2011), all the sequences were clustered using MEGA v7.0.16 for both nucleotide and amino acid sequences respectively, along with some reference sequences.

2.3. Results

2.3.1. Soil properties

As shown in Table 2.1, E-soil from Pallas was unexpectedly rich in WSOC, while the B-soil contained only one-third of the WSOC found in E-horizon soil. A- and B-soils of the larch forest bed in Tomakomai contained relatively high concentration of WSOC. The pH of Pallas soils (E and B) was relatively low (pH 4.9-5.2 in A soil, pH 5.6-6.6 in A soil), likely owing to the prevision of organic acids by leaf litter from the O-horizon to E-horizon soil.

Table 2.1 Soil characteristics

	Horizon	Depth (cm)	WSOC (mg kg^{-1})	pH (H_2O)
Pallas (Spodosol, subarctic)	O	−2 ~ 0	n.d	4.13
	E	0 ~ 5	32.3	4.93
	B1*	5 ~ 10	10.3	5.12
	B2*	10 ~	10.6	5.20
Tomakomai (Inceptisol, volcanic)	O	−5 ~ 0	n.d	5.53
	A*	0 ~ 20	43.3	5.64
	B*	20 ~	16.9	6.58

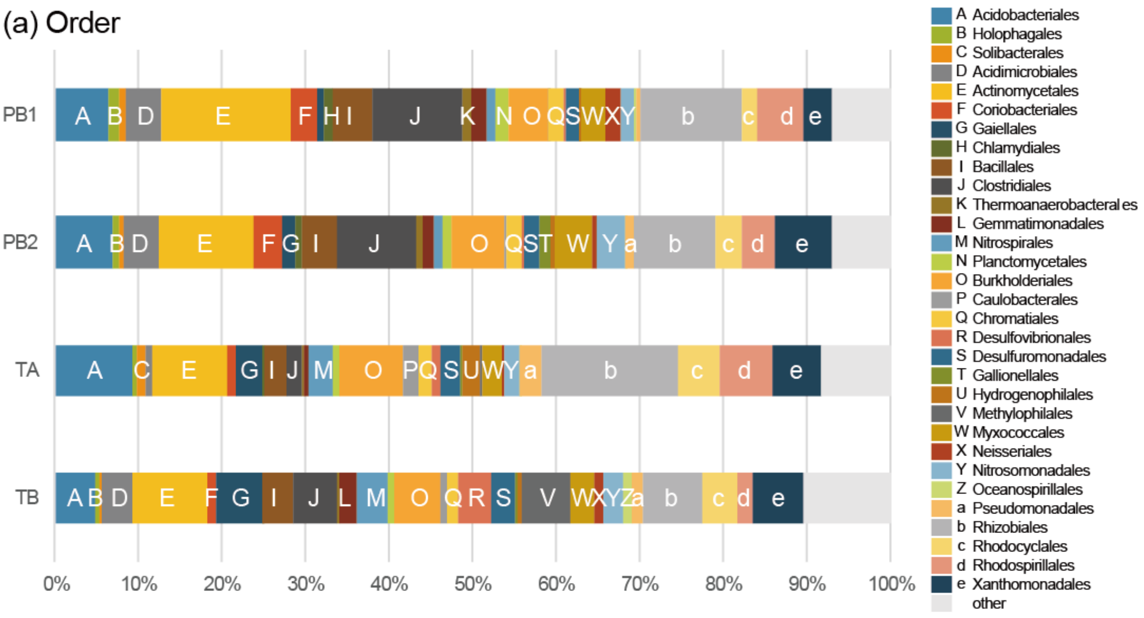
The soil horizons with star* were analyzed in this study. n.d.:no data

2.3.2. Eubacterial community structures of Pallas and Tomakomai forest bed soils analyzed by high-throughput sequencing

Euacterial community structure in soil samples were analyzed by 16S rRNA gene amplicon sequencing under high-throughput sequencing platform. Mapped reads obtained from Ion Torrent PGM was 520624 in total. The eubacterial community in the phylum level was occupied by *Protepbacteria* (50%) followed by *Actinobacteria* (20%), *Firmicutes* (12%), and *Acidobacteria* (9%). In the order level, the community of B soils of Pallas and Tomakomai showed characteristic components, respectively (Fig. 2.3a). At the family level, the eubacterial communities in the B1 and B2 soils from Pallas site showed *Pseudocaldiaceae* (E) and *Peptococcaceae* (K) as major community members, and *Corynebacteriaceae* (D) and *Coriobacteriaceae* (G) were uniquely apparent in the communities. On the other hand, the eubacterial communities in the B soils from Tomakomai site was rich in *Gaiellaceae* (H) and *Methylophilaceae* (U), the latter of which was found as unique community member in this site together with *Sphingosporangiaceae* (F) (Fig. 2.3b).

Potential nitrogen-fixing eubacterial communities were extracted from the metagenomics sequencing result at the order and family level. Similar compositional patterns were obtained from both B-soil of Tomakomai site and B1-and B2-soil of Pallas site (Fig. 2.4). Among the potential nitrogen-fixers, order *Clostridiales* (main family members were *Peptococcaceae*), order *Burkholderiales* (main family members were *Oxalobacteriaceae* and *Burkholderiaceae*), order *Rhizobiales* (main family members were *Bradyrhizobiaceae*), and order *Bacillales* (main family members were *Paenibaciaceae*) occupied relatively large proportions in the community structure. The community structure of A-soil of Tomakomai site showed slightly different from that of B-soils, family *Mollaxellaceae* of order *Pseudomonadales* and family *Comamonadaceae* of order *Burkholderiales* were unique but major community member. Clear difference among the eubacterial communities of nitrogen-fixers from the B-soils of two sites was their proportions: those of Pallas B-soils occupies 23-24% of the soil eubacterial proportion, while only 15% in that of Tomakomai B-soil.

(a) Order



(b) Family

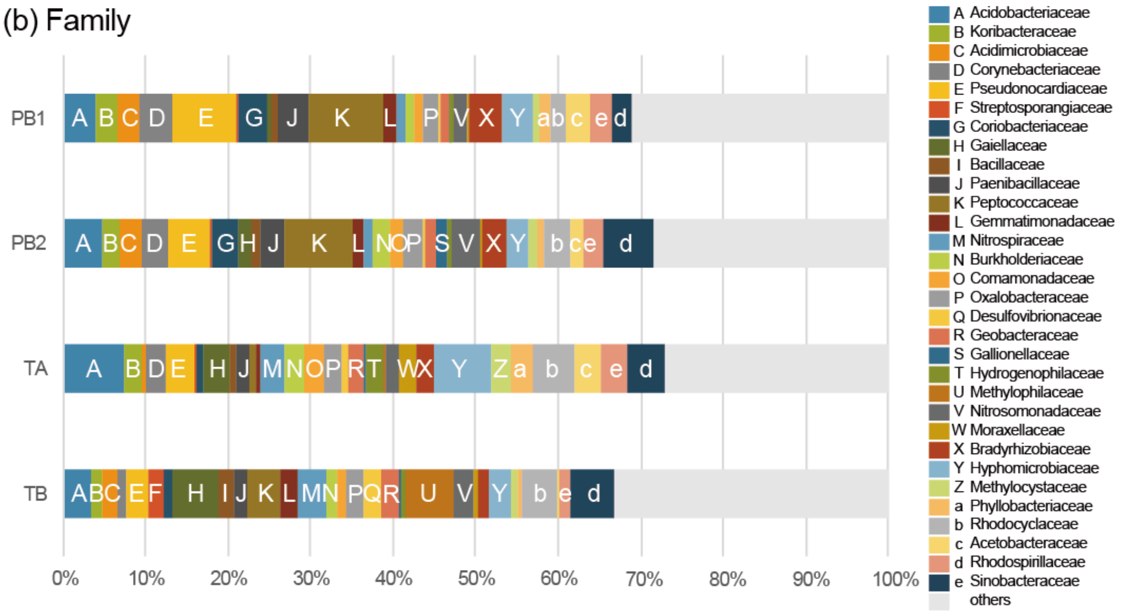


Figure 2.3 High-throughput analysis of eubacterial 16S rRNA gene fragment of Pallas and Tomakomai soils.

Eubacterial 16S rRNA gene fragment were sequenced using Ion Torrent PGM system. (a)order level and (b) family level Abbreviations: PB1, Pallas B1; PB2, Pallas B2; TA, Tomakomai A; TB, Tomakomai B.

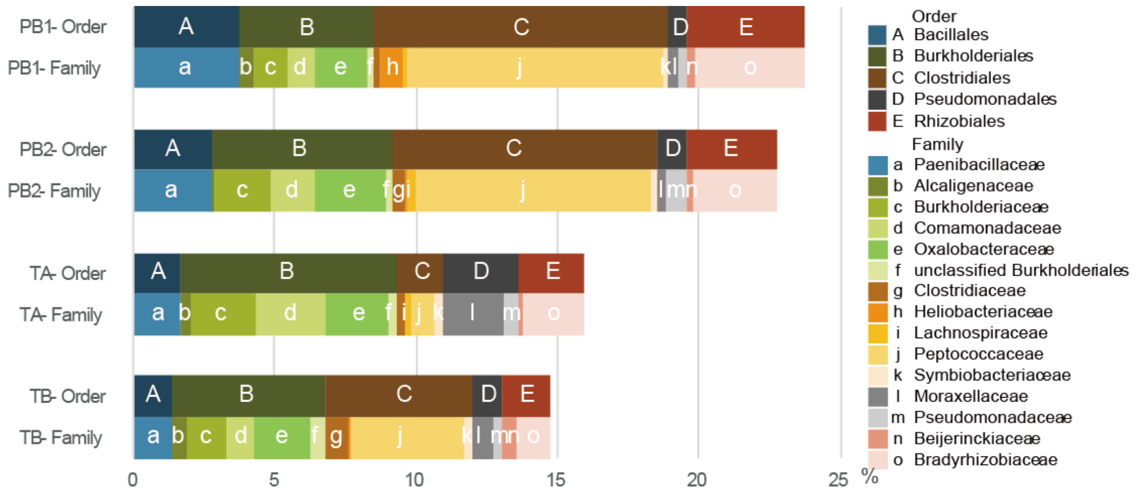


Figure 2.4 Estimated nitrogen fixing communities in Pallas and Tomakomai soils

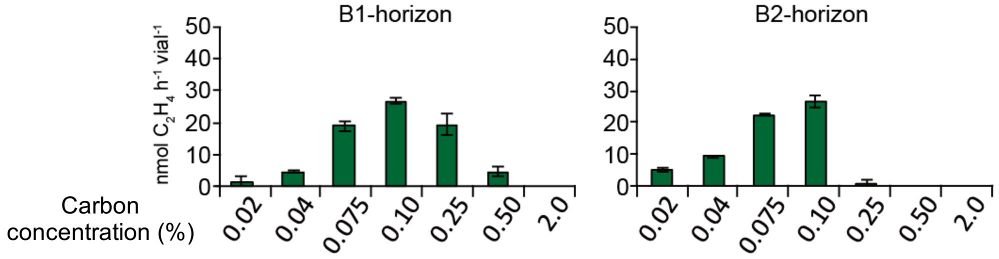
Potential nitrogen fixing communities were extracted from eubacterial community. Abbreviations: PB1, Pallas B1; PB2, Pallas B2; TA, Tomakomai A; TB, Tomakomai B.

2.3.3. Soil culturing in nitrogen-poor gellan gum media and acetylene reduction

The culture soil of B-soil form Pallas site showed a remarkable level of acetylene reduction greater then 19 nmol C_2H_4 h^{-1} $vial^{-1}$ in 0.075, 0.10, and 0.25% mixed carbon medium, while it was less than 5.0 nmol C_2H_4 h^{-1} $vial^{-1}$ in 1.0 and 0.04% carbon source medium (Fig. 2.5, horizon bar charts of the top two). In B2-soil from Pallas site, a maximum of 27 nmol C_2H_4 h^{-1} $vial^{-1}$ was detected from the culture with 0.1% carbon source medium. At carbon concentrations of 0.1% or less, acetylene reduction by soil microbiota from both B1- and B2-soils was significantly higher than that of microbiota cultured in 0.5 to 2.0% mixed carbon medium.

In contract to the B-soils in Pallas site, the A-soil from Tomakomai showed the highest acetylene reduction activity of 103 nmol C_2H_4 h^{-1} $vial^{-1}$ at 0.5% mixed carbon medium (Fig. 2.5, two bar charts at the bottom).

(a) Pallas



(b) Tomakomai

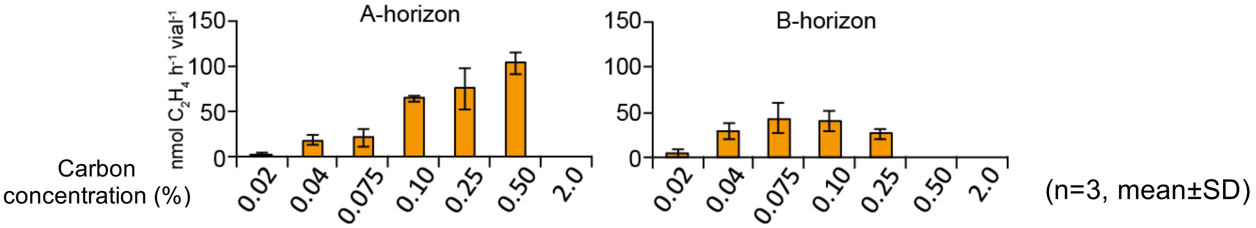


Figure 2.5 Acetylene reduction of cultured soils in different carbon source concentrations.

The acetylene reduction assay was carried out in triplicate in gellan gum media of Winogradsky's mineral solution at pH 5.0 containing a carbon source mixture at concentration of 0.02, 0.04, 0.075, 0.025, 0.50, or 2.0%. Pallas B1, Pallas B2, Tomakomai A, Tomakomai B-horizon soils were inoculated to the medium and incubated for 7 days, then assayed for acetylene reduction (bar, ± standard deviation).

2.3.3. Archaeal community structure of Pallas and Tomakomai forest bed soils analyzed by high-throughput sequencing

The high-throughput short-read sequencing analysis of archaeal 16S rRNA gene fragment revealed high abundance of *Thaumarchaeota* in archaeal community structures (Fig. 2.6, Fig. 2.7). The thaumarchaeal groups were consisted of 3 clusters; FHMa11 terrestrial group, SAGMCG-1 (South Africa gold mine group 1), and SCG (soil crenarchaeotic group), all of which are a major group in phylum *Thaumarchaeota,* except for the marine group (MG). Soil archaea of the FHMa11 terrestrial group were one of the most abundant groups of soil archaea in B1- and B2-soils of Pallas. The mesophilic thaumarhcaeal group of SAGMCG-1 was dominant *Thaumarchaeota* in both A- and B-soils from

Tomakomai site. The A-soil of Tomakomai showed unique archaeal community structure as that highly abundance of SAGMCG-1 and SGC. Notably, SAGMCG-1 accounted for approximately 50% of archaeal community in the A-soil. The neighbor-joining phylogenetic tree of the soil archaea in the high-throughput sequencing data showed that archaeal members of these groups are highly adapted to organic carbon-poor conditions.

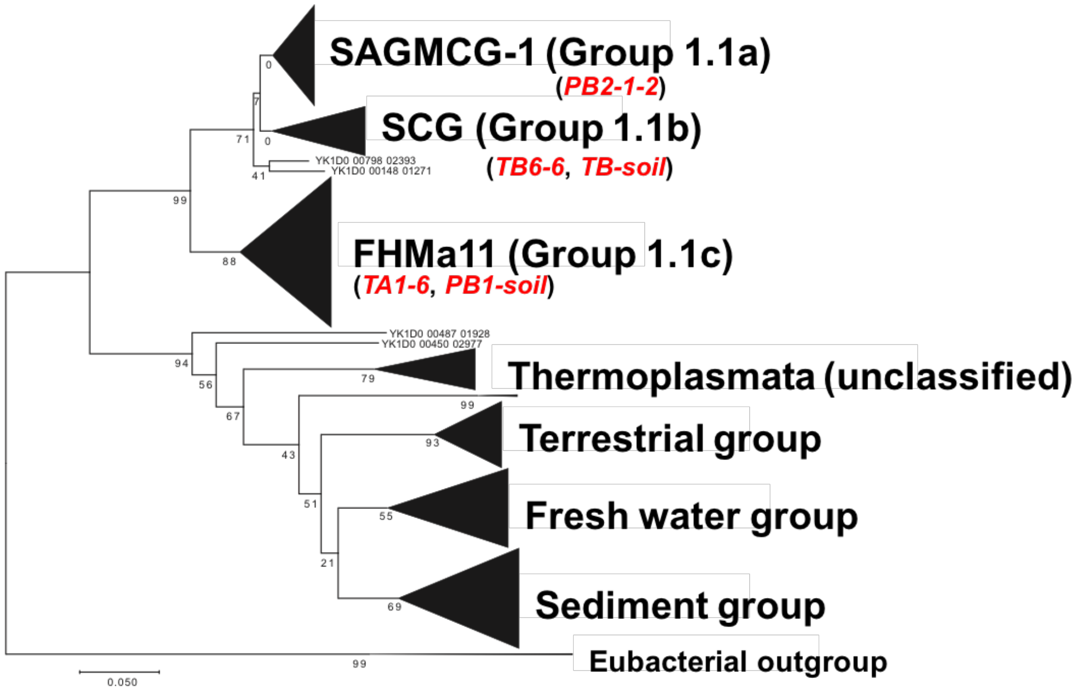


Figure 2.6 Phylogenetic position of archaeal 16S rRNA gene sequence obtained from high-throughput short read sequences.

Archaeal 16S rRNA gene (400 bp) were sequenced by Ion Torrent PGM system. The Mothur software package was used for data processing. The resulting unique sequences were aligned in all pairwise combination in MEGA7, and the distance matrix was clustered using neighbor-joining with 1000 bootstrap replications. *S. griseus* and *E.coli* were used for the bacterial outgroup. Red letters were the sequences obtained from DGGE bands.

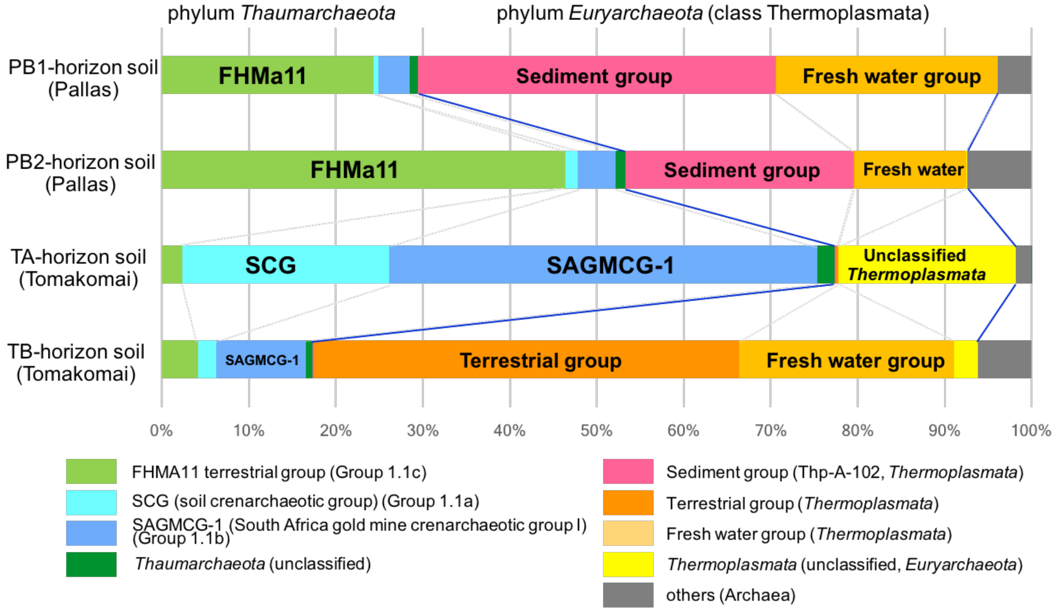


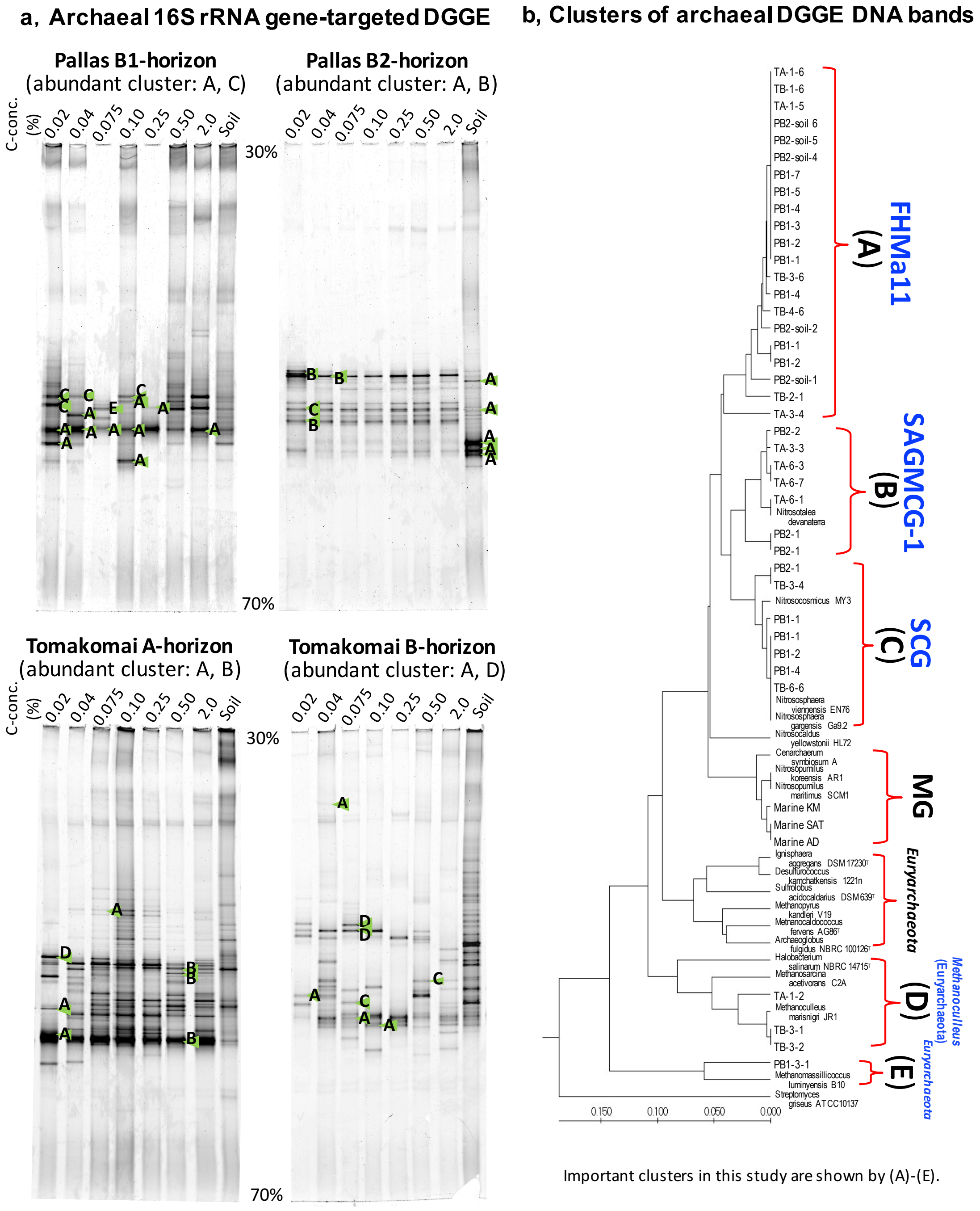
Figure 2.7 High-throughput analysis of archaeal 16S rRNA gene fragments from Pallas and Tomakomai mineral soils.

Archaeal 16S rRNA gene (400 bp) were sequenced by Ion Torrent PGM system. The Mothur software package was used for data processing. Sequences longer than 370 bp with quality score>20 without Ns were classified using SILVA reference. Blue solid lines show the boundary between phylum Thaumarchaeota and class Thermoplasmata of phylum Euryarchaeota, or Thermoplasmata and other archaea.

2.3.4. Archaeal community structure and phylogenetic analysis by DGGE

From archaeal 16S rRNA gene-targeted DGGE, 39 band sequences were obtained (Fig. 2.8a). These sequences were clustered in to 6 groups: FHMa11 cluster, SAGMCG-1 cluster, SCG cluster, class *Methanoculleus* cluster, and unclassified *Euryarchaeota* cluster (Fig. 2.8b). The FHMa11 cluster was the largest group among obtained sequences, and FHMa11 is group I.1c *Thaumarchaeota* which was found mainly from boreal forest soil. The cluster SAGMCG-1 cluster was abundant in the soil microbiota of B2-soil from Pallas site and those of A-soil from Tomakomai site, and SAGMCG-1 is I.1a associate *Thaumarchaeota* which include *Nitrosotalea* group harboring ammonia oxidizing activity. Conversely, the cluster SCG cluster was dominated by B1-soil form Pallas site, and SCG is I.1b *Thaumarchaeota* including Nitrososphaera group also harboring ammonia oxidizing activity.

PB2-1-2 amplicon (1,205 bp, at position 88-1295) obtained from culture of B2-soil from Pallas showed 96% homology with an obligate acidophilic ammonia-oxidizer '*Candidatus* Nitrosotales devanaterra' that was found in agricultural soils in Scotland (Lehtovirta-Morley et al., 2011). In contrast, the 1,494-bp sequences (positions 16-1524) of *TB-soil* from Tomakomai B-soil showed 94% homology with a mesophilic AOA, '*Candidatus* Nitrososphaera gargensis' (Spang et al., 2012). The 1429-bp sequences of *TB6-6* (positions 17-1464) obtained from 0.5%-carbon source containing soft gel medium inoculated with the Tomakomai B-soil was also clustered in SCG, previously recognized as Nitrososphaera cluster (group 1.1 b). Conversely, 1429-bp sequences of *PB1-soil* (position 32-1503) from Pallas B1-soil DNA matched with known AOAs as less than 85% homology. 748-bp sequences of *TA1-6* (positions 84-813) from culture of Toamkomai A-soil was also showed a low homology with known AOAs. These 5 sequences were subjected to phylogenetic analysis with some reference sequences (Fig. 2.9). Subsequently, these longer sequences were combined with the high-throughput short-read sequencing data of archaeal 16S rRNA gene showed *PB2-1-2* is clustered in SAGMCG-1, whereas *TB6-6* and *TB-soil* were identified as members of SCG. *PB1-soil* and *TA1-6* were uniquely characterized as soil archaeal clones of group FHMa11 abundant in *B-soil* of Pallas forest soil (Fig. 2.7, shown in red letters).



Important clusters in this study are shown by (A)-(E).

Figure 2.8 Archaeal 16S rRNA gene-targeted DGGE profiles of Pallas and Tomakomai

(a)Archaeal 16S rRNA gene region were amplified from each DNA extracted from cultured soils and intact soils. Each lane represents medium carbon concentration. DGGE was performed on 30%-70% gradient gel. The bands with same identification were described with same alphabet (A, B, C, D, E). (b)Calculation for the phylogenetic analysis were performed using the neighbor-joining method with 1000 bootstrap replications.

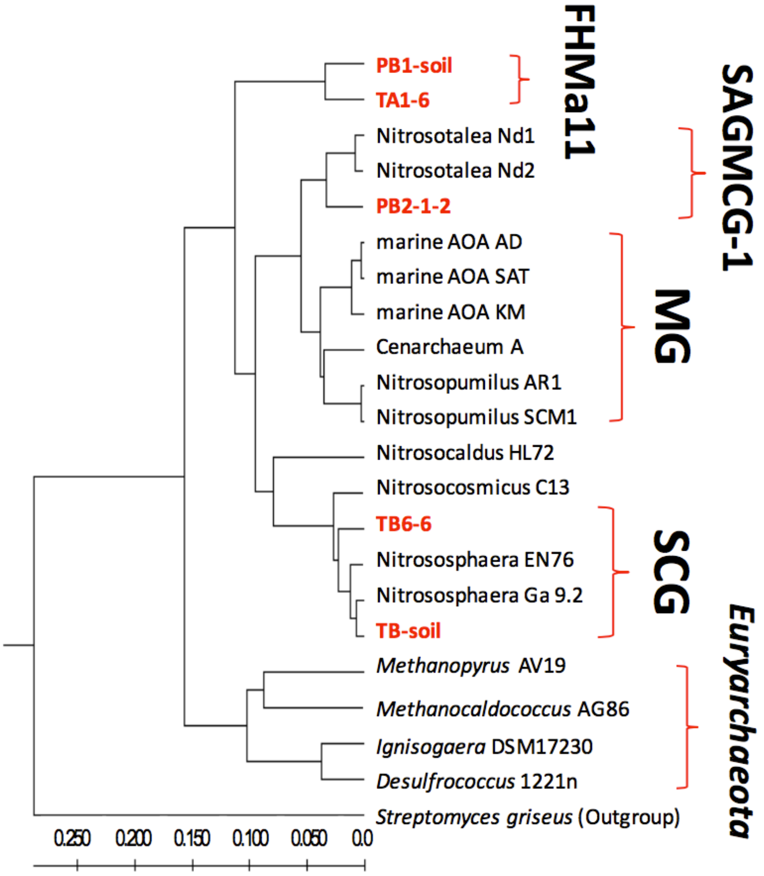
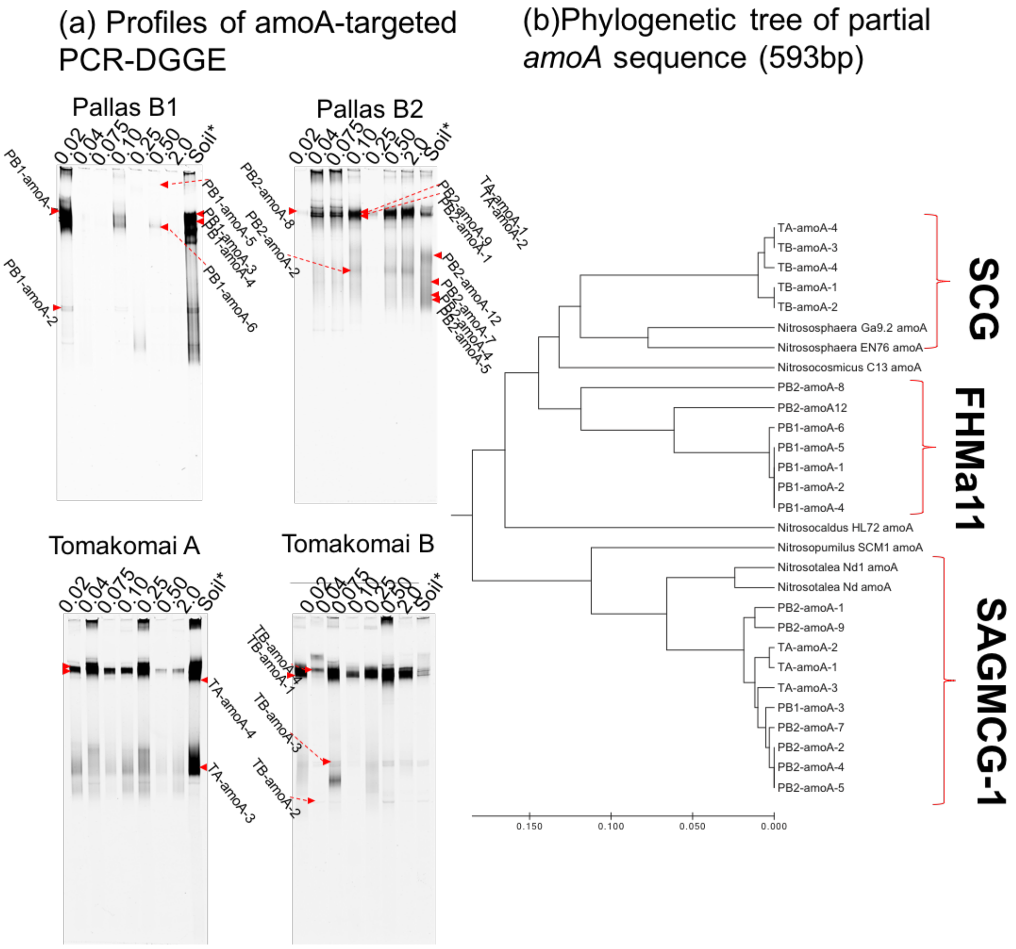


Figure 2.9 Phylogeny of longer 16S rRNA gene sequences from Pallas and Tomakomai soils.

All of the clones obtained as longer 16S rRNA gene sequences were grouped in the member of phylum Thaumarhcaeota. For the phylogenetic analysis, the length and regions of the nucleotide sequence (760 pb) were adjusted to that of *M. fervens* AG85^{T} at positions 84-813, without gap removal. *Streptomyces griseus* ATCC 10137was used for the outgroup.

2.3.5. Phylogenetic analysis of archaeal 16S rRNA gene and *amoA* gene

As mesophilic AOAs generally harbored *amoA* gene, the archaeal *amoA*-targeted-DGGE analysis was performed (Fig. 2.10a). Archaeal *amoA* diversity was relatively low; however, the band intensities were higher than those of archaeal 16S rRNA DGGE profiles. The *amoA* gene sequence from DGGE were clustered in to 3 groups (Fig. 2.10b). A set of 22 nucleotide sequences obtained from DGGE were converted into amino acid sequences for the ammonia monooxygenase subunit A. The resulting amino acid sequences (position at 69-128, 60 amino acids) were aligned with some reference sequences, which was highly conserved (Fig. 2.11). The phylogenetic tree of amino acid sequences showed 3 clusters respectively: FHMa11, SAGMCG-1, and SCG (Fig. 2.12).


(a) Profiles of amoA-targeted PCR-DGGE

(b)Phylogenetic tree of partial *amoA* sequence (593bp)

Figure 2.10 DGGE profile of the partial *amoA* gene for each soil and soil cultures and phylogeny of *amoA* gene sequences

(a)Archaeal *amoA* gene region were amplified from each DNA extracted from cultured soils and intact soils. Each lane represents medium carbon concentration. DGGE was performed on 30%-50% gradient gel. (b)Calculation for the phylogenetic analysis were performed using the neighbor-joining method with 1000 bootstrap replications.

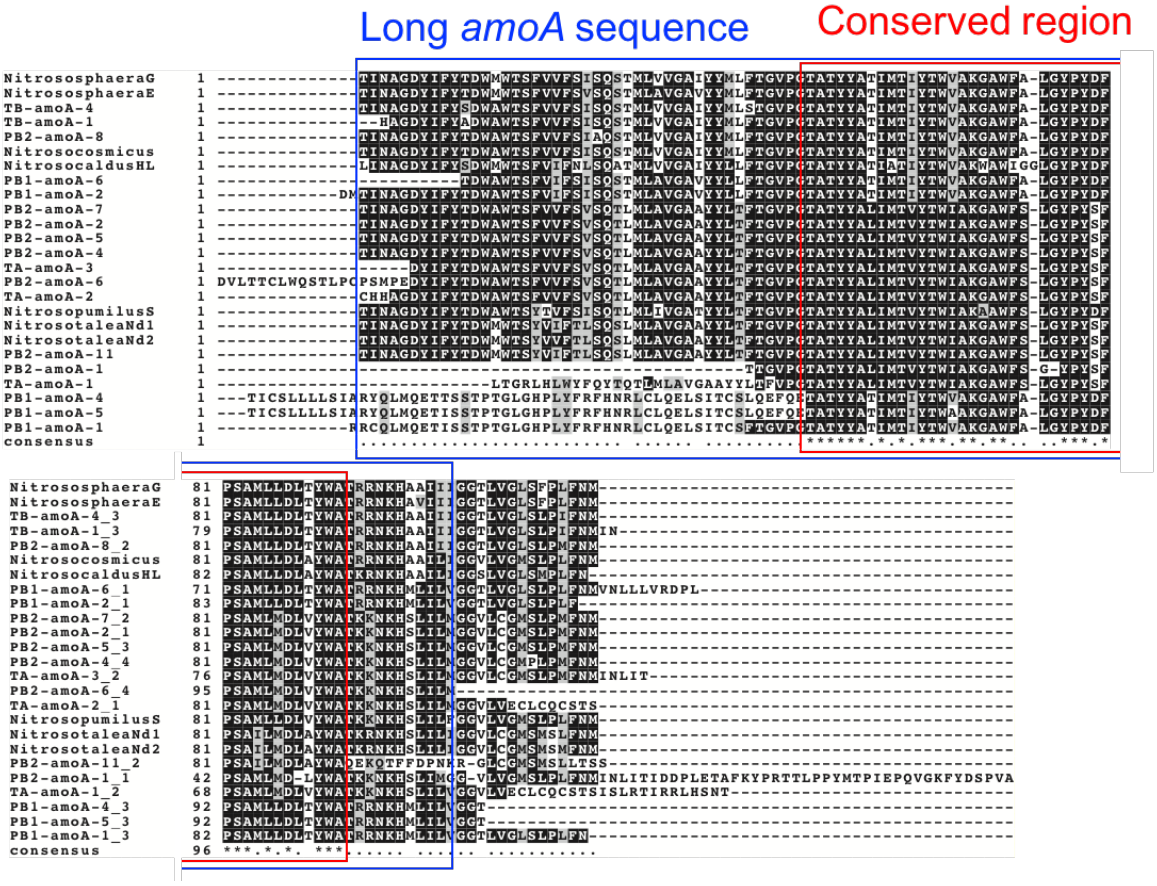


Figure 2.11 Partial amino acid sequence alignments of archaeal ammonia monooxygenase.

Sequence region of *amoA* protein (120 amino acid) were aligned. 60 amino acid highlighted with red solid line were highly conserved among *amoA* sequence obtained from PCR-DGGE.

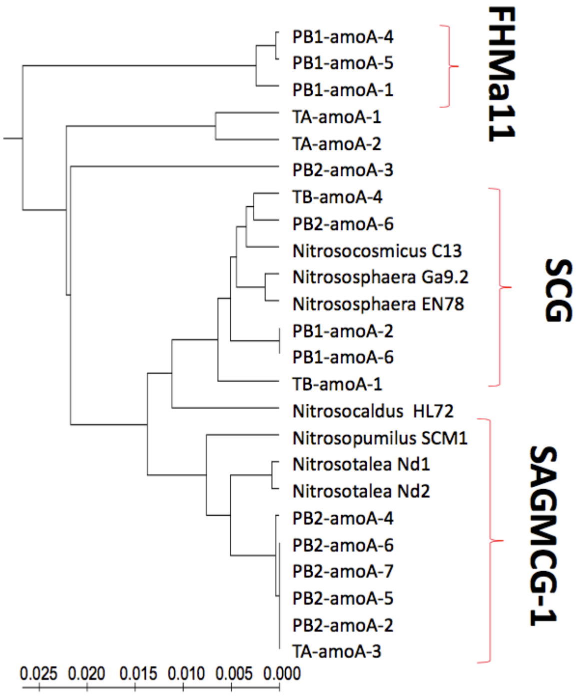


Figure 2.12 Phylogeny of amino acid sequence of *amoA*.

Calculation for the phylogenetic analysis were performed using the neighbor-joining method with 1000 bootstrap replications.

2.4. Discussion

The composition of bacterial communities was investigated using high-throughput sequencing analysis. Both the B-soils from Pallas open spruce forest with poor in organic carbon supply and B-soil from Tomakomai planted larch forest showed similar community composition of estimated nitrogen-fixers, while A-soil from Tomakomai showed clearly different profiles. This suggested that a large amount of WSOC was supplied from thick leaf litters during summer season, and hence nitrogen fixers are highly affected its composition to show high nitrogen-fixing potentials.

It is indicated that much of the WSOC provided to deeper mineral soils from O-horizon, from the acceleration of litter decomposition by increased temperature in summer, affected microbial community balance via the proliferation of not only functional nitrogen fixers, but also of eubacterial that interfere with nitrogen fixation.

The accelerated litter decomposition in soils would also accelerate C-N imbalance, leading to potent nitrogen fixation in the mineral soil (Wild et al., 2014). Thus, the possibility of spikes in nitrogen fixation is a major concern to give high impacts to the soils ecosystems (Han et al., 2013). Further investigations are required to determine the contributions of increased WSOC supply and the activation of WSOC-responsive nitrogen fixers to the predicted changes in nitrogen cycles in the soil.

The comparison of the *amoA* genes can serve as a clear indicator of the unique dynamics of AOAs at different sites and depth of mineral soils, reflecting the overall local soil conditions. The mesophilic AOAs in soil might relay on nitrogen cycles in the terrestrial ecosystems (Hernández et al., 2014). Such mesophilic AOAs associated with a nitrogen cycle could help to uncover the "missing link of nitrogen in boreal forest ecosystems".

Chapter 3

Relation Between Vegetation and Soil Microbial Community Structure in Subarctic Tundra in Northern Sweden

3.1. Background

Tundra landscape exhibits a large topological relation with vegetation and soil properties across short distance. Sometimes within meters of distance, the tundra landscape can have large effects on above- and belowground properties in arctic tundra. Such differences are expressed aboveground in plant community composition and diversity and in belowground properties such as nutrient cycling, soil chemical properties and functional differences in the microbial community such as the population ratio of fungi and bacteria in soil. For example, the relationship of vegetative distribution and soil properties has been reported that there are wide range of pH in the valley from 3.4-8.0, which is closely related with the vegetation types in Kärkevagge, located in northern Sweden (Darmody et al., 2004). However, there are only few studies that have detailed how this is reflected in the microbial community structure and how it relates to the heterogeneity in above- and belowground properties across the tundra (Sundqvist et al., 2014), and these reports are focused only limited vegetation, not to cover all the tundra regions.

Kärkevagge is a 5-km-long U-shaped glacial valley located in approximately 200 km north of the Arctic Circle in northern Sweden, where numbers of different vegetation types can be found that are separated mainly by their topographic position in the landscape (Fig. 3.1). There are five major vegetation types in the valley, which represent Scandinavian alpine tundra plant communities. Along the hill slopes, meadow type vegetation is dominating named mesic meadow (MM). In slope areas with a stronger calcareous influence *Dryas* heath (DH) can be found. On ridges and elevated sites along the slopes, *Empetrum*-heath named mesic heath (MH) is found. The valley also contains numbers of large boulders with isolated plant communities of *Empetrum*–heath named boulder heath (BH). At the bottom of the valley, grassland (GL) is found. The landscape includes a range of vegetation types with functionally very different plant communities and different soil properties. In a small area, the controlling factors of plant community structure are mainly moisture content and temperature, both of which are related with snow depth and duration of snow cover (Djukic et al., 2010).

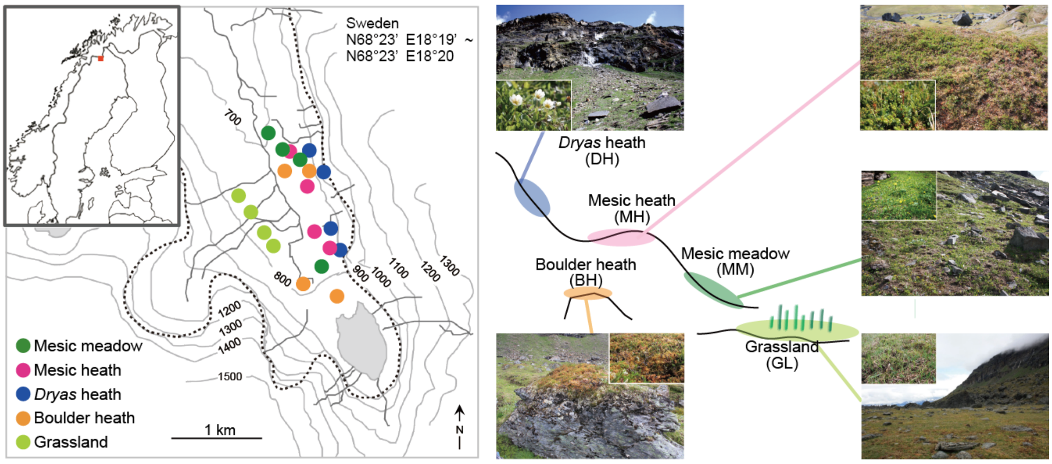


Figure 3.1 Location of sampling site and topological features in Kärkevagge valley, Sweden.

As described in general introduction, available nitrogen is limited in the subarctic/alpine tundra due to retarded organic matter decomposition. Cyanobacteria-associated nitrogen fixation is one of the nitrogen source in tundra ecosystems (Rousk et al., 2015). In addition, the geologic nitrogen, nitrate stored in the bedrock, might be one of the nitrogen source especially in Arctic and alpine ecosystems (Dixon et al., 2012). However, total potentials of nitrogen suppliers are relatively low, hence nitrogen source in arctic tundra remains mystery.

The aims of this study are to understand how this small-scale variation reflects the bacterial community structures across different vegetation type in subarctic tundra landscape. Moreover, nitrogen-fixing potentials were evaluated by culturing method using soil-mimicking gellan gum medium.

3.2. Materials and Methods

3.2.1. Site description and soil sampling

The soil samples were collected from Kärkevagge located in northern Sweden (N68°23′ E18°19′). Major vegetation types are mesic meadow (MM), mesic heath (MH), *Dryas* heath (DH), boulder heath (BH), and grassland (GL). *Dryas octopetala* is major plant species in *Dryas* heath, located near calcareous bed rock is exposed. Boulder heath which is typical heath type vegetation on the boulders isolated from surrounding vegetation. Grassland is located bottom of the valley distributed forbs and graminoid. The location of each plot is shown in Fig. 3.1.

Soil samples were collected in late July in 2015 from four replicated patches of each vegetation type. In each patch, humus soils were sampled with five representative locations using 10 cm-diameter stainless core and then homogenized together with removing leaf litter and root using 4 mm mesh.

Permission to import the banned item (27Y581) to Japan was obtained from Minister of Agriculture, Forestry and Fisheries via the Sapporo Branch of

Yokohama Plant Protection Station.

3.2.2. Soil properties

Following soil properties were determined based on the method in Sundqvist et al., 2011: pH, water content, NO_3-N and NH_4-N. Soil pH was determined using soil suspension with 6 g of homogenized soil into 50 mL MilliQ water after shaking overnight at 200 rpm. The shaken sample were stand for 1 h before measurement. Water content was measured gravimetrically by 70°C for 3 days. To determine the concentration of NO_3-N and NH_4-N, 5 g fresh weight soil was extracted in 80 mL 1 M KCl with in 24 h after sampling.

3.2.3. Soil cultured medium, Acetylene reduction assay and DNA extraction

Nitrogen-fixing potential was evaluated using acetylene reduction assay combined with culture method. A 300-mg portion of soil was inoculated to the 10 mL Winogradsky's nitrogen poor medium with 0.5% mixed carbon source adjusted at pH 5.0 solidified with 3 g L^{-1} of gellan gum as described in 2.2.7. with 10 replications for each soil from 5 plots with 4 replicates. After 1 week of pre-incubation at 15°C, 2.3 mL volume of acetylene gas was injected into the headspace. A 50 µL of headspace gas was analyzed after 3-day additional incubation following same analytical condition written in 2.2.7.

DNA was extracted from each homogenized soil sample using ISOIL Large for Beads ver.2 (Nippon Gene), with a 10-fold reduction in the scale of standard protocol. A 0.5 g portion of each soil sample was subjected to the DNA extraction just after sampling. DNA was also recovered from cultured medium using for acetylene reduction assay with ISOIL for Beads Beating (Nippon Gene) following the same protocol described in 2.2.3. Both DNA recovered from the homogenized soil and cultured medium were quantified.

3.2.4. Short read sequencing of bacterial 16S rRNA gene on the Ion Torrent platform and sequence analysis

The metagenomics analysis of soil bacterial community structure was performed using next-generation sequencing system, Ion Torrent PGM (Life Technologies). Bacterial 16S rRNA gene region were amplified using the Ion 16S Metagenomics Kit (Life Technologies) using a primer pair of V2-4-8 and V3-6, 7-9 under the same PCR condition in 2.2.6. The library and template preparation were conducted under the same condition described in 2.2.6.

3.3. Results

3.3.1. Soil properties

All environmental factor except NO_3-N concentration varied between vegetation types (Table. 3.1). The soil pH was ranged between 4.7 to 7.0. DH showed significantly high pH at 6.9 in average of 4 replicated plots, while BH showed significantly low pH at 4.9 in average. DH and MM showed relatively high concentration of NH_4-N. DH showed relatively low water content.

Table 3.1 Soil properties in Kärkevagge valley.

	pH	Water content ($g\ H_2O/g$ soil)	%Corg	%N	C/N	NO_3-N (mg/L)	NH_4-N (mg/L)
F-value	25.4^{***}	4.3^{*}	9.9^{***}	2.6^{NS}	3.1^{*}	2.2^{NS}	27.7^{***}
DH	6.9 ± 0.1^{b}	0.5 ± 0.0^{e}	20.9 ± 3.3^{a}	1.1 ± 0.2^{b}	20.2 ± 2.4^{a}	0.0 ± 0.0	1.4 ± 0.5^{b}
MM	6.4 ± 0.4^{a}	0.6 ± 0.0^{d}	21.5 ± 2.3^{a}	1.6 ± 0.2^{a}	13.5 ± 1.0^{a}	0.1 ± 0.1	3.2 ± 0.7^{a}
GL	5.4 ± 0.3^{c}	0.6 ± 0.1^{c}	17.2 ± 4.0^{a}	1.1 ± 0.4^{c}	17.6 ± 4.9^{a}	0.0 ± 0.0	0.7 ± 0.4^{c}
MH	5.2 ± 0.4^{d}	0.7 ± 0.0^{a}	35.8 ± 4.8^{a}	1.3 ± 0.1^{c}	27.0 ± 2.9^{a}	0.0 ± 0.0	0.0 ± 0.0^{d}
BH	4.9 ± 0.2^{c}	0.7 ± 0.0^{b}	29.0 ± 5.4^{a}	1.0 ± 0.3^{c}	32.3 ± 15.3^{a}	0.0 ± 0.0	0.1 ± 0.1^{d}

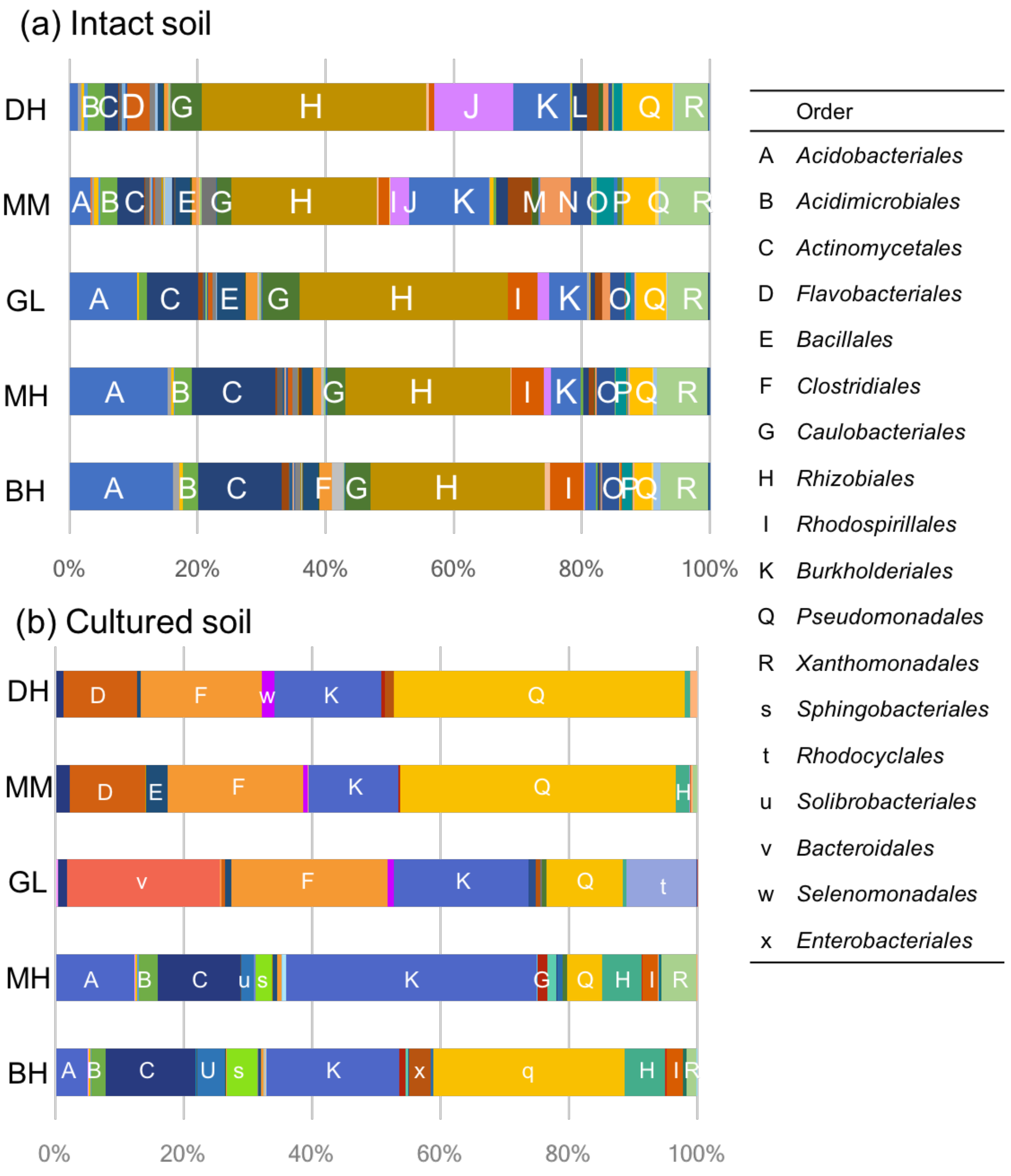
Statistic differences (F-values) and means (±s.d., n=4) of environmental factors
Significance levels: $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$; ^{NS}, nonsignificant
Small letters indicate differences between induvial means assessed by Tukey HSD tests ($P<0.05$). Abbreviation: %Corg, percentage of organic carbon content in dry weight.

3.3.2. Soil bacterial community

We obtained 505974 mapped reads in total (Fig. 3.2a). The bacterial communities in phylum level were dominated by *Proteobacteria* (69%) followed by *Actinobacteria* (12%), *Acidobacteri* (10%), *Firmicutes* (4%), and *Bacteroidetes* (2%). In order level, *Rhizobiales* (H) (phylum Proteobacteria, class Alphaproteobacteria) dominated among all the vegetation types (23-35%) (Fig. 3.2a). *Sphingomonadales* (J) (phylum Proteobacteria, class Alphaproteobacteria) was the second dominated order in DH. *Acidobacteriales* (A) (phylum *Acidobacteria*, class *Acidobacteria*) and *Actinomycetales* (C) (phylum *Actinobacteria*, class *Actinobacteria*) were major in GL, MH and BH, 10-16% and 13% respectively. *Flavobacteriales* (D) (phylum *Bacteroidetes*, class *Bacteroidetes*) was a specific order in DH occupying 4% of its community.

Principal component analysis revealed the relation between vegetation types and community composition (Fig. 3.3a). PC1 explained 20.7% of the variables while PC2 explained 9.1% of the variables. Soil bacterial community derived from the same vegetation type showed similar community structure.

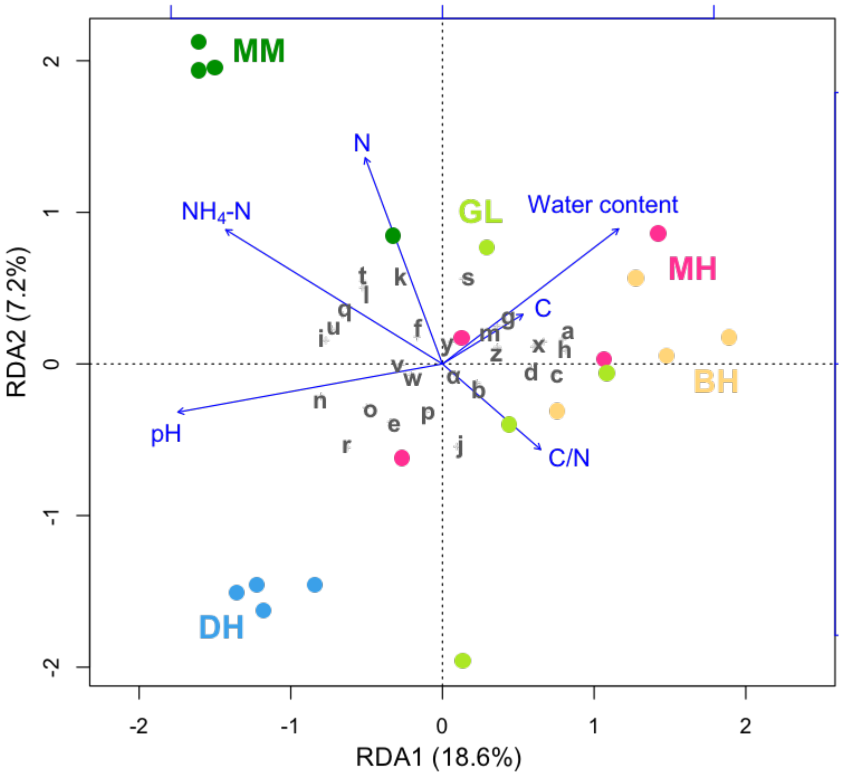
MH and BH showed similar community composition each other characterized by *Acidobacteriales* (A), *Actinomycetales* (C), and *Rhodospirillales* (I) (phylum *Proteobacteria*, class *Alphaproteobacteria*) (Fig. 3.3)


(a) Intact soil

(b) Cultured soil

Figure 3.2 High-throughput analysis of 16S rRNA gene fragment of Kärkevagge soils.

Bacterial 16S rRNA gene were sequenced by Ion Torrent PGM system. Ion Reporter based on Qiime software was used for data processing. (a) bacterial community of intact soil and (b) bacterial community of cultured soil used for acetylene reduction assay were compared. Abbreviation: DH, Dryas heath; MM, mesic meadow; GL, grassland; MH, mesic heath; BH, boulder heath.



	Order		
a	*Acidobacteriales*	o	*Pseudomonadales*
b	*Holophagales*	p	*Rhizobiales*
c	*Actinomycetales*	q	*Rhodocyclales*
d	*Coriobacteriales*	r	*Sphingomonadales*
e	*Flavobacteriales*	s	*Xanthomonadales*
f	*Sphingobacteriales*	t	*Nitrospirales*
g	*Bacillales*	u	*Desulfovibrionales*
h	*Clostridiales*	v	*Acidimicrobiales*
i	*Burkholderiales*	w	*Gaiellales*
j	*Caulobacteriales*	x	*Rhodospirales*
k	*Chromatiales*	y	*Solibacteriales*
l	*Desulfruomonadales*	z	*Thermoanaerobacteriales*
m	*Myxococcales*	α	*Rhodobacterales*
n	*Nitrosomonadales*		

Figure 3.3 Redundancy analysis of soil bacterial community

Redundancy anasysis was conducted using intact soil bacterial community. For this analysis, R software was used together with vegan package. Each alphabet showed characteristic bacterial.

3.3.3. Nitrogen-fixing potentials and bacterial communities in cultured soils

Nitrogen-fixing potential was varied between vegetation types. DH, MM, and GL showed relatively higher acetylene reduction while MH and BH showed slight or no acetylene reduction (Fig. 3.4). This tendency was common among 4 replicates derived from the same aboveground plant community. MM showed the highest acetylene reduction at 86.4 nmol C_2H_4 h^{-1} $vial^{-1}$.

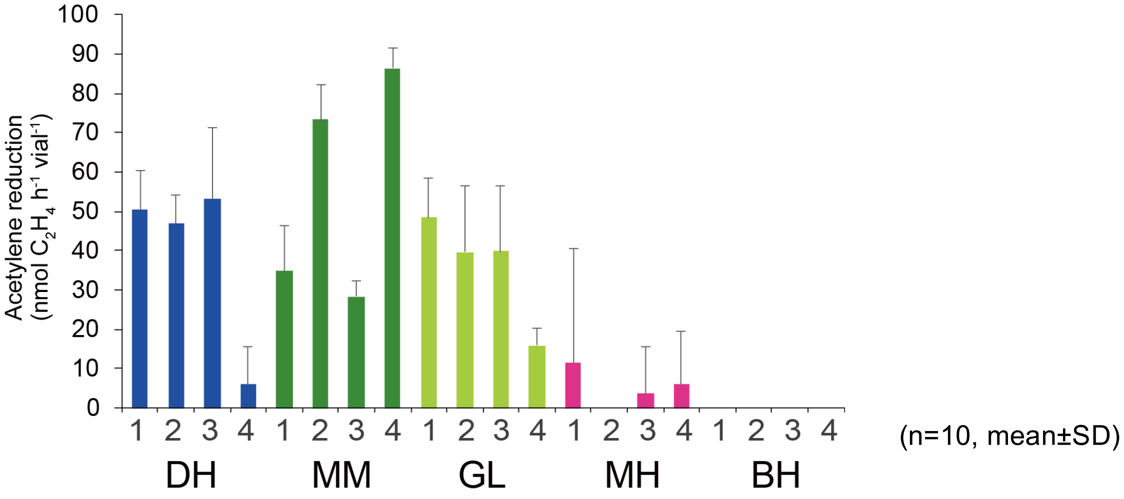
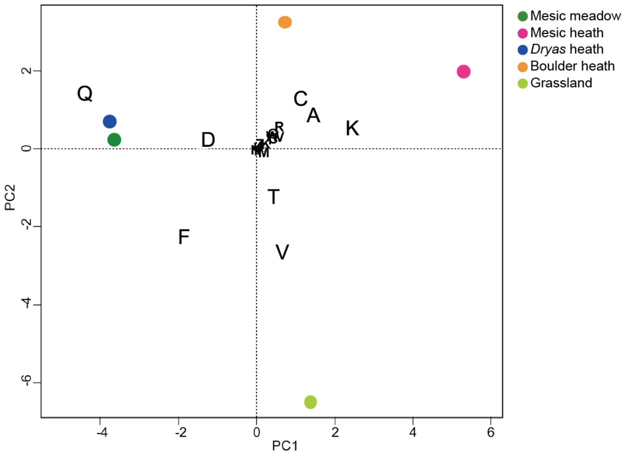


Figure 3.4 Acetylene reduction of cultured soil of Kärkevagge in 0.5% carbon source concentration.

The acetylene reduction assay was carried out in gellan gum media of Winogradsky's mineral solution at pH 5.0 containing a carbon source mixture at concentration of 0.5%. Each soil was inoculated to the medium and incubated for 7 days, then assayed for acetylene reduction (n=10, bar; ± standard deviation).

The bacterial community structure of cultured soil using acetylene reduction assay was also investigated. The bacterial communities of cultured soil in phylum level were dominated by *Proteobacteria* followed by *Firmicutes* (15%), *Bacteroudetes* (11%), *Actinobacteri* (8%), and *Acidobacteria* (3%). In order level, *Pseudomonadales* (Q) (phylum *Proteobacteria*, class *Gammaoriteobacteria*) was the most dominant in cultures inoculated with soil of DH, MM, and BH (Fig. 3.2b). *Burkholderiales* (K) (phylum *Proteobacteria*, class *Gammaoriteobacteria*) was dominated in GL, MH, and BH. *Bacteroidales* (V) (phylum *Bacteroidetes,* class *Bacteroidetes*) was uniquely occupied large abundance in GL community. *Clostridiales* (F) (phylum *Firmicutes*, class *Clostridia*) was the most major order in DH, MM, and GL occupying 19-24% of their community, while *Acidobacteria* (A) (phylum *Acidobacteria*, class *Acidobacteria*) and *Actinomycetales* (C) (phylum *Actinobacteria*, class *Actinobacteria*) were appeared uniquely in MH and BH.

Principal component analysis showed some trends in microbial community composition (Fig. 3.5). DH and MM showed highly similar bacterial community structure characterized by a large population ratio of *Pseudomonadales* (Q) and *Flavobacteriales* (D) (phylum *Bacteroidetes*, class *Bacteroidetes*). The communities of MH and BH shared *Acidobacteriales* (A), *Actinobacteriales* (C), and *Burkholderiales* (K). The community of GL was characterized by *Bacteroidales* (V) and *Rhodocyclales* (T) (phylum *Proteobacteria*, class *Betaproteobacteria*). *Clostridiales* (F) was also abundant in GL, same as those of DH and MM.



	Order
A	*Acidobacteriales*
C	*Acidimicrobiales*
D	*Actinomycetales*
F	*Flavobacteriales*
K	*Caulobacteriales*
T	*Rhodospirillales*
Q	*Pseudomonadales*
V	*Xanthomonadales*

Figure 3.5 Principle component analysis of eubacterial community in cultured soils.

Principle component analysis was conducted using cultured soil bacterial community. For this analysis, R software was used together with vegan package.

3.4. Discussion

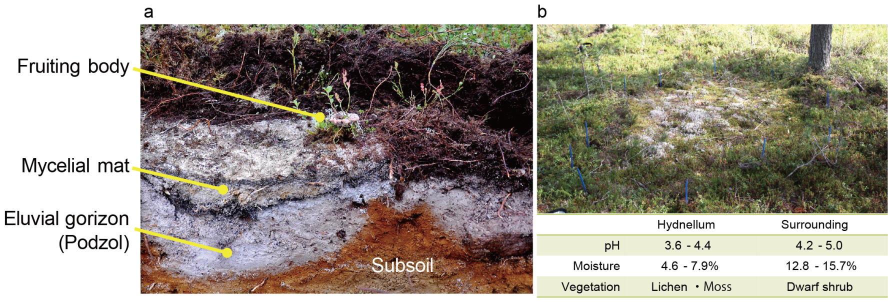
Soil bacterial community structure in each vegetation type was unique along with the pH gradient in the soil (Table. 3.1, Fig. 3.2). The principal component analysis of intact soils showed the plots of GL communities are converged in the central position, which reflects the geological location of grassland at the bottom of the valley, because grassland is a water catchment flown the streams pass through the other vegetation and bedrock. Nitrogen-fixing potentials of cultured medium was related with the aboveground plant community (Fig. 3.4). There were clear differences in the bacterial communities of culture medium depending on with or without nitrogen-fixing potentials (Fig. 3.4, 3.5). One of the most characteristic bacterial orders in cultured soils with high nitrogen-fixing potentials, DH, MM and GL was *Clostridiales*, which suggested its contribution to nitrogen fixation. *Acidobacteriales* and *Actinomycetales* were the characteristic orders in cultured medium of MH and BH, showing a low or no nitrogen-fixing potential. The nitrogen-fixing potentials were also related with the vegetation and concentration of NH_4-N in the intact soils (Table. 3.1, Fig. 3.4).

Chapter 4

A Role of Microbial Community under a Mycelial Mat of *Hydnerrum Ferrugineum*

4.1. Background

In boreal forest, the low temperature and the acidic condition lead podzol development as described in the general introduction. Hintikka in 1967 suggested that an ectomycorrhizal fungus *Hydnellum ferruginum* influenced on the podzolization in Finnish coniferous forest (Hintikka & Näykki, 1967). *H. ferrugineum* is known as a fairy ring-forming ectomycorrhizal fungus usually distributed throughout the pine forest in the area from western Europe to western Siberia. It forms 1-5 cm thick felt-like mycelial mat with fruiting bodies on the edge (Fig. 4.1a). The impact of this fungus on the vegetation can be recognized as lack of dwarf shrubs on the mycelial mat, of which green moss-abundant circles are approximately 50 cm long in diameter in the relatively young colonies but 1-2 m or larger in the old and mature colonies (Fig. 4.1b). The mycelium grows 2-3 cm outwards annually while gradually dying at the center of the mycelial mat.



	Hydnellum	Surrounding
pH	3.6 - 4.4	4.2 - 5.0
Moisture	4.6 - 7.9%	12.8 - 15.7%
Vegetation	Lichen • Moss	Dwarf shrub

Figure 4.1 A colony of *H. ferrugineum* and soil profiles

(a) The fruiting body in the edge of the mycelial mat and thick eluvial soil were observed underneath. (b) the site of mycelial mat of *H. ferrugineum*, which is characterized by lack of dwarf shrubs, mosses on the mat, and lichens on the decomposed mat.

One of the most characteristic features of *H. ferrugineum* is that highly developed eluvial soil known as podzol is observed just under the mat (Fig. 4.1a). The soils under the mycelial mat show less moisture content compared with the soils surrounding the mycelial colonies due to the mat preventing the downwards movement of rain water. The eluvial soil under the mat contains less iron oxide, phosphate, and organic matters due to severe leaching.

The organic acids are one of the driving agents for the mineral leaching from the soil, which is produced by living plants as exudates from the roots, decomposed plant residues by saprophytic microbes, and ectomycorrhizal or ericoid mycorrhizal fungi directly (Lundström et al., 2000). Acidic substances detected from the top soils are mainly low molecular organic acids, such as oxalic acid, citric acid, and malic acid, and humic substances including fulvic and humic acids. Crystalline of Ca oxalate has been observed on outer surface of the fungal hypha growing into litter layer (Graustein et al. 1977). Besides, a high concentration of oxalic acid was detected from ectomycorrhizal mat (Griffiths et al., 1994). Moreover, some ectomycorrhizal fungi have been reported as "rock-eating fungi" which penetrate their mycelia into rocks with exuding oxalic acid and some other organic acids, leading to acceleration of rock weathering (Jongmans et al., 1997).

Based on my hypothesis that bacteria and archaea associated with *H. ferrugineum* mycelial mat play certain key role in progress of podsolization and development of eluvial horizon, the soil microbial communities along the succession of mycelial mat were investigated using PCR-DGGE technique and high-throughput sequencing system in this study. In addition, the bacterial isolation was conducted under oxalate condition. Since the report by Hintikka in 1967, there has been no report concerning the relation between *H. ferrugineum* and the boreal forest ecosystems. This study aimed at understanding contribution of the fungal mat and microbial communities under the mat to podzol formation throughout in the boreal conifer forest.

4.2. Materials and Methods

4.2.1. Site description and soil sampling

The study sites are located at research forest near Ruttjeheden, northern Sweden (N65°43' E18°44') where *Pinus sylvestris* and *Picea abies* are dominant (Fig. 4.2). The sampling plots were selected with 3 replications, where the fruiting body of *H. ferrugineum* were observed on the edge of fairy ring with the mycelial mat below the vegetation. The soil samples were collected from 3 positions: the center of the mat (CP), the front edge of the mat (MP), and 50 cm outside of the edge (OP). From each position, litter (O_1), humus (O_2), podzol (E), and subsoil (B) were sampled from each position respectively. Sampling was done in late August in 2014. Official permission was obtained from Silver museum, Arjeplog and permission to import the banned item (26Y624) to Japan was obtained from Minister of Agriculture, Forestry and Fisheries via the Sapporo Branch of Yokohama Plant Protection Station.

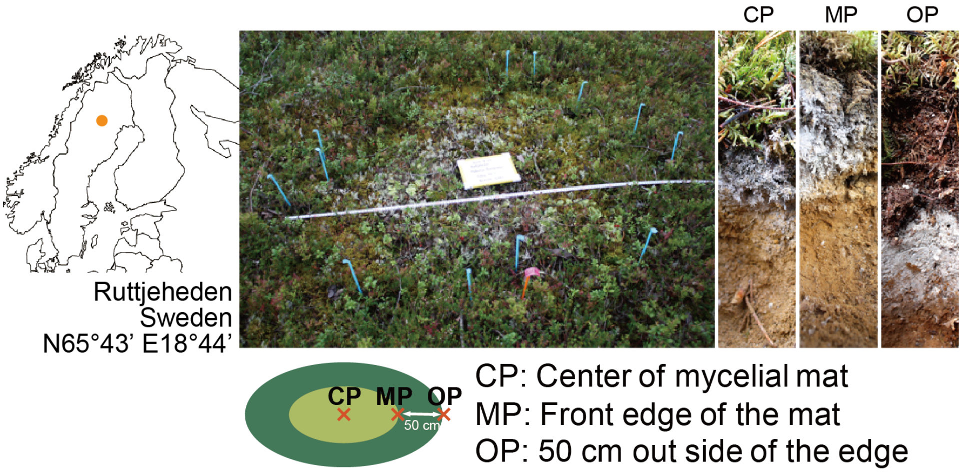


Figure 4.2 Sampling site location and sampling design

4.2.2. DNA extraction

Soil DNA was extracted from each soil sample and a piece of mycelial mat using ISOIL Large for Beads ver.2 (Nippon Gene) with the same procedure described in 2.2.4. The soils were stored in 4°C until DNA extraction with in 2-3 days.

4.2.3. Amplification of fungal ITS region and PCR-DGGE analysis

Fungal ITS was amplified with primer pair of *ITS1* and *ITS4* and purified amplicons were nested with *ITS1-gc* and *ITS2* (Gardes & Bruns, 1993). AmpliTaq Gold 360 master mix (Life Technologies) was used for the amplification under a PCR condition as follows for primary amplification: denaturing at 95°C for 30 sec, annealing 55°C for 30 sec, extension at 72°C for 1.5 min with repeating 25 cycles. For second amplification, PCR condition was same as primary amplification except for extension for 30 sec.

DGGE was performed by DCode Universal Mutation Detection System (Bio-Rad) with linear gradient of 20-50% under same condition in 2.2.5. Major bands were excised and subjected to PCR using a primer pair of *ITS1* and *ITS2* under the same condition with second PCR described above and then sequenced using ABI Prism 310 genetic analyzer (Applied Biosystems). The resulting DNA sequence data were subjected to BLASTn search.

4.2.4. Bacterial 16S rRNA gene analysis on high-throughput sequencing platform

The bacterial community analysis was performed on the Ion Torrent PGM platform using Ion 16S Metagenomics Kit (Life technologies). The library and template preparation were conducted under the same protocol in 2.2.7. Also, the same workflow was used for the data analysis.

4.2.5. Bacterial isolation and identification

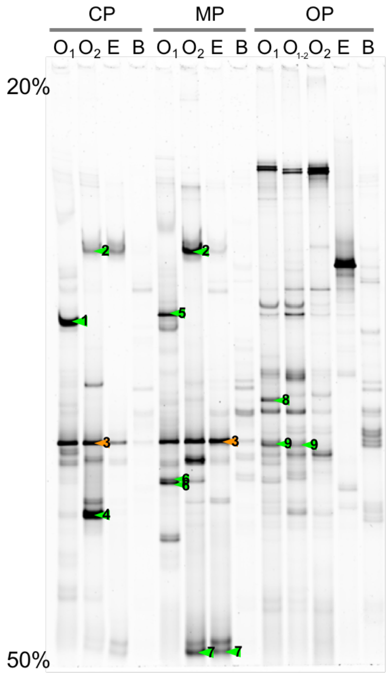
For bacterial isolation, Winogradsliy's medium was used combination with limiting dilution method. The first enrichment culture contained 0.02% mannitol with 0.001% oxalic acid or 0.01% of citric acid using 30 mL vial. The second culture was same composition without mannitol solidified with 3 g L^{-1} gellan gum. The plate medium was also prepared with the same composition with second culture solidified with 20 g L^{-1} gellan gum. A 1-mg portion of MP-B soil was inoculated to 20 mL of the first enrichment culture. After 10-days incubation at 15°C, the culture solution was inoculated to the second enrichment culture by 10000-fold dilution. From this point forward, dilution inoculation was repeated for 3 times, and 100 µL of cultured solution was inoculated on the plate medium.

Some colonies were isolated and subjected colony PCR. The resulting amplicons were subjected to sequencing.

4.3. Results

4.3.1. Fungal community analysis using PCR-DGGE

Fungal communities were analyzed using PCR-DGGE targeting ITS region (Fig. 4.3). in O_1 and O_2 soils showed higher diversity then in E and B soils among all plots. The community structure of CP and MP were similar between same soil properties. *H. ferrugineum* was detected from O_1, O_2 and E soils of CP and MP where the mycelial mat was observed, but not from OP where the mycelial mat has not invaded yet.



1	*Sistotremastrum suecicum*
2	*Mortierella*
3	***Hydnellum ferrugineum***
4	*Hypocrea rufa*
5	*Galerina arctica*
6	*Hyaloscyphaceae*
7	*Sagenomella humicola*
8	*Basidiomycota*
9	*Helotiales*

Fungal ITS region: *ITS1-gc/ITS2*

Figure 4. 3 Fungal ITS region-targeted DGGE of intact soils

Fungal ITS region was amplified from each DNA extracted from intact soils. Each lane represents soil horizon. DGGE was performed on 20%-70% gradient gel. Site description: CP, center position; MP, edge of mycelial mat; OP, outer position.

4.3.2. Bacterial community analysis using high-throughput sequencing system

Bacterial communities were analyzed using Ion Torrent PGM platform (Fig. 4.4). The major bacterial community in O_1 soils were *Burkholderiales* (A), *Acidobacteriales* (B) and Rhodospirillales (E). *Burkholderiales* (A) was the most dominant eubacteria among O_2 soil of CP and MP. *Acidobacteriales* (B) and *Actinomycetales* (C) were major in O_2 soil of OP. *Actinomycetales* (C) and *Rhizobiales* (D) were the dominant order in E soil of CP and MP. *Acidobacteriales* (B) was one of the major order in E soil of OP. The community structure in B soil was similar among OP and CP due to the composition of *Actunomycetales* (C) and *Rhizobiales* (D) as the major order. *Acidobacteriales* (B) was uniquely dominant in B soil of MP. The bacterial community in the mycelial mat also showed unique pattern: *Oscillatoriales* (G) as the most dominant order.

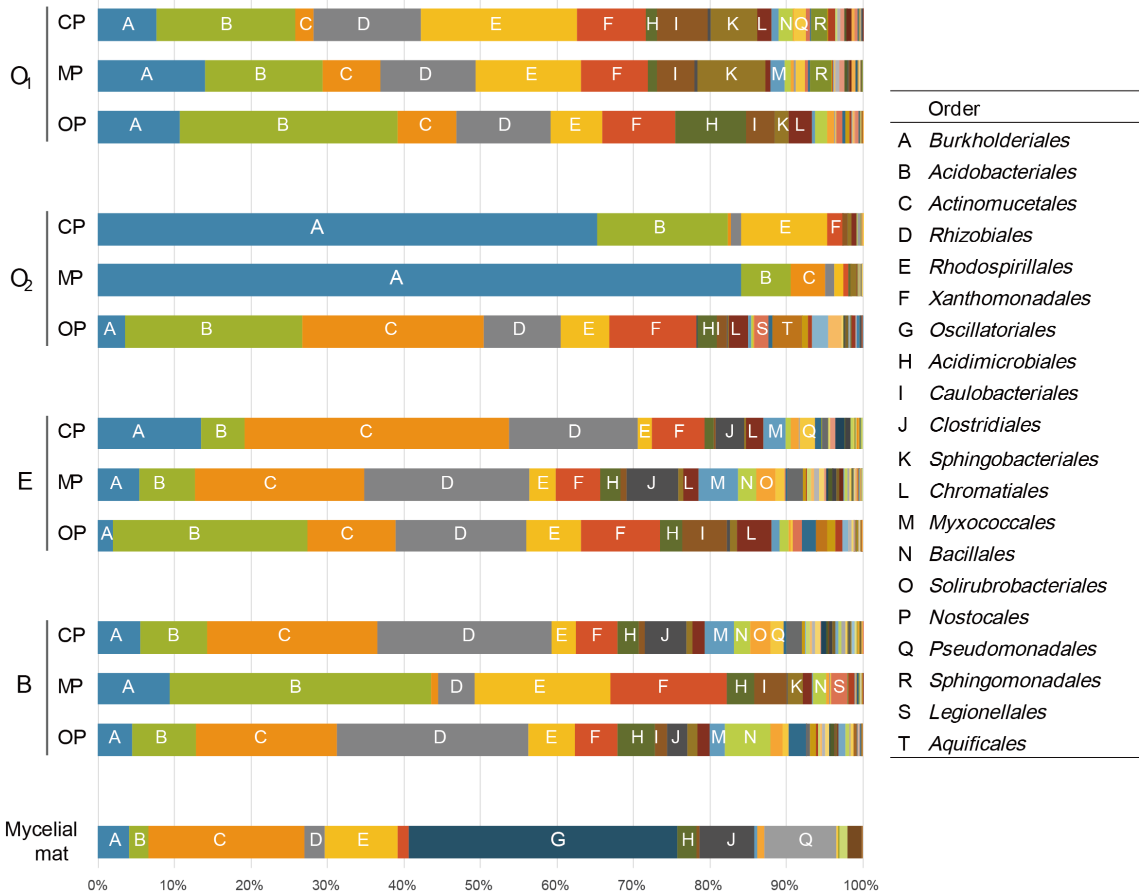


Figure 4.4 Bacterial community structure

Bacterial 16S rRNA gene region were amplified from each DNA extracted from intact soils and mycelial mat, then sequenced by Ion Torrent PGM. Ion Reporter based on Qiime software was used for data processing. Each alphabet (O_1, O_2, E, B) represents soil horizons. Site description: CP, center position; MP, edge of mycelial mat; OP, outer position.

The nMDS analysis revealed the clustering of bacterial communities each soil profile and plot (Fig. 4.5). The bacterial communities of O_1 soils showed similar patterns among CP, MP, and OP. The communities of CP- and MP-O_2, located immediately above the mycelial mat, were the same cluster with that of mycelial mat. OP-E was clustered in a different group from CP- and MP-E. CP-B was clustered in the same group with OP-B.

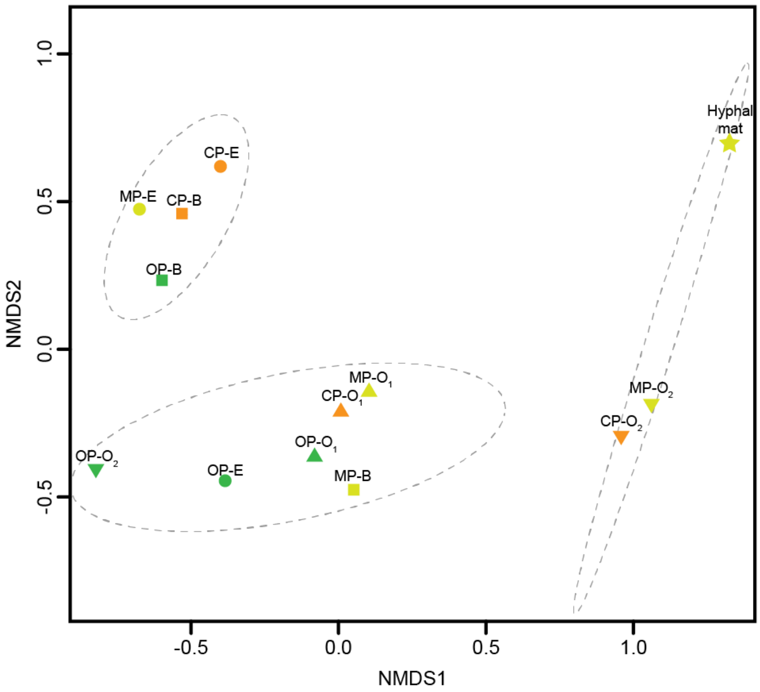


Figure 4.5 Clustering of bacterial community using nMDS

nMDS analysis was conducted using intact soil bacterial community. For this analysis, R software was used together with vegan package. First two letters represent site description and the subsequent alphabet (O_1, O_{1-2}, O_2, E, B) represent soil horizons. The shape of plot was as fallows; Triangle, O1 horizon; inverted triangle, O2 horizon; circle, E horizon; square, B horizon. Each color represents site description: orange, CP; light green, MP; dark green, OP.

4.3.3. Bacterial isolation and identification

Eleven isolates were obtained from soft-gel and plate medium (Fig. 4.6, Table. 4.1). Two strains of *Collimonas* sp. were obtained by an enrichment culture, after 4-times repetition of subculturing in the Winogradsky's mineral solution-based, oxalic acid- and citric acid-containing soft-gel medium, respectively. The subcultured soft gel medium was spread over an oxalic acid-containing Winogradsk's mineral solution-based agar plate, and eventually obtained three isolates of *Collimonas* sp. from the subculturing media containing oxalic acid.

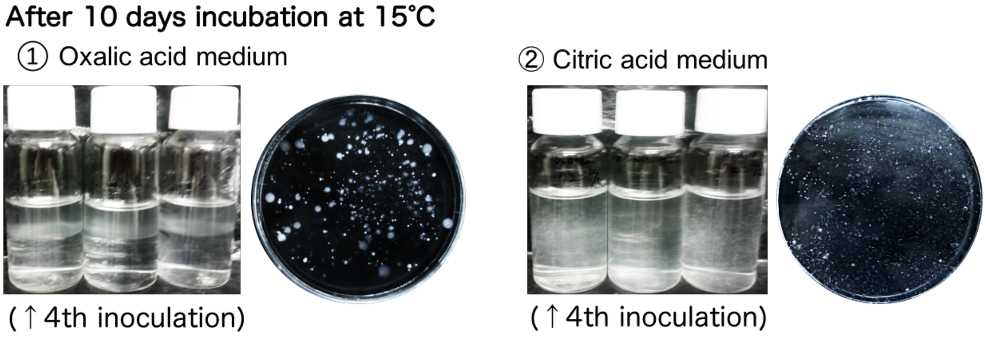


Figure 4.6 Cultured medium after 10-day incubation of 4th inoculation

MP-B soil was inoculated to Winogradsky's mineral solution base oxalic acid medium or citric acid medium.

Table 4.1 Result of isolation

Medium	Description	Number of isolates
① soft-gel	***Collimonas* sp.**	2
① plate	***Collimonas* sp.**	3
	Paenibacillus sp.	1
	Burkholderia sp.	1
② soft-gel	***Collimonas* sp.**	2
② plate	***Collimonas* sp.**	1
	Phyllobacetrium sp.	1

①0.001% oxalic acid ②0.01% citric acid

4.4. Discussion

There were clear vegetative gradients along the development of mycelial mat, which were also related with soil profiles. In this study, the soil bacterial communities showed different patterns in each plot. This indicates that not only vegetation and soil profiles but also microbial community was influenced by the mycelial mat.

The mycelial mat of *H. ferrugineum* itself plays important role in podzolization in boreal conifer forest bed soil. Here I isolated *Collimonas* sp. which had been reported to an ability of dissolving inorganic phosphorous and also mobilizing iron (Uroz et al., 2007; Leveau et al., 2010). This may suggest that some of soil bacteria also contribute to development of pozdol and mineral supply. In addition, *H. ferrugineum* was reported as a rock weathering fungus (Hintikka and Näykki, 1967). Combination of *H. ferrugineum* and some of the soil bacteria may accelerate podzolization of the conifer forest bed soils in boreal ecosystems.

Chapter 5

Total discussion

5.1. Ecological role of bacterial and thaumarchaeal community members in mineral horizons of soils in boreal and cold-temperate conifer forests

Three major thaumarchaeal groups were detected from mineral horizons of Pallas and Tomakomai. SCG including *Nitrosotalea* group, defined as the I.1a *Thaumarchaeota*, was the second dominant group among *Thaumarchaeota* in B-horizon of Tomakomai site (Auguet et al., 2010; Lehtovirta-Morley et al., 2011). SAGMCG-1 defined as the I.1b *Thaumarchaeota,* including *Nitrososphaera* sp. (Auguet et al., 2010; Spang et al., 2012), was the most dominant group in A-horizon of Tomakomai site. Conversely, FHMa11 defined as the I.1c *Thaumarchaeota* which has been found in acidic soils mainly from boreal forests (Yrjälä et al., 2004; Nicol et al., 2007; Bomberg et al., 2011) was most dominant in Pallas B-horizons of soils. Both I.1a associate and I.1b are ammonia oxidizers but I.1c is not (Weber et al., 2015). Although coherence of 16S rRNA gene and *amoA* gene of this group members have been reported, any FHMa11 terrestrial group (I.1c *Thaumarchaeota*) has not yet been successfully obtained as pure culture (Oton et al., 2016). Hence, the ecological function of FHMa11/I.1c group *Thaumarchaeota* remains unclear.

In some studies, the ecological function of I.1c *Tahumarchaeota* has been discussed, such as relation with mycorrhizal fungi and contribution to degradation process of soil organic matters (Bomberg and Timonen., 2009; Rinta-Kanto et al., 2016). The possibility of contribution of archaea to decomposition of organic matters in soil was proposed by Bomberg (Bomberg, 2016). The I.1c *Thaumarchaeota* showed relatively rapid growth compared with other thaumarchaeal strains in shallower mineral soils. Mineral soils, particularly that of A-horizon, from Tomakomai would contain easily degradable organic carbon, and hence I.1c *Thaumarchaeota* may outcompete I.1a associate and I.1b. If organic carbon of small molecular size is supplied to the nutrient-poor mineral horizon soil in boreal forest bed, thaumarhcaeal community structure might be altered by the carbon sources to be FHMa11-rich to SCG and SAGMCG-rich via nitrogen-fixation by bacteria leading to ammonia provision to the soil ecosystem.

5.2. Ecological role of soil microbial community in different type of vegetation in subarctic tundra in northern Sweden

The bacterial community structure along the subarctic tundra vegetation demonstrates the similar community member across the valley and the different composition for each vegetation type. The relationship between soil bacterial communities and soil pH has been reported that the bacterial community structure is predictable across large spatial scale with soil pH as a predictor (Lauber et al., 2009; Kaiser et al., 2016). Leuber et al. showed bacterial community structure with pH range of less than 4 to over 8 using high-throughput sequencing, covering tropical forest to tundra. In this study, these community structures showed a convergence almost at the same pH range (Lauber et al., 2009). In addition, some other studies also suggested that soil pH is important for compositional diversity of bacterial community (Shi et al., 2015). Richness of plant species is also influenced by pH (Gough et al., 2000). Another study showed that the communities of soil microbes in Alaskan tussock and shrub tundra were regulated by plant communities, determining the quantity and chemical quality of the litter (Wallenstein et al., 2007).

The influence of vegetation on bacterial community may result in further extent to establishing vegetation, depending on the topological differences. The vegetation distribution of Kärkevagge is largely counted for its topological and geological features. For example, grassland is located on a water catchment of the valley, therefor the streams flown from calcareous bedrock and iron sulfate bedrock make the soil pH at the neutral region. Interestingly, the bacterial community structure covers all the features of community structures of other vegetation.

Taken together, vegetation and bacterial community structure may not have direct correlation between them, but both are highly influenced by soil pH. The bacteria community structure in Kärkevagge showed clear gradient match with the gradient of pH value. The abundance of *Acidobacteriales* and *Actinomycetales* was increased while pH value decreased. *Acidobacteriales* and *Actinomycetales* are often found from humus soil in boreal forest and subarctic tundra (Chu et al., 2011; Ganzert et al., 2014).

The nitrogen fixing potential was found from 3 vegetation types: *Dryas* heath, mesic meadow, and grassland. Order *Clostridiales* was the common bacterial member from these cultured soils. In some subarctic ecosystem, *nifH* gene of *Clostridiales*, *Rhizobiales*, *Pseudomonadales*, and *Desulfuromonadales* were detected (Duc et al., 2009; Deslippe et al., 2005). Furthermore, vegetation and *nifH*-harboring bacteria were associated. Indeed, the bacterial community of 3 vegetation types with high nitrogen-fixing potential covered these bacterial order, as did those of 2 heath-type vegetation. It is possible that some secondary metabolites from heath-type plants inhibit or activate nitrogenase activity and/or bacterial growth.

5.3. Ecological role of microbial community including mycelial mat of *Hydnerrum ferrugineum* and bacteria in the eluvial horizon soil

As described in general introduction, there are two main theories of the mechanism of podzolization. One is the precipitation theory: relatively higher-molecular-weight, water-soluble organic acids caused by the decomposition of organic matter from O-horizon dissolve Fe and Al at the surface to shallow soils of mineral horizon. The other is the biological degradation theory: organic acids with low-molecular weight produced by microbiomes form metal complexes with Fe and Al leading to soil leaching. Besides, Breemen proposed another hypothesis that ectomycorrhizal fungi contribute to the podzolizaion (van Breeman et al., 2000). This theory was supported by a report that the annual fluxes of Fe, Al and Si from E to B horizons covered 70% of the annual fluxes of the mineral from O to E horizons (Giesler et al., 2000). This suggested that Fe, Al and Si released from mycorrhizal hyphal tips.

"Rock-eating fungi" are ectomycorrhizal fungi which can dissolve sandy mineral particles by organic acid exuded from their hyphae (Jongmans et al., 1997). *H. ferrugineum* has a feature of rock-eating fungi (Hintikka and Näykki, 1967), and thus their contribution to plant nutrition and biogeochemical cycle has been suggested (van Schöll et al., 2008). Moreover, mineral weathering bacteria have been reported (Uroz et al., 2009). I have isolated one of the mineral weathering bacteria *Collimonas* sp. from B-horizon soil in soft gel medium containing oxalate as sole carbon source. In addition, I successfully detected *Collimonas* sp. using high-throughput sequencing from soils under the mycelial mat. Thus, I showed reliable evidences that *H. ferrugineum* and *Collimonas* sp. may contribute mineral weathering and podzolization in boreal conifer forest.

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