Archaea in the Mycorrhizosphere of Boreal Forest Trees

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To Riku,

My thoughest critic and fiercest supporter,
My champion and companion

Contents

Sammanfattning

Arkéerna antogs länge vara en uråldrig typ av bakterier som bebodde endast extrema miljöer. Med hjälp av DNA sekvenseringsmetoder och molekylär fylogeni påvisades det dock att arkéerna utgjorde en egen organismgrupp och att livsformerna på jorden indelades i tre domäner, nämnligen Archaea, Bacteria och Eucaryota. Sedan början av 1990-talet hittades arkéer också i vanliga miljöer och det har påvisats att dessa icke-extremofila arkéer är mycket allmännare än de extremofila. De icke-extremofila arkéerna tillhörande Grupp 1 faller fylogenetiskt med fylat Crenarchaeota. De vanligaste arkéerrna i jord hör till undergruppen 1.1b, men de arkéer som hittas i boreal skogsjord inom Fennoscandien hör till grupp 1.1c.

Det organiska ytskiktet, humusen, i de boreala skogarna domineras av svamphyfer som tillhör mykorrhizasvampar. Dessa koloniserar praktiskt taget trädens alla finrötter och har påvisats ha speciell bakterieflora som skiljer sej från den i humusen. Också arkéer har påvisats leva både i skogshumus och på växtrötter.

I den här forskningen studerades arké-populationerna i boreala skogsträds ektomykorrhizosfär i mikrokosmer. Arkéer tillhörande 1.1c Crenarchaeota samt Euryarchaeota tillhörande *Halobacterium* och *Methanolobus* hittades. Arkéerna koloniserade alla habitat som omfattade svampmycel och mykorrhizor, framom trädrötter utan mykorrhizasvamp. Arten av mykorrhizasvamp var av betydelse för sammansättningen av arké-samhällena. Euryarkeoter hittased särskilt i mykorrhizor i tallens rötter. Populationen av Crenarchaeota var mer sporadisk, men påvisade en högre mångfald än euryarkeoterna. I motsats till de andra ektomykorrhizasvamparna påvisade pluggskivlingens (*Paxillus involutus*) mykorrhizor en mångfaldig population av 1.1c Crenarchaeota och bara få Euryarchaeota.

Trädrötterna innehöll i allmänhet bara låga mängder av olika arkéer, men alens rötter utgjorde ett undantag. Mångfalden i alens arké-bestånd i finrötterna var högre än den mångfald som påvisats i mykorrhizorna. I de fylogenetiska analyserna på arkéernas 16S rRNA gensekvenser bildade dessa arkéer en grupp som skilde sej från de grupper med arké-sekvenser från pluggskivlingens mykorrhizor trots att de föll inom grupp 1.1c. När rötterna hos de olika trädarterna koloniserats av pluggskivlingen minskade trädartens inverkan på arkéerna och arké-bestånden blev likartade oberoende av trädarten.

Både cren- och euryarkeoter anrikades under odlingsförhållanden där C-1 substrat tillsatts. 1.1c crenarkéer växte anaerobt i mineralmedium vars enda kolkällor var CH_4 och CO_2, samt i ett jästextraktmedium med CO_2 och CH_4 eller H_2. Diversiteten hos de aerobt anrikade grupp 1.1c crenarkeoterna, som växte i mineralmedium med antingen CH_4 eller CH_3OH som kolkälla, var dock större. Euryarkeoter tillhörande *Halobacterium* växte anaerobt i jästextraktmedium med låg salthalt samt CH_4, tillsatt eller producerat av den ursprungliga mikrob-floran närvarande i provet. CH_4-produktionen i mykorrhizaproven var mer än 10 gånger (en storleksordning) högre än i humusproven utan svamphyfer. Autofluorescerande metanogena arkéer påvisades också genom mikroskopering och vid analys av 16S rRNA gener från desamma erhölls sekvenser tilhörande släktet *Methanolobus.*

Dessa resultat indikerar att arkéerna har en viktig funktion i de mikrobsamhällen som direkt underhålls av ektomykorrhizasvampar samt att både träd- och svamparterna påverkar arkéflorans sammansättning. Resultaten tyder också på att cren- och euryarkeoterna har skilda ekologiska funktioner i de olika delarna av de boreala skogsträdens rotsystem och mykorrhizosfär. Genom att tillämpa dessa reultat kan det vara möjligt att isolera 1.1c crenarkéer, icke-halofila *Halobacterium*-

arter och även aerotoleranta metanogener. Dessa organismer kunde få en användning som indikatororganismer för att registrera förändringar i skogsjordens ekosystem, förorsakade av olika faktorer, såsom intensivt skogsbruk men också den globala temperaturökningen. Mera information om viktiga mikrobgrupper med troligtvis låg populationstäthet men stor ekologisk betydelse, som t.ex. metanogenerna i skogsjorden, kan vara av stor betydelse för att kunna minska de miljöeffekter människan förorsakar dessa globalt viktiga ekosystem.

Abstract

Archaea were long thought to be a group of ancient bacteria, which mainly lived in extreme environments. Due to the development of DNA sequencing methods and molecular phylogenetic analyses, it was shown that the living organisms are in fact divided into three domains; the Archaea, Bacteria and the Eucarya. Since the beginning of the previous decade, it was shown that archaea generally inhabit moderate environments and that these non-extremophilic archaea are more ubiquitous than the extremophiles. Group 1 of non-extreme archaea affiliate with the phylum Crenarchaeota. The most commonly found soil archaea belong to the subgroup 1.1b. However, the Crenarchaeota found in the Fennoscandian boreal forest soil belong to the subgroup 1.1c.

The organic top layer of the boreal forest soil, the humus, is dominated by ectomycorrhizal fungal hyphae. These colonise virtually all tree fine root tips in the humus layer and have been shown to harbour distinct bacterial populations different from those in the humus. The archaea have also been shown to colonise both boreal forest humus and the rhizospheres of plants.

In this work, studies on the archaeal communities in the ectomycorrhizospheres of boreal forest trees were conducted in microcosms. Archaea belonging to the group 1.1c Crenarchaeota and Euryarchaeota of the genera *Halobacterium* and *Methanolobus* were detected. The archaea generally colonised fungal habitats, such as ectomycorrhizas and external mycelia, rather than the non-mycorrhizal fine roots of trees. The species of ectomycorrhizal fungus had a great impact on the archaeal community composition. A stable euryarchaeotal community was detected especially in the mycorrhizas, of most of the tested Scots pine colonising ectomycorrhizal fungi. The Crenarchaeota appeared more sporadically in these habitats, but had a greater diversity than the Euryarchaeota. *P. involutus* mycorrhizas had a higher diversity of 1.1c Crenarchaeota than the other ectomycorrhizal fungi.

The detection level of archaea in the roots of boreal trees was generally low although archaea have been shown to associate with roots of different plants. However, alder showed a high diversity of 1.1c Crenarchaeota, exceeding that of any of the tested mycorrhizas. The archaeal 16S rRNA genes detected from the non-mycorrhizal roots were different from those of the *P. involutus* mycorrhizas. In the phylogenetic analyses, the archaeal 16S rRNA gene sequences obtained from non-mycorrhizal fine roots fell in a separate cluster within the group 1.1c Crenarchaeota than those from the mycorrhizas. When the roots of the differrent tree species were colonised by *P. involutus*, the diversity and frequency of the archaeal populations of the different tree species were more similar to each other.

Both Cren- and Euryarchaeota were enriched in cultures to which C-1 substrates were added. The 1.1c Crenarchaeota grew anaerobically in mineral medium with CH_4 and CO_2 as the only available C sources, and in yeast extract media with CO_2 and CH_4 or H_2. The crenarchaeotal diversity was higher in aerobic cultures on mineral medium with CH_4 or CH_3OH than in the anaerobic cultures. Ecological functions of the mycorrhizal 1.1c Crenarchaeota in both anaerobic and aerobic cycling

of C-1 compounds were indicated. The phylogenetic analyses did not divide the detected Crenarchaeota into anaerobic and aerobic groups. This may suggest that the mycorrhizospheric crenarchaeotal communities consist of closely related groups of anaerobic and aerobic 1.1c Crenarchaeota, or the 1.1c Crenarchaeota may be facultatively anaerobic. Halobacteria were enriched in non-saline anaerobic yeast extract medium cultures in which CH_4 was either added or produced, but were not detected in the aerobic cultures. They may potentially be involved in anaerobic CH_4 cycling in ectomycorrhizas. The CH_4 production of the mycorrhizal samples was over 10 times higher than for humus devoid of mycorrhizal hyphae, indicating a high CH_4 production potential of the mycorrhizal metanogenic community. Autofluorescent methanogenic archaea were detected by microscopy and 16S rRNA gene sequences of the genus *Methanolobus* were obtained.

The archaeal community depended on both tree species and the type of ectomycorrhizal fungus colonising the roots and the Cren- and Euryarchaeota may have different ecological functions in the different parts of the boreal forest tree rhizosphere and mycorrhizosphere. By employing the results of this study, it may be possible to isolate both 1.1c Crenarchaeota as well as non-halophilic halobacteria and aerotolerant methanogens from mycorrhizospheres. These archaea may be used as indicators for change in the boreal forest soil ecosystem due to different factors, such as exploitations of forests and the rise in global temperature. More information about the microbial populations with apparently low cell numbers but significant ecological impacts, such as the boreal forest soil methanogens, may be of crucial importance to counteract human impacts on such globally important ecosystems as the boreal forests.

List of original articles

Paper I
Bomberg M, Jurgens G, Saano A, Sen R, Timonen S (2003) *Nested PCR detection of Archaea in definedned compartments of pine mycorrhizospheres developed in boreal forest humus microcosms*. FEMS Microbiology Ecology **43**: 163-171.

Paper II
Bomberg M and Timonen S (2007) *Distribution of Cren- and Euryarchaeota in Scots Pine Mycorrhizospheres and Boreal Forest Humus.* Microbial Ecology **54**: 406-416.

Paper III
Bomberg M and Timonen S (2008) *Effect of tree species and mycorrhizal colonisation on the archaeal population of boreal forest rhizospheres*. Manuscript.

Paper IV
Bomberg M, Montonen L and Timonen S (2008) *Anaerobic Eury- and Crenarchaeota inhabit ectomycorrhizas of boreal forest Scots pine*. Manuscript.

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Author's contribution to each publication

Paper I
Malin Bomberg planned the experiment together with Sari Timonen, Robin Sen and Aimo Saano. She produced the mycorrhizospheres and samples, and did the laboratory work. She analysed the results together with the other authors, with the exception of the phylogenetic analyses performed by German Jurgens. She wrote the paper together with Sari Timonen with contribution from all authors.

Paper II
Malin Bomberg planned the experiment together with Sari Timonen. She produced the mycorrhizospheres and samples and performed all laboratory and phylogenetic analyses. She analysed the results and wrote the paper together with Sari Timonen.

Paper III
Malin Bomberg planned and performed the work and wrote the article under supervision by Sari Timonen.

Paper IV
The experiment was discussed and planned by all authors. The laboratory work was performed by Malin Bomberg and Leone Montonen. Malin Bomberg performed the phylogenetic analyses and wrote the article with contributions from Leone Montonen and Sari Timonen.

Abbreviations

AM	arbuscular mycorrhiza
ECM	ectomycorrhiza
O_2	oxygen
CO_2	carbon dioxide
H_2	molecular hydrogen
CH_4	methane
CH_3OH	methanol
Fr	non-mycorrhizal fine root
Mm	mature mycorrhiza
Em	external mycelium
Hh	hyphal humus = humus colonised by fungal hyphae
Uh	uncolonised humus = humus devoid of fungal hyphae
16S rRNA	16S subunit ribosomal ribonucleic acid
DNA	deoxyribonucleic acid
F*IS*H	fluorescent *in situ* hybridisation
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
DGGE	denaturing gradient gel electrophoresis
OTU	operational taxonomic unit
bp	base pair
fw	fresh weight
C-1	one carbon compound
NaCl	Sodium Chloride
RC	Rice Cluster
LGT	Lateral gene transfer
FFSB	Finnish forest soil archaea type B
FFSC	Finnish forest soil archaea type C

1. Introduction

1.1 The Archaea

According to structural differences between the organisms inhabiting Earth, life was divided into two domains, the Procaryota (comprising the bacteria) and Eukaryota (the rest). The main difference between these domains was the defined membrane enclosed cell nucleus, which was absent in the first group and present in the latter. However, due to the pioneering 16S rRNA gene sequencing work done on methanogens by Woese and Fox in 1977, it was discovered that this group of microbes was very different from all other bacteria. Carl Woese and colleagues recognized the Archeabacteria (later Archaea) as a separate group (see e.g. Sapp, 2005; Garrett and Klenk, 2007). They were given the name Archaebacteria to distinguish them from the true bacteria. The Archaebacteria appeared to be a form of ancient micro-organisms found only in extreme environments, such as highly saline brines, hot springs and anaerobic habitats. In 1989, two independent studies on the phylogenetic relationships between Eubacteria, Archaebacteria and Eukaryota showed that the Archaebacteria shared an evolutionary history excluding the Eubacteria, i.e. the Archaebacteria were more closely related to the Eukaryota than to Eubacteria (Gogarten *et al.*, 1989; Iwabe *et al.*, 1989) (Fig 1). In 1990 Woese *et al*. suggested the tripartite division of life, presenting the domains Archaea, Bacteria and Eukaryota (Woese *et al.*, 1990). This news was considered of such importance that it even outranked the achievements of cloning the human insulin gene for therapeutic purposes, which was reported at the same time.

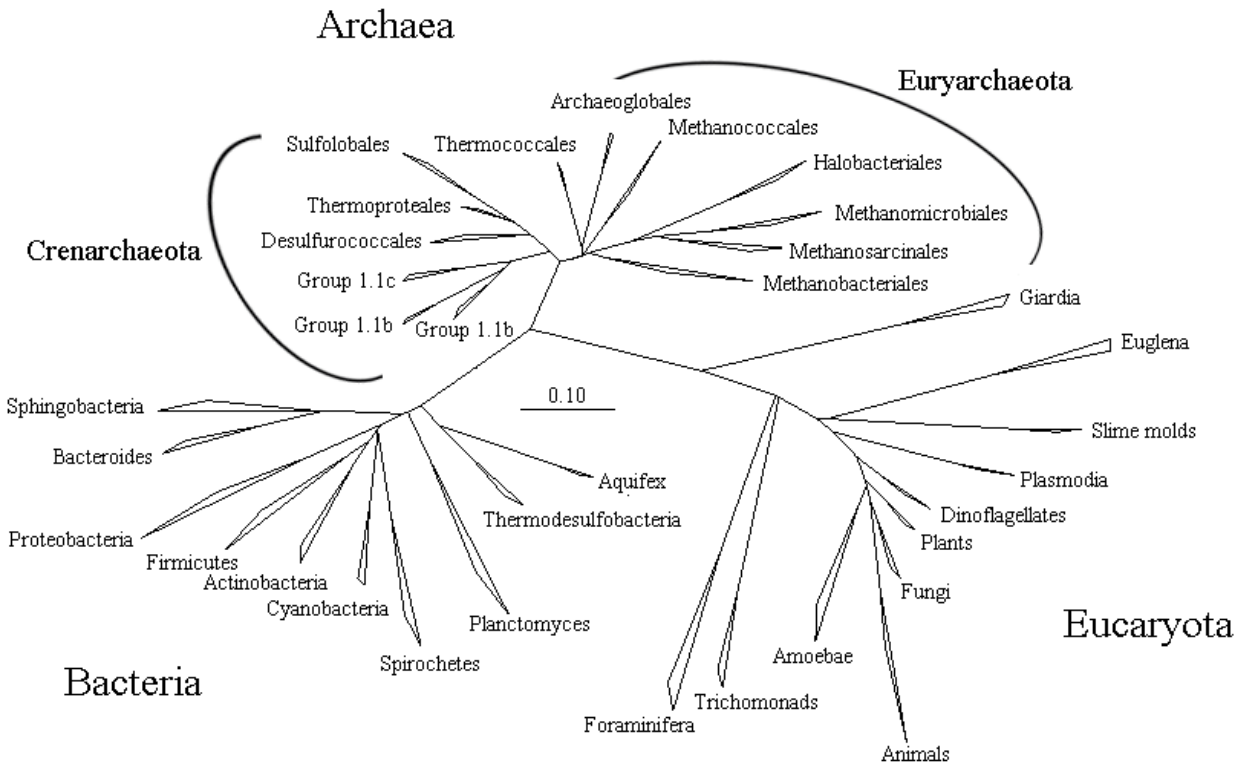

Fig 1. The three domains of life. A 16S rRNA tree of 121 mainly cultivated taxa obtained from the SILVA 93 and 94 databases (Prusse *et al.*, 2007). Sequence data were analysed with the ARB software package (Ludwig *et al*., 2004). The backbone tree was calculated using maximum likelihood on 90 long sequences in combination with filters excluding highly variable positions. Additional sequences were inserted using parsimony without allowing changes to the overall tree topology. Scale bar displays 0.10 changes/nucleotide position. The tree was constructed by Leone Montonen.

The archaea differ from the bacteria in many aspects (e.g. Cavicchioli, 2007; Garrett and Klenk, 2007). One of the major differences is the different lipids found in the membranes of the micro-organisms. While bacteria generally have a double membrane of phospholipids in which the fatty acids and glycerol moieties are ester-linked, the archaeal membranes are built up by branched-chain lipids containing hydrocarbon moieties bound to the glycerol by ether linkage. The archaeal information processing systems are also more reminiscent of the eukaryotal than the bacterial types. The bacterial cell walls contain peptidoglucan, which the archaeal cell walls lack. In stead, the archaea have diverse cell walls built of for example polysaccharides, glycoproteins or pseudopeptidoglucan. An important outcome of this is that antibiotics affecting bacteria rarely harm the archaea.

Today, the Archaea consist of two recognised phyla, the Euryarchaeota and the Crenarchaeota (Fig1). The Euryarchaeota contains all currently known methanogenic micro-organisms, the orders Methanopyrales, Methanococcales, Methanobacteriales, Methanomicrobiales and Methanosarcinales. The Methanopyrales appear to be rapidly evolving organisms and are phylogenetically situated far from the other methanogens (Brochier *et al.*, 2004). The other methanogens are closely related to the extreme halophiles of the order Halobacteriales. Members of the last mentioned orders have recently been detected in moderate environments (e.g. Elshahed *et al.*, 2004; Purdy *et al.*, 2004). Some taxa of the mainly thermophilic order Thermoplasmatales have also been detected in psycrophilic to mesophilic environments (Pesaro and Widmer, 2002). Members of the thermophilic orders Thermococcales and Archaeoglobales have so far only been found in extreme environments (e.g. Cavicchioli, 2007).

The extremophilic Crenarchaeota consist of three orders, the Thermoproteales, Desulfurococcales and the Sulfolobales. These archaea have classically been considered (hyper)thermophilic and sulphur metabolising micro-organisms. However, after the introduction of culture-independent molecular biological tools into microbial ecology, other types of Crenarchaeota were found to be distributed ubiquitously in diverse moderate environments (e.g. Schleper *et al.*, 2005). DeLong (1992) showed that 20% of the microbial 16S rRNA genes extracted from marine surface waters were archaeal. Fuhrman *et al*. (1992) reported a diversity of archaeal plankton at 100 m and 500 m depths of the Pacific Ocean, which resembled thermophilic Crenarchaeota. Hershberger *et al*. published archaeal 16S rRNA genes found in the sediments of Lake Griffy in 1996. The ubiquitous distribution of the archaea was further supported in 1997 with reports on Crenarchaeota in the sediments of Lake Lawrence (Schleper *et al*., 1997) and both Cren- and Euryarchaeota in Lake Michigan (McGregor, 1997). Crenarchaeal 16S rRNA genes were not only restricted to aquatic environments, but were also found in both agricultural (Bintrim *et al*., 1997, Wisconsin; Groβkopf *et al.*, 1998, Italy) and tropical forest (Borneman and Tripplett, 1997, Brazil). Studies in boreal forest soil of southern Finland revealed a unique type of crenarchaeotal 16S rRNA genes not found anywhere else (Jurgens *et al.*, 1997; Jurgens and Saano, 1999). Since these first reports, continuous work has been done to study these organisms.

Although members of the different euryarchaeotal orders have been found in moderate environments, the types of Crenarchaeota detected in these environments form their own monophyletic lineage affiliated with the Crenarchaeota, the Marine Group 1 of uncultured archaea (DeLong, 1998). It was also discovered that the Crenarchaeota from the different moderate habitats differed significantly from each other in their 16S rRNA gene sequences and in 1998 DeLong described the phylogenetic grouping of these non-thermophilic Crenarchaeota. Group 1 of non-thermophilic Crenarchaeota contains three distinct clusters, groups 1.1 (marine plankton, soil, sediments), 1.2 (marine and lake sediments) and 1.3 (lake sediments, palaeozols, anaerobic digesters). Group 1.1 has since been divided into several subgroups. Group 1.1a includes mainly planktonic archaea and group 1.1b mostly archaea from soils and lake sediments. The boreal forest

soil Crenarchaeota were so different from the other soil Crenarchaeota that they formed a separate group on their own, the 1.1c group.

Until recently, the Group 1 Crenarchaeota had no cultivated members. However, Quaiser *et al*. (2002) were able to isolate and sequence a large genome fragment of an uncultured 1.1b crenarchaeote and Hallam *et al*. (2006b) succeeded in enriching the 1.1a crenarchaeote, *Cenarchaeum symbiosum,* together with its sponge host and study its genome. Könneke *et al.* (2005) were the first to grow a crenarchaeote, *Nitrosopumilus maritimus,* in pure culture and this is placed within the Group 1.1a lineage. The growing number of whole genome studies may strengthen the suggestion by Brochier-Armanet *et al*. (2008) that this Group 1 Crenarchaeota actually forms a distinct new phylum, the Thaumarchaeota.

Different types of archaea are continuously detected in both natural and agricultural soil. In water saturated soils, specific clusters of methanogenic Euryarchaeota have been found in rice field soils (e.g. Erkel *et al.*, 2005; Sakai *et al.*, 2007) and peatland soil (Brauer *et al.*, 2006). Indications of the presence of members of the order Halobacteriales in boreal forest soil have been found (Jurgens et al, 1996 - GeneBank submission) and although methanogenic archaea have not been reported in the boreal forest soils, methane emissions from boreal forests are known (Sinha *et al.*, 2007). Different methanogenic taxa have been detected in boreal bogs and mires (e.g. Galand *et al*., 2003; Juottonen *et al*., 2005).

In dry soils Crenarchaeota are the predominant type of archaea. It has been estimated that the relative abundance of crenarchaeotal 16S rRNA genes in agricultural and natural field soils is up to 1 to 2% of the total 16S rRNA gene pool (Buckley *et al.*, 1998; Sandaa *et al.*, 1999) and 0.3-0.5% in sandy soil (Ochsenreiter *et al.*, 2003). The dominating type of Crenarchaeota in most aerated soils, both natural and agricultural, is Group 1.1b (Nicol and Schleper, 2006). Group 1.3 Crenarchaeota have also recently been found in mature Austrian alpine grassland soil and Australian agricultural soil (Nicol *et al.*, 2005; Midgley *et al.*, 2007). The predominant group of Crenarchaeota in the acidic (pH 3.5 - 5) Scots pine dominated forests in southern Finland and in Scotland are the 1.1c (Jurgens *et al.*, 1997; Jurgens and Saano, 1999; Bomberg *et al*., 2003; Yrjälä *et al.*, 2004; Bomberg and Timonen, 2007). Despite a growing number of studies, no other types of Crenarchaeota have yet been found in the boreal forest soil.

The distribution of group 1.1c Crenarchaeota has been suggested to be affected by the pH of soil. In a few studies, both group 1.1b and 1.1c have been reported simultaneously, but 1.1c Crenarchaeota have with only one exception (Yrjälä *et al.*, 2004) not been found in soil with a pH above 5.1. In addition, this approaches the pH minimum in which 1.1b Crenarchaeota have usually been detected (Nicol *et al.*, 2003a; Oline *et al.*, 2006; Hansel *et al.*, 2008). The 1.1c Crenarchaeota are not specific to the boreal forest soils and have been detected in various mature and unmanaged grassland soils where the soil pH was below 5 (Nicol *et al.*, 2003a; Nicol *et al.*, 2003b; Ochsenreiter *et al.*, 2003; Nicol *et al.*, 2005; Hansel *et al.*, 2008).

1.2 The boreal forests

Coniferous forests grow in the northern parts of the Earth's northern hemisphere, the boreal regions. Boreal forests cover one third of the global forest areas and are thereby the largest forests in the world. They have a profound impact on global atmospheric CO_2 concentrations and the greenhouse effect. In fact, during the relatively short northern summer their intense photosynthesis rate even reduces the global atmospheric CO_2 content (Bonan, 1991). The boreal forests store about 60% of the global forest soil carbon and about 25% of the forest tree carbon (Dixon *et al.*, 1994). The boreal forest soils are podzolised and typically consist of a top layer of organic humus (O) under which a thin eluvial (E) mineral soil horizon lies on top of the illuvial (B) mineral soil (e.g. Mokma

et al., 2004, and references therein). In the boreal forests, the humus layer is typically thick because of the limited time of sufficiently warm temperatures for decomposers to function. The highest respiration rates are found in the humus layer where particularly fungi and soil animals degrade organic litter, such as tree leaves and dead plant roots rich in lignin and cellulose (Lundström *et al.*, 2000, and references therein). These degradation processes support the whole biological community of plants, animals and microbes. This microbial community further benefits the plants by forming diverse symbiotic relationships, such as mycorrhizas and nitrogen-fixation (Tugel *et al*., 2000).

Boreal forests are typically dominated by coniferous trees. Typical boreal forests in the Fennoscandian region consist of Scots pine, *Pinus sylvestris* (L.) and Norway spruce, *Picea abies* (L) H. Karst. They have thick moss layers and are classified according to the under storey vegetation into *Oxalis-Myrtillum* type (OMT) which is the most humid type, to *Myrtillum* type (MT), *Vaccinum* type (VT), *Calluna* type (CT) and the driest *Calluna-Lichenes* type (ClT) forests (Cajander, 1926). Some deciduous tree species are also able to reproduce in the boreal areas. Of these the silver birch (*Betula pendula* Roth.) is the most common, although other birch species, such as paper birch (*B. pubescens* Ehrh.) can also be found.

Different tree species influence the physical, chemical and biological conditions of the soil in their vicinity by different means (Priha and Smolander, 1997). The pH of the soil in a coniferous forest is generally acidic and the typical Finnish dry pine forest soils exhibit a pH between 4 and 5. Conifers, especially spruce, may decrease the pH of the soil as low as 3.5, whereas birch has been shown to increase the soil pH up to 5.7 and enhance the cycling of nutrients (Mikola, 1985). The deciduous litter contains carbon which is more easily leached, whereas coniferous litter is acidic and contains more stable, recalcitrant material such as waxes, phenolics and lignin (Harris and Safford, 1996; Prescott *et al.*, 2004). Plants attract specific microbial communities to their rhizospheres by secreting different types of root exudates into the soil. Exudates, such as sugars, organic acids and amino acids attract among others, the mycorrhizal fungi living in the soil.

1.3 Mycorrhizal fungi

The term ‘mycorrhiza’ is derived from the Greek words ‘myces’ (fungus) and ‘rhiza’ (root). It was first used by Albert Bernhard Frank in 1885 (Frank, 2005, English translation). He reported that ectomycorrhizas are common in the root systems of woody plants in many types of soils. He suggested a symbiotic relationship between the plant host and fungus in which the fungus brings nutrients extracted from the soil to the plant and the plant nourishes the fungus by providing photosynthetically derived carbon substrates. His theories were much opposed by the scientific community of the day, but nearly all of his major hypotheses have been proved correct (reviewed by Trappe, 2005).

Mycorrhizas are roughly divided into three groups; endo-, ecto- and ectendo-mycorrhiza (e.g. Smith and Read, 1997). Endomycorrhiza, especially the arbuscular mycorrhiza (AM) are the most abundant type of mycorrhiza (Harley and Smith, 1983), and AM symbioses have been estimated to occur in over 80% of the flowering plant species on land (Harley and Smith, 1983). AM fungi penetrate the walls of the cortical cells of the plant roots and differentiate on the intracellular side of the cell wall into highly branched structures called arbuscules, without disrupting the plant cell membrane. Hardly any external signs of AM fungal colonisation is seen. Other types of endomycorrhiza are more rare. The most typical mycorrhizal type in the roots of boreal forest trees are the ectomycorrhizas (ECM). The ECM mycelium surrounds the cortical cells of the roots, forming the so called Hartig net, without penetrating the cell walls of the plant root. ECM mycelium typically forms a thick mantle around the colonised root tip. ECM are characteristic for most conifers, such as *Pinus* and *Picea*, and some Cupressaceae (e.g. *Juniperus*). They are also common in several dicot families such as the Betulacece, Fagaceae and Salicaceae. ECM have the

ability, lacking in higher plants, to absorb and utilise ammonium directly from decaying organic material (Finlay *et al.*, 1992; van Hees *et al.*, 2006). They are mostly formed by basidiomyceteous fungi, but some Ascomycetes have also been reported to form ECM. The ECM fungi have co-evolved with plants on land to utilise a complex variety of organic substrates.

Mycorrhizal fungi have had a profound influence on the terrestrial colonisation success of plants. There is fossil evidence for the presence of fungi in the roots of the earliest land colonising plants (Redecker *et al.*, 2000) and it has been shown that AM fungi were already present 460 million years ago, which predates the appearance of vascular plants (Wilkinson, 2001). The ECM fungi appeared when sclerophyllous vegetation developed around 200 million years ago and the organic matter content of the ancient soils increased (Cairney, 2000). Phylogenetic analyses show that the Basidiomycota have both gained and lost the ectomycorrhizal condition repeatedly during their evolution. Even today, more than 90% of all plant species are mycorrhizal.

ECM root tips are primarily formed in the uppermost organic horizon and the interphase between the organic and mineral horizon of the podsolized soil of boreal forests (Smith and Read, 1997). Up to 90% of the ECM root tips are found in the O horizon (Jonsson *et al.*, 2000). More than 1000 species of fungi are estimated to form ECM in the roots of the few tree species in the Fennoscandian boreal forests (Knudsen and Hansen, 1991). However, the tree rhizospheres are typically dominated by only a few key species, while the whole of the fungal diversity is composed of rare fungal species (e.g. Horton and Bruns, 2001).

The mycorrhizal fungi have important impacts on the global carbon cycle. They have been shown to increase the rate of photosynthesis in the plants they colonise (Högberg *et al.*, 2008) thereby increasing the amounts of carbon the plant assimilates into its biomass and allocates into the soil. Not only is a considerable part of the plant derived carbon transported to the external mycelium, but carbon is also bound in the fungal biomass. The greatest part of the fungal carbon is in the form of membrane lipids, which are rapidly metabolised by other microbes when the fungal hyphae die. Another large proportion of fungal carbon is found in the chitin containing cell walls. These structures can be very resistant to microbial decomposers and be stored in the soil for years. The amount of fungal hyphae in the organic soil layer is considerable. It has been estimated that 125 to 200 kg of fungal hyphae are found per hectare of forest soil (Wallander *et al.*, 2001) and all ectomycorrhizal mycelia (including ectomycorrhizal mantle structures) were estimated to be as high as 700 to 900 kg ha^{-1}. The ECM fungal hyphae have been shown to conduct over 50% of the microbial respiration in boreal forest soil (Högberg *et al.*, 2001).

1.4 Mycorrhizosphere/rhizosphere concept

Mycorrhizospheres dominate nearly all natural soil ecosystems. However, pure, non-mycorrhizal rhizospheres can be found in nature. Non-mycorrhizal plant species are often the pioneers colonising land that has been disturbed by different means, such as forest fires and melting of glaciers (Allen, 1991). Terrestrial plant species, which survive without mycorrhizal symbionts, are usually found in relatively recent plant families such as Brassicaceae (e.g. *Arabidopsis thaliana*), Chenopodiaceae, Cyperaceae and Juncaceae (Brundrett, 1991).

The rhizosphere was described by Hiltner (1904) to be the soil adjacent to and influenced by plant roots. This compartment differs remarkably from the surrounding ‘bulk’ soil in pH, as well as concentration and quality of nutrients, micro-environments, moisture and oxygen levels (Nye, 1981; Wang and Zabowski, 1998). The mycorrhizosphere was first described by Linderman (1988) as a term for the volume of soil influenced by the mycorrhizal fungus colonising the roots of a plant. Thus, the mycorrhizal fungi radically increase the amount of soil that can be exploited by a plant (Smith and Read, 1997).

Mycorrhizal plants are often dependent on their fungal partners. There are studies where the symbiotic fungus has been shown to protect the plant partner from different environmental stresses, such as drought, pathogens and heavy metal pollution (e.g. Harley and Smith, 1983; Lehto, 1992; Smith and Read, 1997). The benefits of the symbiotic fungus for the host plant and plant community are numerous. The diameter of the mycorrhizal fungal mycelium is only approximately one tenth of that of the root. Thus, the fast growing mycelium can access pores in the soil, which would be impossible for the plant to use. The fungi may participate in decaying processes and release and take up nutrients for both itself and the plant. There are even cases were the fungus preys on soil fauna and utilises the released nitrogen compounds (Klironomos and Hart, 2001). One mycorrhizal fungus can connect plants of both the same and different species (Simard *et al.*, 1997). These networks can allocate nutrients from photosynthetically active individuals to sustain tree seedlings and shaded trees.

Soil is a complex and heterogeneous habitat (e.g. Paul and Clarck, 1996; Lavelle and Spain, 2003). It consists of various sizes of aggregates of both organic and inorganic soil matter intertwined with gaseous and aqueous pore spaces. Depending on microbial activities as well as the substrates present, the concentrations of gases, such as O_2 and CO_2, varies significantly in the microhabitats in the soil. In contrast to aqueous environments, there is relatively little mixing of the soil matrix, which would reduce these variations and restricts the ability of soil microorganisms to move to more preferential locations when resources are used. This makes soils mosaic-like environments. The interiors of soil aggregates can be anaerobic due to microbial activities whereas the surfaces are aerobic. Soils also contain a lace of fungi, plant roots and earth fauna. When these soil organisms die, they form hotspots for decomposers and leave cavities and tunnels for water and gas exchange. This revives the nutrient cycles and allows for new organisms to colonise the empty areas.

Rhizospheres and mycorrhizospheres actively contribute to the heterogeneous structure of soil. Trees allocate 40%-70% of their photosynthetically assimilated carbon to the roots and 2-10% of this is further secreted as root exudates into the rhizosphere (reviewed by Grayston *et al.*, 1997). Mycorrhizas have been shown to substantially increase the amounts of C the plant allocates to the roots and the plants often replace this carbon loss by increasing their level of photosynthesis. Low molecular weight, water soluble exudates, such as sugars, amino and organic acids, hormones and vitamins are diffused from the root tips into the surrounding soil (reviewed by Bertin *et al.*, 2003) or are taken up by the mycorrhizal fungi directly from the plant root cells. The colonisation of the roots by mycorrhizal fungi alter both quality and quantity of exudates released into the soil. The fungus metabolises the plant derived compounds into fungal metabolites, such as trehalose, mannitol and arabitol (Söderström *et al.*, 1988) and they also produce lactic and oxalic acid. These compounds are secreted at the fast growing and metabolically active mycelial tips further modifying the soil habitats at the margins of the mycorrhizosphere (Finlay and Read, 1986).

1.5 Funcrions of the rhizosphere micro-organisms

Ectomycorrhizal fungi constitute the largest microbial biomass in forest soils. Although there are numerous species of mycorrhizal fungi able to colonise the roots of a tree species, the community is often dominated by only a few species of mycorrhizal fungi taking care of the everyday maintenance of the host (Heinonsalo *et al.*, 2007). Fungi often found in the tree mycorrhizospheres in the Fennoscandian boreal forest topsoils are species of e.g. *Suillus*, *Paxillus*, *Tomentellopsis*, *Piloderma* (e.g. Kåren *et al.*, 1997; Timonen *et al.*, 1997).

Bacteria are highly abundant in soil and the rhizosphere. The highest bacterial abundances are found closest to the roots and are often relatively plant specific (Griffiths *et al.*, 1999; Duineveld *et al.*, 2001). Typical cultured bacteria found in the ectomycorrhizosphere of Fennoscandian boreal

forest Scots pine are species of *Burkholderia*, *Paenibacillus* and *Bacillus* (Timonen *et al.*, 1998; Timonen and Hurek, 2006). Fluorescent pseudomonads and *Bacillus* spp. are common in nursery forests in temperate regions (reviewed in Garbaye, 1994). The different habitats of the mycorrhizosphere, such as the mycorrhizas and external mycelial tips, have been shown to harbour bacterial communities differing in both numbers and activity (Timonen *et al.*, 1998; Heinonsalo *et al.*, 2001). In a microcosm experiment, Timonen *et al.* (1998) showed that the bacterial communities of mycorrhizospheres have potential to use different carbon sources than the ones in the forest humus uncolonised by mycorrhizal fungal hyphae. It was also shown that the bacterial communities inhabiting Scots pine-*Suillus bovinus* or -*Paxillus involutus* mycorrhizospheres favoured different carbon sources. The *S. bovinus* communities especially favoured mannitol whereas the *P. involutus* communities preferred fructose.

Some bacteria have been shown to have great impact on the mycorrhization efficiency of fungi by producing substrates such as IAA to attract fungi (reviewed by Garbaye, 1994; Frey-Klett *et al.*, 2007). These bacteria were termed Mycorrhization Helper Bacteria, or MHBs. They represent diverse lineages and appear to show some specificity to types and species of mycorrhizal fungi. Bacteria in the mycorrhizosphere have been shown to promote germination of fungal spores (Xavier, 2003). Some bacteria have also been shown to increase the production of fungal mycelium and promote the branching of roots, which enhances the formation of mycorrhizas (Duponnois, 2006).

Non-symbiotic rhizosphere bacteria are known to promote plant growth, i.e. plant growth promoting rhizobacteria or PGPRs (reviewed in Zhuang *et al.*, 2007). These bacteria may enhance plant growth by degrading organic compounds into suitable substrates for the plant. They reduce environmental stresses and protect the plants from pathogens. By production of gaseous substances, such as ethylene, these bacteria enhance root growth. They degrade environmental pollutants, such as PCB and PAHs. The Rhizobia are nitrogen fixing bacteria, which inside root nodules can live in symbiosis with leguminous plants. These microbes promote plant growth by fixing atmospheric nitrogen into NO_3^- and NH_4^+, which they release to the plant in return for constant and protected habitats with plant-provided carbon (Prell and Poole, 2006). They are also found freely in the rhizospheres of many non-nodulating plants (Maunuksela *et al.*, 1999). The mycorrhizoshere and rhizosphere bacterial communities have also been reported to be involved in uptake and release of phosphorus from insoluble minerals (Leyval and Berthelin, 1993) and in degradation processes they are able to completely mineralize the compounds. The above mentioned activities of the bacteria in the rhizosphere are only a small part of the vast functional and taxonomical diversity found so far.

1.5 Archaea in roots and rhizosphere soil

Group 1.1b Crenarchaeota have been shown to inhabit the root system of tomato plants grown in agricultural soil (Simon *et al.*, 2000). A 10-fold higher number of crenarchaeotal cells were detected by *FISH* on senescent roots compared to young roots. Although not as dramatic, bacteria were also more abundant on senescent rootlets compared to young roots. A few Group 1.1a Crenarchaeota were detected by culture independent methods from maize roots (Chelius and Triplett, 2001), although this group has been more commonly found in aquatic ecosystems. Both above mentioned studies were performed on agricultural soil from Wisconsin. Other studies have examined archaeal communities in the rhizospheres of plants growing in more undisturbed soils (Sliwinski and Goodman, 2004; Nicol *et al.*, 2005) with the only type of archaea found were group 1.1b Crenarchaeota. In the study by Sliwinski and Goodman (2004), there were no differences in the populations of archaea inhabiting the roots or rhizosphere soil between plants of different genera growing in the same location. The same was seen in the study by Nicol *et al.* (2005), when they examined the rhizosphere soil of dominant plant species colonising the different locations of a

gradient of maturing soil in front of a receding glacier. Both studies concluded that there was a greater variability of the archaea in the soil between sites than in the rhizosphere of different plant genera within a site. Recent studies have shown that the soil Crenarchaeota carry genes, which are involved in ammonia oxidation (Treuch *et al*., 2003) and Leininger *et al*. (2006) have found that the ammonia oxidizing archaea predominate over bacteria in soil. However, in comparison to what is known about soil, plant and fungal associated bacteria, very little is known about the archaea found in these habitats.

2. Aims and hypotheses

The aims of this study were to identify the archaeal groups in different mycorrhizospheric compartments of the organic top soil layer of the boreal forest soil ecosystem. This study also aimed to investigate the association between ectomycorrhizal fungi and the archaea in the soil.

In this study, the following hypotheses were tested:

1. The frequency and diversity of non-extreme archaea is higher in the boreal forest tree mycorrhizospere than in the non-mycorrhizal tree roots and humus (Paper I & II)

2. The species of ECM fungi colonising Scots pine roots have an impact on the archaeal community structure of the mycorrhizosphere (Paper II)

3. Archaeal populations are affected by the tree species and the colonisation of the tree roots by the ECM fungus *Paxillus involutus* (Paper III)

4. The boreal forest mycorrhizosphere archaea have a role in both anaerobic and aerobic C-1 cycling in boreal forest soil (Paper IV)

3. Material and methods

3.1 Sampling site (Papers I, II, III, IV)
Sample humus for all publications and the naturally grown seedlings for paper IV were collected from an approximately 70-80 years old dry Scots pine (*Pinus sylvestris* L.) dominated forest of *Vaccinium*-type (VT) in southern Finland (60°28' N, 23°45' E) .

3.2 Plant and fungal material
Tree species, all of which are common in Finnish forests

Scots pine *Pinus sylvestris* L.	Paper I, II, III, IV
Norway spruce *Picea abies* (L.) H. Karst.	Paper III
Silver birch *Betula pendula* Roth	Paper III
Common alder *Alnus glutinosa* (L.) Gaertner	Paper III

EM fungi used in the study

Suillus bovinus (L.: Fr.) O. Kuntze, isolate SBH1	Paper I, II
Paxillus involutus Batsch: Fr., isolate PIL1	Paper I, II, III
Tomentellopsis submollis (Svrcek) Hjortstam	Paper II
Telephora terrestris Pers.: Fr.	Paper II
Piloderma fallax (Libert) Stalpers, isolate PC MIK	Paper II
Mixed indigenous mycorrhiza	Paper IV

3.3 Synthesis of test mycorrhizospheres (Papers I, II, III)
Sterilized seeds were germinated on glucose (10%) agar plates, protected from light at 20ºC between 10-14 days before they were planted in sterile 100 ml test tubes containing sterile expanded clay pellets (Timonen *et al.*, 1993). To produce the mycorrhizospheres, roots of the small seedlings were aseptically inoculated with ECM fungal mycelium. These seedlings and corresponding non-inoculated seedlings were grown in light with the roots in dark for 1.5 months before being transferred to humus. Fully developed mycorrhizospheres were produced in thin microcosms (as in Finlay and Read, 1986) containing natural, non-sterilized sieved boreal forest humus (Fig 2). The microcosms were grown for 1.5 – 2.5 months with a 20 h photoperiod, photon influence rate 250 µmol m^{-2} s^{-1} and temperature of 15-20ºC for shoots and 10ºC for roots. Microcosms were kept moist with distilled water spray.

3.4 Anaerobic processing of samples (Paper IV)
The three young Scots pine seedlings used in Paper VI were collected together with a 15 cm diameter soil core 10 cm deep through the humus layer. The soil cores were immediately put in anaerobic jars with oxygen removing GasPak Pouch® System. The soil cores were kept at +4°C over night and processed the next day in an anaerobic chamber. Composite samples of indigenous mature mycorrhizas and humus uncolonised by visible fungal hyphae were sampled.

3.5 Anaerobic enrichment cultures (Paper IV)
Mycorrhizal samples were homogenized in an anaerobic chamber in microcentrifuge tubes with sterile quartz sand before each sample was transferred to a sterile anaerobically prepared test tube, with a butyl rubber stopper, containing 9 ml anaerobic water. The tubes were vortexed three times for one minute to release the microbes in the water before 0.1 ml samples were anaerobically and aseptically removed with a syringe and needle. The samples were immediately injected into sealed 120-ml infusion bottles through the butyl rubber stopper. The bottles contained 50 ml liquid growth medium. A yeast extract medium and a mineral medium was used for the cultivations. The yeast extract medium was only used for anaerobic enrichments (with H_2+CO_2 or CH_4 added to the atmosphere), but the mineral medium was tested both anaerobically (with H_2+CO_2 or CH_4) and

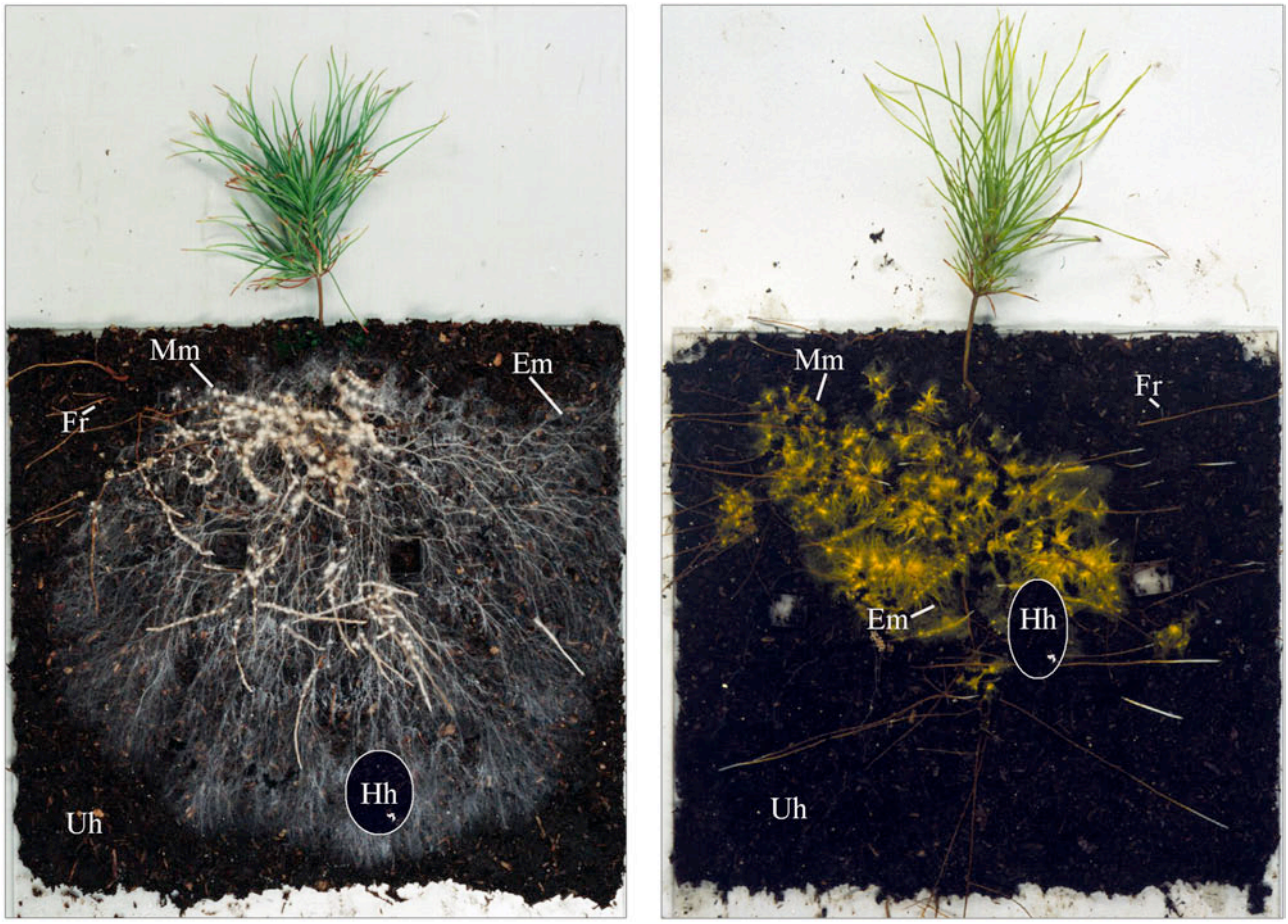

Fig 2. Two Scots pine microcosms used in the study, to the left with *Suillus bovinus*, and to the right with *Piloderma fallax*. Fr=fine root, Mm=mature mycorrhiza, Em=external mycelium, Hh=hyphal humus, i.e. the humus below the hyphal layer, Uh=humus uncolonised by mycorrhizal fungakl hyphae.

aerobically (with CH_4 or CH_3OH). Resazurin (0.01%) was used as redox indicator in the anaerobic enrichments. The sealed enrichment cultures were incubated for a total of 93 days at 18°C (150 rpm).

3.6 Sampling for DNA extraction (Papers I-IV)
Replicate microcosms, five or ten of each type, were sampled (Papers I-III). A composite sample consisting of 10 mycorrhizal root tips (mature mycorrhiza, Mm, 0.02 g fw) were collected with sterile forceps from each mycorrhizal microcosm. Additionally, for papers I and II, ECM mycelium (Em) over an area of 10 cm^2, was collected from the mycorrhizal microcosms. A composite sample of 10-15 non-mycorrhizal root tips (fine roots, Fr, 0.02 g fw) were collected from each non-mycorrhizal microcosm. Of the mycorrhizosphere-soil interface, i.e. the hyphal humus (Hh), and the uncolonised humus (Uh) outside the mycorrhizoshere, 0.2 g (fw) samples were collected. All samples were kept at -80ºC until DNA extraction. From the enrichment cultures of the mycorrhizal samples, 1 ml subsamples were retrieved for DNA extraction on nine time points (Paper IV). The samples were immediately centrifuged in order to collect the microbial cells. The culture media were discarded and the microbial cell pellets were resuspended in 50 µl sterile double distilled water and frozen at -20°C until use after all samples were collected after 93 days.

3.7 DNA extraction (Papers I-IV)
DNA was extracted from the plant root, fungal and soil samples used in Papers I-III, and samples of the H_2+CO_2 treated mycorrhizal enrichment cultures (Paper IV) with the MoBio Soil DNA Extraction kit (Cambio) according to the manufacturer's instructions, except that the Fr, Mm and

Em samples were ground with quarts sand in 50 µl sterile double distilled water to disrupt the plant and fungal tissues prior to DNA extraction. The humus samples did not require any pre-extraction treatment. The prepared and stored samples of the enrichment cultures were boiled at 105°C for ten minutes to release the DNA prior to PCR. All samples were frozen at -20°C between uses.

3.8 PCR (Papers I-IV)
A nested PCR approach was used throughout the study to detect archaeal 16S rRNA genes. Four different combinations of primer sets were used (Table 1). PCR reactions were performed with Red Hot (AB) or Phusion PCR polymerase (Finnzymes) according to the manufacturer's instructions. The size and quality of the PCR products were checked on 1% agarose with ethidium bromide according to standard protocols (Sambrook and Russell, 2001).

3.9 Cloning and RFLP (Paper I)
PCR products of desired size were cleaned with a Wizard PCR purification kit (Promega) and cloned with the pGem-T cloning kit with $CaCl_2$ competent JM109 cells (Promega) according to the manufacturer's instructions. Up to 30 clones from each sample were screened. The plasmids were isolated by alkaline lysis (Sambrook and Russell, 2001). The inserts were reamplified with primers 3f and 9r and subsequently digested with restriction enzymes *Hinf*I, *Msp*I and *Rsa*I (Promega). The digests were electrophoresed in a 2.3% Metaphor agarose gel (SeaKem) in 1xTAE at 100V for 3 h, stained with ethidium bromide and imaged. The RFLP patterns were visually evaluated and divided into groups. One representative of each group was chosen for sequencing.

3.10 Denaturing Gradient Gel Electrophoresis analysis (Papers II-IV)
Nested PCR products of approximately 800 bp in length were separated by denaturing gradient gel electrophoresis (DGGE) in a 6% acrylamide/bis (37.5/1) gel with a 40-60% urea-formamide gradient. The electrophoresis was run at 65 V for 17 h at 60°C in a DCode Universal Mutation Detection System (Bio-Rad). The gels were stained with silver nitrate (Papers II and VI) or SybrGreen II (Promega) (Paper III). Bands were divided into groups of operational taxonomic units (OTUs) according to their mobility compared to a 1 kb molecular size standard (Invitrogen). DGGE bands chosen for sequencing were excised from the gels with a sterile scalpel and the DNA was eluted using the 'crush and soak' method (Peters *et al.*, 2000).

3.11 Sequencing (Papers I-IV)
For paper I, plasmids were isolated from overnight 5-ml liquid cultures with a Qiagen plasmid mini kit and the inserts were sequenced with primers T7 and Sp6. For papers II to IV, isolated DGGE fragments were reamplified for sequencing with different methods. In paper II and IV, two different PCR reactions, dividing the PCR fragment in two, one with primers 3f and 514r (Jurgens *et al.*, 2000), and one with 344f (Rinçón *et al.*, 2006) and 9r, were used. In paper III, the same nested primer pair used for the original amplifications (A109a - A934b or A109a - Ar9r) was used. The sequencing reactions were performed with primers Ar514r and Ar344f (See Table 1 for primer references).

After an initial sequencing of one representative band from each OTU group, two or more parallel bands from different samples were chosen for sequencing to test the reproducibility of the DGGE.

The sequences were edited with the program package Vector NTI® (InforMax, Invitrogen) and compared to all archaea and bacteria 16S rRNA gene sequences in the DDBJ/EMBL/GenBank databases with Blastn. The sequences were tested for chimeras with the Chimera Check tool of the Ribosomal Database Project II (http://rdp.cme.msu.edu/index.jsp). Possibly chimeric, unspecific and bacterial sequences were subtracted from further analysis.

Table 1. The primer combinations used in the PCRs.

combination	Primers	Primer sequence	Position *E. coli* numbering	Reference	Used in publications
1	Archaea Ar4f Ar958r	 5'-TCYGGTTGATTCTGCCRG-3' 5'-YCCGGCGTTGAVTCCAATT-3'	 8-25 918-936	Hershberger *et al*., 1996 DeLong, 1992	Paper I
	Archaea Ar3f Ar9r	 5'-TTCCGGTTGATCCTGCCGGA-3' 5'-CCCGCCAATTCCTTTAAGTTTC-3'	 7-26 906-927	 Jurgens *et al*., 1997 Jurgens *et al*., 1997	
2	Archaea Ar3f Ar9r	 5'-TTCCGGTTGATCCTGCCGGA-3' 5'-CCCGCCAATTCCTTTAAGTTTC-3'	 7-26 906-927	 Jurgens *et al*., 1997 Jurgens *et al*., 1997	Paper II, III[a2] & IV[b1]
	Archaea A109a A934b	 5'-ACKGCTCAGTAACACGT-3' 5'-GTGCTCCCCCGCCAATTCCCT-3'	 109-125 915-934	 Groβkopf *et al*., 1998 Stahl and Amann, 1991	
3	Universal 8f (fD2) 1512r (rP2)	 5'-AGAGTTTGATCATGGCTCA-3' 5'-CCGGCTACCTTGTTACGACTT-3'	 8-26 1492-1512	 modified from Weisburg, 1991	Paper III[a1] & IV[b2]
	Archaea A109a A934b	 5'-ACKGCTCAGTAACACGT-3' 5'-GTGCTCCCCCGCCAATTCCCT-3'	 109-125 915-934	 Groβkopf *et al*, 1998 Stahl and Amann, 1991	
4	Archaea Ar3f A934b	 5'-TTCCGGTTGATCCTGCCGGA-3' 5'-GTGCTCCCCCGCCAATTCCCT-3'	 7-26 915-934	 Jurgens *et al*., 1997 Stahl and Amann, 1991	Paper III[a3]
	Archaea A109a Ar9r	 5'-ACKGCTCAGTAACACGT-3' 5'-CCCGCCAATTCCTTTAAGTTTC-3'	 109-125 906-927	 Groβkopf *et al*, 1998 Jurgens *et al*., 1997	

[a1] primer combination 1 in Paper III [b1] primer combination 1 in Paper IV
[a2] primer combination 2 in Paper III [b2] primer combination 2 in Paper IV
[a3] primer combination 3 in Paper III

3.12 Phylogenetic analyses (Papers I-IV)

The phylogenetic analyses of the sequences for Paper I were done using ARB (http://www.arb-home.de/). The 16S rRNA gene sequences in papers II-IV were aligned using the ClustalW alignment tool (http://www.ebi.ac.uk/) and the alignments were checked and manually edited using BioEdit alignment editor (version 7.0.5 © Tom Hall). The maximum parsimony and maximum likelihood analyses on the sequences in Paper II were performed with both WinClada (Nixon, 1999) and Paup 4.0b 10 (Swofford, 2003) (Sinauer Associates, Sunderland, MA, USA) and in Papers III and VI only with Paup 4.0b 10. The heuristic search parameters in Papers II-VI consisted of 10 repeats of step-wise addition of random sequence with a tree-bisection-reconnection (TBR) branch-swapping algorithm. A maximum of 10,000 trees was set for the maximum parsimony analyses. A consensus bootstrap tree was calculated for both maximum parsimony and maximum likelihood cladograms on 1,000 bootstrap replicates.

3.13 Statistical analyses (Papers II-IV)
The statistical analyses were performed with the InStat package (GraphPad Software Inc.). The statistical significance of differences between the frequencies of occurrence of OTUs in different sample types in Papers II and III was analysed by Kruskal-Wallis nonparametric one-way analysis of variance (ANOVA) and posthoc analysis by Dunn's multiple comparisons test. The Mann-Whitney test was performed on the differences in methane production in mycorrhizal samples compared to uncolonised humus (Paper IV). The multidimensional scaling (MDS) analysis in Paper II was performed with the PAST programme package (Hammer *et al.*, 2001).

3.14 Detection of methanogens in culture suspensions by microscopy (Paper IV)
Methanogenic archaea were detected by fluorescence microscopy as described by Doddema and Vogels (1978). A 0.5 ml sample was taken from the anaerobic yeast extract enrichments with H_2+CO_2 of the mycorrhizal samples on day 4. The microbial cells were concentrated by centrifugation prior to microscopy. *In vivo* detection of methanogens in the cultures was performed by epifluorescence microscopy with a Zeiss Axioscope 2 plus, equipped with UV light and a no. 31016 filter (Chroma Technology). The auto-fluorescent coenzyme F_{420}, which is involved in methanogenesis, has a strong blue fluorescence when exited with 420 nm UV light. Presence of methanogens was registered as strong blue autofluorescence.

3.15 Production of methane (Paper IV)
The methane production in the mycorrhizal and humus enrichment cultures on yeast extract medium amended with H_2+CO_2 was examined on days 0, 7, 14, 35 (Mm only) and 93. A 1 ml sample was collected from each bottle with a syringe and needle and was injected into a Venoject (Terumo®, Medicool, Inc.) vacuum blood collection tube for subsequent analysis. Methane was measured by gas chromatography (Varian 3700, GenTech) using a 1 m long Porapack T 80/100-mesh column. Helium was used as carrier gas, and the injector, column and detector were worked at 150°C, 40°C and 200°C, respectively. The methane amount of each gas sample was compared to three standards, 0.1%, 1% and 10%.

4. Results and Discussion

4.1 Influence of the primer choice on the detected archaeal diversity

During the course of these experiments, several different primer combinations were used in a nested PCR approach (Table 1). The combination of primers had a great impact on the numbers and types of archaea detected. Primer combination 1 (Paper I) was specific for archaea and provided very stringent PCR conditions. This resulted in a PCR product from only a low number of samples and a low diversity of archaeal 16S rRNA gene sequences. However, no unspecific PCR amplification was detected. With primer combination 2 (used in Paper II) a PCR product was obtained from a high number of the different mycorrhizospheric samples of most of the tested ECM fungi. A considerable diversity of archaeal 16S rRNA genes was obtained, with only a low degree of unspecific amplification. However, the *P. involutus* mycorrhiza and external mycelium did not appear to have any archaea when tested with primer combination 2. *P. involutus* mycorrhizas and fine roots of four different forest trees were tested with three different primer sets in Paper III. The least stringent primer set 3 revealed a great diversity of Crenarchaeota in the *P. involutus* mycorrhizosphere of all tested trees, whereas the more stringent primer sets 2 and 4 did not. However, the drawback was the contaminating detection of bacterial 16S rRNA genes in these samples. Primer set 4 was the primer combination chosen for the most optimal detection of archaeal 16S rRNA genes, with relatively high specificity to archaea, although both primers A109a and A934b amplified bacterial 16S rRNA genes when used in primer combination 3. However, primer combination 4 resulted in archaeal PCR products only from non-mycorrhizal alder roots. The diversity of DGGE bands obtained from the alder roots with primer combination 4 exceeded that of even the mycorrhizas. Interestingly, primer set 4 did not detect any archaeal 16S rRNA genes in any other type of sample, including the alder mycorrhiza. In Paper IV, all sequenced DGGE bands obtained with primer set 2 were archaeal. However, in contrast to the results in Paper III, primer combination 3 resulted in mostly bacterial sequences, with only a few archaea, all of which were Euryarchaeota. Many samples, especially the bulk soil and non-mycorrhizal fine roots, did not display any PCR products with any of the primer combinations used. Humus generally contains high levels of PCR inhibiting agents, which might have been the reason for the low PCR success with the used primer sets. However, most of these samples were also amplified with universal bacterial primers 986f and 1378r (Heuer *et al.*, 1997) and resulted in PCR products (data not shown). The results presented above clearly show, as it has been suggested before, that to obtain a conclusive picture of the uncultured microbial community, several primer pairs should be used (Banning *et al.*, 2005; Juottonen *et al.*, 2006; Mahmood *et al.*, 2006).

4.2 Frequency and diversity of archaea in the mycorrhizospheres of boreal forest soil

Archaeal 16S rRNA gene sequences were most readily detected in mycorrhizas and external mycelia. The number of archaeal cells in the mycorrhizosphere of boreal forest trees was not directly estimated. However, a hypothetical estimation of the minimum number of archaeal cells detectable with the primer combinations used may be drawn from the diversity of archaeal OTUs detected in the samples. The DNA of each mycorrhizal sample (0.02 g fw) was extracted into 50 µl solution and 1 µl extract was used in each PCR reaction. The mycorrhizal samples generated a mean number of five different DGGE bands in the DGGE run, although some showed over 10 archaeal DGGE bands while others considerably less. If each DGGE band originated from at least one archaeal cell, the 1 µl DNA sample for the PCR would have contained the 16S rRNA genes from at least five archaeal cells, if the archaea have only one copy of the 16S rRNA gene and the PCR amplified fragments of these genes were optimally resolved in the DGGE. This would mean that the original composite sample of mycorrhizas may have contained at least 250 archaeal cells and subsequently a 1 g (fw) sample 1.25×10^4 archaeal cells. This is an extremely conservative

estimation based on direct DNA extraction giving the absolute minimum number of archaeal cells, and is not comparable with analyses done with standardized quantitative methods. In enrichment cultures, the archaea that appear below the detection limit for the methods employing direct DNA extraction, grow in numbers and can be detected in the DGGE analysis. The enrichment cultured in Paper IV displayed a higher number of DGGE bands compared to the direct DNA extraction method combined with PCR amplification and DGGE used in papers II and III. The samples used in the enrichment cultures in paper IV showed at least 30 different archaeal OTUs obtained from different enrichment conditions. Following the reasoning above, the enrichment cultures point to an archaeal cell number of 1.3 x 10^5 per g mycorrhiza. The non-mycorrhizal pine roots and humus uncolonised by fungal hyphae generally had the lowest archaeal populations. None of the five replicate samples in paper I, and only one sample in paper II, revealed an archaeal 16S rRNA gene sequence. The calculations used above leads to an estimation that the non-mycorrhizal pine roots, where DGGE bands were detected, harboured a maximal archaeal population of 2.5 x 10^3 cells per gram (fw) sample. However, most non-mycorrhizal pine root samples had no archaeal PCR products and this suggests a lower archaeal population in this habitat. Nevertheless, the non-mycorrhizal roots of alder (Paper III) displayed an archaeal population of the same magnitude (10^4) as for the mycorrhizas in papers II and III. The archaeal number in uncolonised humus was low. The 0.2 g humus samples only rarely displayed archaeal PCR products, but occasionally, a humus sample presented a high number of archaeal DGGE bands (Paper II). If the 0.2 g sample contained 1-2 archaeal DGGE bands, and one band was derived from at least one cell, the uncolonised humus would contain only 2.5-5 x 10^2 cells per gram (fw) sample. However, since the humus samples were collected from artificial microcosms, the results do probably not display the situation of natural, pristine soil. In the forest soil, mycorrhizal fungi are not the only group of organisms affecting soil microbial communities, as decomposing fungi also play an important role in the release of nutrients to the nutrient cycles. However, only the mycorrhizal fungi were added to the microcosms, while the indigenous decomposers and soil animals were excluded from the interpretation of the results. An interesting experimental design for future research would be to compare the impact of the mycorrhizal fungi, which spread the primary produced carbon compounds into the soil, to that of decomposing fungi, which release nutrient from organic material, on the archaeal populations in forest humus. These estimations of archaeal numbers in mycorrhizospheric, rhizospheric and soil habitats are extremely speculative and are highly affected by the primers used in each study. However, they may give some indication of the archaeal abundance in the mycorrhizosphere and humus, and help in the outlining of further experiments.

The hypothetical estimations of archaeal cell numbers in the ectomycorrhizisphere and boreal forest soil microcosms are several magnitudes smaller than the numbers of archaeal 16S rRNA genes detected in other studies. Kemnitz *et al.* (2007) calculated by quantitative PCR that the number of archaeal 16S rRNA genes in the upper layers of a temperate mixed deciduous forest in Germany to be as high as 0.5 to 3.9 x 10^8 per g dry soil. These numbers are not directly comparable to the crude estimations based on DGGE results as the quantitative PCR is a more accurate method designed for quantification. Sandaa *et al.* (1999) calculated by FISH, that a Norwegian agricultural field had between 2.6-4.2 x 10^7 archaeal cells per gram soil, corresponding to 1-2% of the microbial population. The same level of archaeal relative abundance has been reported in other agricultural and field soils as well (Buckley *et al.*, 1998; Ochsenreiter *et al.*, 2003). In the German deciduous forest soil, Kemnitz *et al.* (2007) showed that archaea constituted a considerably greater part of the prokaryotic community (12-38%) than that detected in the agricultural field soil. No comparisons between the archaea:bacteria ratio in boreal forest soils have yet been reported.

The majority of the archaeal 16S rRNA genes detected in the boreal forest tree mycorrhizospheres and humus belonged to 1.1c Crenarchaeota. This group was also the most abundant type of archaea detected by Kemnitz *et al.* (2007) and constituted up to 85% of the archaeal community in the

deciduous forest soil. However, the majority of archaea found in other types of soils, such as tropical forest soil, emerging glacier front soil, sandy grass field soils, agricultural soil, belonged to 1.1b Crenarchaeota (e.g. Buckley *et al.*, 1998; Ochsenreiter *et al.*, 2003; Nicol and Schleper, 2006). In a study by Burke *et al*. (2002) the effect of AM mycorrhiza on the abundance of rhizosphere microorganisms was tested with *Spartina patens* growing in a salt marsh. Archaeal cell numbers of a magnitude of 10^7 cells per gram soil dw were estimated by FISH. It was also found that the abundance of AM fungi in the salt marsh soil had little effect on the number of archaeal cells. The type of archaea was not identified by Burke *et al*., but compared to the study by Purdy *et al*. (2004) in a similar habitat, it is likely that the archaea detected in the hybridisation experiments were euryarchaeotal.

Simon *et al*. (2000) used epifluorescence microscopy to show that the population of 1.1b Crenarchaeota on tomato roots was as high as 200 crenarchaeotal cells per rootlet. However, only the senescent tomato roots reached this number. Generally, the rootlets presented between three and ten crenarchaeotal cells per rootlet. This is in agreement with the estimations for ectomycorrhizas and non-mycorrhizal alder roots presented above.

In the microcosm experiments, archaea were found in all compartments of the tree rhizosphere, mycorrhizosphere and humus. There was a clear difference in archaeal community composition and detection frequency between the different compartments. With only a few exceptions, the detection frequency and diversity of archaeal OTUs (RFLP groups or DGGE bands) was highest in the mycorrhizas and lowest in the non-mycorrhizal fine roots and humus devoid of fungal hyphae. The average number of different archaeal OTUs in the mycorrhizas and some non-mycorrhizal roots was between five and 10. Our results agree with those of Sliwinski and Goodman (2004) who found similar diversity of crenarchaeal OTUs in plant roots. As in Sliwinski and Goodman (2004), our results indicated a lower diversity of archaeal 16S rRNA genes in the bulk soil than in the rhizosphere and mycorrhizosphere, although a higher number of bulk soil samples would be needed for definitive conclusions. The non-mycorrhizal roots of boreal forest trees have been reported to sustain a high diversity of bacteria (e.g. Timonen *et al.*, 1998; Timonen and Hurek, 2006) that was generally not the case with archaea.

4.3 Phylogeny of boreal forest mycorrhizosphere archaea

All Crenarchaeota found in the boreal forest soil and mycorrhizosphere so far belong to the group 1.1c Crenarchaeota although the archaea generally found in soils belong to the 1.1b group (Fig 3). The found 1.1c Crenarchaeota divide on three major groups of which the majority cluster with FFSB type Crenarchaeota. This is the most commonly found type of Crenarchaeota detected in the boreal forest soil which were first found in clear-cut and burned coniferous forest soil (Jurgens and Saano, 1999). A few of the sequences detected in this study also fell with the group FFSC which contains sequences originally found in humus from an undisturbed coniferous forest (Jurgens and Saano, 1999), and with the so-called 1.1c associated group FFSB6, both of which appear to be much rarer than the FFSB group. Only little research has so far been performed on the 1.1c Crenarchaeota, because their global distribution is limited to mostly acidic forest or field soils, where their population densities usually are low. The crenarchaeotal 16S rRNA gene sequences obtained from non-mycorrhizal fine roots of the different tree species fall into a separate cluster of the 1.1c Crenarchaeota than the ones from mycorrhizas (Fig 3). This further implies that the archaeal populations in the different rhizospheric and mycorrhizospheric habitats may have different functions.

Two types of euryarchaeotal 16S rRNA gene sequences were found in the boreal tree mycorrhizospheres. The most frequently detected euryarchaeotal genes showed high similarity to


Thermophilum pendens, X14835
VAL151, boreal forest lake, AJ131316
ARR29, rice rhizosphere, Aj227941
pGrfC26, freshwater lake sediment, U59986
ZmrA1, maize roots, AF226271
ZmrA4, maize roots, AF226270
Ar9, marine sponge, AF186424
Cenarchaeum symbiosum, marine sponge, U51469
WHARQ
Nitrosopumilus maritimus, marine water, DQ085097
KBSCul1, soil, AF058719
SCA1145, soil, U62818
TRC132 9, tomato roots, AF227639
SCA1173, soil, U62818
SCA11, soil, U62820
TRC23_10, tomato roots, AF227640
54D9, calcerous grassland soil, AY278106 AY27
PsPiHh17, Scots pine mycorrhizosphere, DQ460001
PsPiHh18, Scots pine mycorrhizosphere, DQ460002
GFS1 9500i, glacier front soil, AY601285
FFSB6, boreal forest soil, X96693
FFSA2, boreal forest soil, Y08985
FFSA1, boreal forest soil, Y08984
MFT10-C2, boreal coniferous afforestated humus, DQ449486
MFT8-A5, boreal coniferous afforestated humus, DQ449491
FFSB7, boreal forest soil, X96694
FFSB4, boreal forest soil, X96694
PsPfHh16, Scots pine mycorrhizosphere, DQ460000
BpSr2-1, Silver birch
FHMa19, boreal forest humus, AJ428040
FFSC3, boreal forest soil, AJ006921
FSBH03-24, boreal forest humus, AJ419924
PsSbHh19, boreal forest humus and mycorrhizosphere, DQ460003
FHMa22, boreal forest humus, AJ428043AJ
PsMm-AnYH2-11, Scots pine
PsSbHh21, Scots pine mycorrhizosphere, DQ460005
AgFr46-2, Alder, AM903367
Uh35, boreal forest humus, DQ460015
FHMa10, boreal forest humus, AJ428032
AgFr10-2, Alder, AM903371
FFSB10, boreal forest soil, X96695
PSPIMM06-4, boreal forest mycorrhizosphere, AJ419922
AgFr41-2, Alder, AM903370
AgFr43-2, Alder, AM903368
FHMa13, boreal forest humus, AJ428034
PaFr1-1, Norway spruce, AM903366
AgFr8-2, Alder, AM903369
PSPIMM06-11, boreal forest humus and mycorrhizosphere, AJ419918
FFSB11, boreal forest soil, X96696
AgFr68-3, Alder, AM903364
AgFr66-3, Alder, AM903365
AgFr80-3, Alder, AM903363
Alder root
PSPIHH01-10, boreal forest humus and mycorrhizosphere, AJ419923
FHMa17, boreal forest humus, AJ428039
FHMa5, boreal forest humus, AJ428027
PSSBEM10-2, Scots pine mycorrhizosphere, AJ419925
PSPIHH28-7, boreal forest mycorrhizosphere, AJ419920
PSPIMM06-4, boreal forest mycorrhizosphere, AJ419922
Uh6, boresl forest humus, DQ460016
PsSbMm6, Scots pine mycorrhizosphere, DQ459997
PsSbMm5, Scots pine mycorrhizosphere, DQ459996
PsTsMm2, Scots pine mycorrhizosphere, DQ459995
PsSbEm22, Scots pine mycorrhizosphere, DQ460017
PsMm-AnYH2-1, Scots pine
AgPiMm20, Alder, AM903350
PsPfHh, Scots pine mycorrhizosphere, DQ460007
PsPfHh, Scots pine mycorrhizosphere, DQ459998
GFS8i 9500, glacier front soil, AY601302
GRU11, freshwater lake, AY278095
PsMm-OxMCH4-1,I Scots pine
ArcB_cG01, soil, EU306965
GRU10, freshwater, AY278081
GFS10-9500ii, glacier foreland soil, AY601306
JG36-GR-140, mining waste, AJ535136
PsPfHh24, Scots pine mycorrhizosphere, DQ460008
PsMm-AnYCH4-5, Scots pine
PsSbHh27, Scots pine mycorrhizosphere, DQ460011
PSPIHH28-2, boreal forest mycorrhizosphere, AJ419921
PsSbHh28, boreal forest mycorrhizosphere, DQ460012
PsSbHh, Scots pine mycorrhizosphere, DQ460009
PsPfHh, Scots pine mycorrhizosphere, DQ460006
GFS9 135i, glacier foreland soil, AY601303
PsPbHh14, Scots pine mycorrhizosphere, DQ459999
Elev_16S_arch_1367,Trembling aspen soil, EF022050
Elev_16S_arch_1331, Trembling aspen soil, EF022015
Elev_16S_arch_1461,Trembling aspen soil, EF022133
FFSB1, boreal forest soil, X96688
Elev_16S_arch_1415, Trembling aspen soil, EF022092
FRC0, Front Range soil, Colorado, AY016487
Ha5, soil, DQ195672
FRD38, Alpine tundra soil, Colorado, AY01650
PsPiMm73-1, Scots pine mycorrhiza, AM 903348
S248-7, German forest soil, AY278100
A109-10, temperate deciduous forest soil, AM291984
A109-07, temperate deciduous forest soil, AM291983
PsSbHh29, Scots pine mycorrhizosphere, DQ460013
A109-05, temperate deciduous forest soil, AM291982
PsPiMm75-1, Scots pine mycorrhiza, AM903347
PsPiMm76-1, Scots pine mycorrhiza
PsPiMm70-1, Scots pine mycorrhiza
PsMm-OxMCH4-11, Scots pine
PsMm-OxMCH4-9, Scots pine
PsMm-AnYH2-3, Scots pine
PsMm-OxMCH4-12, Scots pine
PsMm-OxMCH4-10, Scots pine
PsMm-AnYCH4-8, Scots pine
PsMm-OxMCH3OH-15, Scots pine
PsMm-OxMCH3OH-1, Scots pine
PsMm-OxMCH4-13, Scots pine
PsMm-OxMCH4-8, Scots pine
PsMm-AnYCH4-13, Scots pine
AgPiMm13-1, Alder, AM903360
PsMm-OxMCH4-3, Scots pine
PsMm-OxMCH3OH-7, Scots pine
PsMm-AnYH2-5, Scots pine
PsPiMm9-1, Scots pine mycorrhiza, AM903359
PsMm-AnYH2-9, Scots pine
AgPiMm19-1, Alder, AM903352
PaPiMm24-2, Norway spruce, AM903353
BpPiMm15-1, Silver birch, AM903361
PsMm-OxMCH4-7, Scots pine
PsMm-AnYH2-10, Scots pine
AgPiMm25-1, Alder, AM903356
BpPiMm16-1, Silver birch, AM903351
PsPiMm11-1, Scots pine mycorrhiza, AM903357
PsMm-AnYH2-7, Scots pine
PaPiMm10-1, Norway spruce, AM903354
AgPiMm3-1, Alder, AM903355
BpPiMm16-1, Silver birch, AM903351
PsPiMm7-1, Scots pine
PsPiMm5-1, Scots pine
PsMm-AnYH2-6, Scots pine
PsMm-AnYH2-2, Scots pine
PsMm-AnYCH4-4, Scots pine
PsMm-OxMCH3OH-17, Scots pine
PsMm-AnYH2-4, Scots pine
PsMm-OxMCH4-2, Scots pine
PsPiMm6-1, Scots pine
BpPiMm37-1, Silver birch, AM903349
10
1.3
1.1a
1.1b
FFSB6
FFSC
Root
Mycorrhiza
1.1c Crenarchaeota

the 16S rRNA gene sequences belonging to the genus *Halobacterium*. The sequences of putatively moderate halophilic Euryarchaeota retrieved from other moderate environments, Zodletone Spring (Oklahoma, USA) (Elshahed et al., 2004) and open mud pans of Colne estuary marshes (Essex, UK) (Purdy et al., 2004), did not cluster with the sequences from this study. The other type of euryarchaeotal 16S rRNA gene sequences found belonged to the genus *Metanolobus*. This genus belongs to the order Methanosarcinales, which also includes the genera *Methanosaeta* and *Methanosarcina*. This is one of the most versatile groups of methanogens and they are able to use many different substrates for methanogenesis (Smith and Ingram-Smith, 2007). These methanogens have been shown to be aerotolerant to some extent (Erkel et al., 2006) and are often encountered in soils, which may be exposed to oxygen, such as rice (Lueders and Friedrich, 2000) and cereal fields (Poplawski et al., 2007).

4.4 Specific effect of ECM fungal species of on the population of archaea

The species of ECM fungus colonising the roots of forest trees had a great effect on the population of archaea harboured in the mycorrhizospheres. Without an ECM fungus, the Scots pine did not support any detectable archaeal populations on its fine roots. However, when the Scots pine rhizosphere was colonised by ECM fungi, the detection rate of archaea increased. This is interesting, since from studies on bacteria in the roots of Scots pine, the fine roots growing in humus harbour extensive populations of bacteria (Timonen *et al.*, 1998; Timonen and Hurek, 2006).

The most commonly detected archaeal 16S rRNA gene sequence in the habitats provided by the different types of ectomycorrhizal fungi belonged to the genus *Halobacterium* (Paper II). This was the case with all other mycorrhizal fungi tested, except *Paxillus involutus*, where most of the archaeal 16S rRNA gene sequences belonged to the 1.1c Crenarchaeota. The *Halobacterium*-like 16S rRNA genes were exclusively detected in fungal samples, i.e. mycorrhizas, external mycelium and hyphal humus, and never in humus uncolonised by mycorrhizal fungal hyphae, or on non-mycorrhizal fine roots. The halobacterial populations in the mycorrhizospheric compartments were stable, showing the same halobacterial OTUs in several replicate samples. The crenarchaeotal population dynamics were sporadical and displayed mostly unique crenarchaeotal OTUs, i.e. the diversity of Crenarchaeota in hyphal and non-hyphal humus was relatively high although the detection frequency was low. Both the Cren- and Euryarchaeota detected in ectomycorrhizospheres appeared to specifically inhabit mycorrhizal fungi containing habitats. This is in contrast to studies on the frequently detected soil inhabiting 1.1b Crenarchaeota. These Crenarchaeota have been detected on the roots of plants, but do not appear to have specific preferences to certain plant species and they are present in the vicinity of both mycorrhizal and non-mycorrhizal plants (Sliwinski and Goodman, 2004; Nicol *et al.*, 2005).

4.5 Specific effect of tree species on the (mycor)rhizospheric archaeal population

The different tree mycorrhizospheres formed with *P. involutus* all had similar archaeal populations (Paper III), but the tree species tested in this study had profoundly different impacts on the archaeal populations in their non-mycorrhizal roots. The Scots pine fine roots did not harbour any detectable populations of archaea, whereas the alder roots showed the highest diversity of archaeal OTUs detected in this experiment. Alder is known to have nitrogen fixing microbial consortia in its

Fig 3. A maximum parsimony tree on the crenarchaeotal 16S rRNA gene sequences obtained in this study. The tree was calculated using PAUP 4.0 10b. Bootstrap values of 1000 repeats are shown for nodes with > 50% support.

rhizosphere, and to produce root nodules for the purpose, although no nodulation was detected in our experiments. Nevertheless, it has been shown, that alder is capable of capturing nodulating *Frankia* spp. in soils devoid of actinorhizal plants (Maunuksela *et al.*, 1999). It has also been reported that when grown in the same soil, the bacterial population composition in the roots of birch and alder are very different (Elo *et al.*, 2000). Norway spruce and silver birch displayed only a low frequency and diversity of archaea in their non-mycorrhizal fine roots. However, although the Norway spruce and silver birch are known to have very different impacts on the soil in which they grow (Mikola, 1985), their archaeal populations were surprisingly similar. It was clearly shown that the tree species attracted different numbers of archaea and that the archaeal populations were dissimilar in the different tree species. Although the Crenarchaeota inhabiting tree roots cannot yet be determined on species level, the molecular characterisation of the archaeal populations in the different tree roots of this study may still reflect the same results as for the bacteria. When the tree roots were colonised by *P. involutus*, the archaeal communities changed in all tree species into much more similar compositions. Even the Scots pine-*P. involutus* mycorrhizas, which in Paper II did not produce any archaeal OTUs, displayed a considerable archaeal population with the primers used (Table 1).

All archaea found in the non-mycorrhizal roots and most of the ones from the *P. involutus* mycorrhizas were 1.1c Crenarchaeota. However, all sequences from non-mycorrhizal roots fell into a separate cluster from the ones obtained from the mycorrhizas (Fig 3). Additionally, in the phylogenetic analysis, the crenarchaeotal 16S rRNA gene sequences obtained from non-mycorrhizal alder roots formed a separate cluster within the root cluster. These results also agree with those obtained from bacterial studies. Timonen *et al*. (1998) and Timonen and Hurek (2006) have shown that the bacterial communities of non-mycorrhizal boreal forest tree roots are different from the bacterial population in the mycorrhizas. It is likely, that not only the impact of the tree, but also the population of rhizosphere inhabiting bacteria determine the number and composition of the archaeal populations. Archaea and bacteria are known to form syntrophic consortia in other environments (Orphan *et al.*, 2001b; Moissl *et al.*, 2002).

4.6 Anaerobic and aerobic archaea in the boreal forest mycorrhizosphere

Group 1.1c Crenarchaeota were enriched in C-1 cycling microbial communities of boreal forest tree mycorrhizas (Paper IV). They were detected in both aerobic and anaerobic enrichment cultures. The only carbon sources provided were CH_4 or CH_3OH (only aerobic) and CO_2. In anaerobic yeast extract media, to which H_2 or CH_4 as well as CO_2 were added, a higher frequency of 1.1c Crenarchaeota were detected than in the anaerobic mineral media. The aerobic media amended with CH_4 in turn, showed twice the number of DGGE bands, compared to any of the anaerobic enrichments. It appears that the 1.1c Crenarchaeota require more complex growth media for anaerobic growth compared to aerobic growth. The results obtained from the enrichment study suggest that the 1.1c Crenarchaeota may have a role in both anaerobic and aerobic cycling of C-1 compounds. Although no information about carbon metabolism has been presented for the non-thermophilic Crenarchaeota, four different pathways for CO_2 fixiation have been identified in thermophilic Crenarchaeota (Hugler *et al.*, 2003). These metabolic pathways also reflect the phylogenetic affiliation of the organisms. It is possible that the non-thermophilic 1.1c Crenarcaeota harbour yet another type of autotrophic CO_2 fixation. 1.1a and 1.1b Crenarchaeota have been shown to grow aerobically (Simon *et al*., 2005; Könneke *et al*., 2005) and to oxidize ammonia (Könneke *et al.*, 2005) or at least harbour the necessary genes for ammonia monooxygenase (Treusch *et al.*, 2005; Hallam *et al.*, 2006a). Additionally, it has been suggested that the archaea probably play a greater role in the ammonia oxidation in soils than bacteria (Leininger *et al.*, 2006). No such study has yet been reported for the 1.1c Crenarchaeota. It is possible that the 1.1c Crenarchaeota, which are also phylogenetically distant from the other Crenarchaeota, do not participate in the oxidation of

ammonia. The mycorrhizal fungi have a very efficient nitrogen metabolism and the plants also prefer ammonia, which might out-compete the archaeal ammonia oxidisers. This competition for ammonia may be one possible explanation for the lack of the commonly found 1.1b Crenarchaeota in acidic boreal forest soils. The possibility of ammonia oxidation can not be excluded, but the detection of numerous 1.1c crenarchaeotal 16S rRNA genes in the C-1 cycling communities indicated that the 1.1c Crenarchaeota may rather be involved in carbon cycling than in the oxidation of ammonia.

Halobacterial 16S rRNA genes were identified in the non-saline anaerobic yeast extract enrichment cultures when CH_4 was either added or produced. They did not appear in any of the enrichment cultures on mineral media. This is in accordance with studies on extremely halophilic members of the Halobacteriales, which are known to be chemoorganotrophs (e.g. Gruber *et al.*, 2004; Yang *et al.*, 2006). Members of the genus *Halobacterium* are generally extremely halophilic. Moderately halophilic strains of typically halophilic genera have previously been isolated and detected by molecular methods from estuarine mud flats (Purdy *et al.*, 2004) and a fresh water spring (Elshahed *et al.*, 2004), but the isolated strains still required at least 2.5% NaCl (Purdy *et al.*, 2004). None of the 16S rRNA gene sequences of these new isolates clustered with the mycorrhizospheric halobacterial sequences in the phylogenetic analyses. The halobacterial 16S rRNA gene sequences were only detected in anaerobic enrichments on yeast extract media where CH_4 was either added to or produced in the enrichments. These results suggest that the halobacteria have a role in the anaerobic cycling of methane, either by metabolising it directly or by using derivatives produced by other microorganisms in the consortia. The halobacterial 16S rRNA sequences detected in the enrichment study (Paper IV) were all from anaerobic cultures. The Halobacteriales are generally aerobic microorganisms (Oren, 1999). However, the solubility of oxygen in hypersaline waters is low and the water often become anaerobic. Many of the Halobacteriales have been shown to have the capacity for anaerobic growth (Gruber *et al.*, 2004; Muller and DasSarma, 2005; Yang *et al.*, 2006), especially in the presence of certain amino acids. The medium in Paper IV was based on yeast extract, which contains amino acids, and may in the future be used for isolation of the non-halophilic *Halobacterium*-like Euryarchaeota residing in the boreal forest ectomycorrhizas.

The 16S rRNA genes of methanogenic archaea were detected in the mycorrhizas of boreal forest trees. Two 16S rRNA gene sequences, both belonging to the genus *Methanolobus*, were obtained by DGGE. *Methanolobus* belongs to the order Methanosarcinales, which comprises of species able to use, in addition to H_2 and CO_2, acetate as well as other low molecular weight organic acids for methanogenesis. These substrates may be abundant in the rhizosphere, and in the rice roots methanogens have been shown to produce CH_4 directly from root exudates (Lu and Conrad, 2005). CH_4 was produced both from mycorrhizas and from humus. However, the CH_4 production in the enrichments from mycorrhizal samples was over 10 times higher than from humus. In accordance to Lu and Conrad (2005) it may be suggested that also the mycorrhizospheric methanogens may be supported directly by exudates from the mycorrhizas. The CH_4 production started immediately when the enrichment medium was inoculated. This instantaneous high production of CH_4 supports the hypothesis that the mycorrhizospheric methanogens are aerotolerant and that the methanogenic community in the mycorrhizas is active and able to produce CH_4 as soon as favourable conditions occur. The methanogenic populations in the mycorrhizas and the boreal forest soil ecosystem are, however, probably small, because although extensive CH_4 production was observed in the mycorrhizal anaerobic enrichments, only two sequences of methanogenic taxa were obtained. Nevertheless, comparably numerous methanogenic archaea with strong blue autofluorescence were detected by microscopy. Most of the methanogens detected were encapsulated in a matrix together with non-fluorescent bacteria (as in Orphan *et al.*, 2001b). This matrix may have made the DNA extraction difficult and would explain the low detection level of sequences belonging to methanogenic archaea. Members of the Methanosarcinales have shown a certain tolerance to

oxygen and are one of the main groups found in rice field soil, where conditions shift between aerobic and anaerobic periods (Orphan *et al.*, 2001a; Orphan *et al.*, 2001b). Shifts in oxygen availability may also occur in the mycorrhizosphere due to microbial respiration (Li *et al.*, 1992).

4.7 The mycorrhizospheric Crenarchaeota

Certain types of ectomycorrhiza, particularly *P. involutus,* were shown to especially harbour 1.1c Crenarchaeota. They were found in all parts of the boreal forest top soil ecosystem, but at a lower frequency in other sample types than the mycorrhizas. Different tree species appeared to select for different populations of 1.1c Crenarchaeota, but the impact of the tree was less evident when the roots were colonised by ECM fungi. The 1.1c Crenarchaeota may possess functions in both the anaerobic and aerobic cycling of C-1 compounds in the mycorrhizisphere and humus. They are most often found in organic soils with low pH and a steady plant cover (e.g. Ochsenreiter *et al.*, 2003; Oline *et al.*, 2006; Nicol *et al.*, 2007). This may imply that they are directly supported by the plants and mycorrhizospheres. In the Austrian Alps, for instance, 1.1c Crenarchaeota only appeared in the soil when it was inhabited by mycotrophic plant species (Nicol *et al.*, 2005; Nicol *et al.*, 2006). Young soils without plant cover or inhabited by pioneering plants, which usually are non-mycorrhizal or only slightly mycotrophic, were devoid of 1.1c Crenarchaeota. Crenarchaeota of the 1.1b group have been shown to associate with plant roots and exist in soils with ECM and/or AM fungi (Sliwinski and Goodman, 2004). They have commonly been found also in non-vegetated soils, such as recently uncovered glacier front soil (Nicol *et al.*, 2005; Nicol *et al.*, 2006). In the enrichment cultures, 1.1c Crenarchaeota were grown in media with a pH 6.8. Usually, the 1.1c Crenarchaeota are detected in soils with pH below 5.1 and the acidity of the soil has been suggested to be a criterion for the growth of 1.1c Crenarchaeota. Nevertheless, these archaea have been found before in an ash fertilized boreal forest soil with a near neutral pH (Yrjälä *et al.*, 2004). It is possible that the pH is a secondary feature of the habitat, and that the appearance of 1.1c Crenarchaeota depends on other factors such as the presence of mycorrhizal fungi and their exudates.

Both anaerobic and aerobic 1.1c Crenarchaeota were detected in Scots pine mycorrhizas. The diversity of aerobic archaea was higher than that of the anaerobic ones. Phylogenetic analyses, revealed that both the aerobically and the anaerobically growing 1.1c Crenarchaeota fell in the same groups. These results suggest that the 1.1c Crenarchaeota may be facultatively anaerobic, but use different substrates in their different life styles. It is also possible that the boreal forest mycorrhizospheric crenarchaeal community is built up of a tight consortium of closely related crenarchaeotal types, of which some are anaerobic whereas others are aerobic.

In particular, the *P. involutus* ectomycorrhiza were seen to harbour Crenarchaeota. Some 1.1a Crenarchaeota have been shown to live in symbiotic relationships with eucaryotes. The first known case was *Cenarchaeum symbiosum*, which comprised over 65% of the prokaryotic community associated with a marine sponge (Preston *et al.*, 1996) where it possibly oxidizes ammonia (Hallam *et al.*, 2006a; Hallam *et al.*, 2006b). The crenarchaeal C-1 cycling may be the corresponding ecological function in the boreal forest mycorrhizospheres and should be the investigated more thoroughly in the future.

4.8 The mycorrhizospheric Euryarchaeota

Members of the phylum Euryarchaeota were found in the mycorrhizospheres of Scots pine. The most common representatives were members of the genus *Halobacterium*. These were found throughout the study, but the first findings were deemed too uncertain to be published in paper I. However, in paper II, it was seen that all other Scots pine mycorrhizospheres tested supported the *Halobacterium*, except for the *P. involutus*. The halobacterial population appeared very stable and

restricted to ECM fungi. The possibility of halobacteria-like microorganisms, and especially of genus *Halobacterium*, in the boreal forest soil and mycorrhizosphere and in non-saline enrichment conditions contradicts all former knowledge of these usually hyperhalophilic organisms. All isolated strains of this genus are hyperhalophilic and perish without at least 1 M (5.8%) NaCl in their environment (reviewed in Oren, 1999). They adjust their internal salinity to the external one by pumping K^+ ions into the cell. As the *Halobacterium* cell does not maintain turgor pressure, a decrease of the external Na^+ would lead to a dilution of the cell interior. Subsequently, this would result in denaturation of the proteins, which mostly are covered in asparaginic and glutaminic amino acids, which render them functional in high salinity but are denatured in non-saline conditions. This could be prevented by either production or uptake of so called compatible solutes, which is the case for many halophilic microbes living in fluctuating salinity. Compatible solutes are polyols, such as glycerol, arabitol, and sugars or sugar derivatives, such as sucrose, trehalose and glucosylglycerol. Amino acids and their derivatives as well as quaternary amines, also function as compatible solutes. Many of these compounds, e.g. arabitol and trehalose, are abundant in the mycorrhizosphere, and they are known to be synthesised by mycorrhizal fungi whereas the abundant sugars in the rhizosphere are provided by the plant. No uptake or synthesis of compatible solutes has yet been identified for any isolated member of the genus *Halobacterium*, although this function is common in other genera of the Halobacteriales and other halotolerant and halophilic microorganisms. The halobacteria-like organisms detected in the enrichments proliferated and their increase in population richness could be followed by PCR and DGGE. It remains to be investigated whether these halobacteria-like organisms are dependent on the mycorrhizal fungi, since they are only found in close vicinity of the hyphae, or if they perhaps are intracellular symbionts on the mycorrhizal hyphae. Intracellular bacteria have been detected before in the hyphae of the ECM fungus *Laccaria bicolor*, a common ECM forming fungus (Bertaux *et al.*, 2003; Bertaux *et al.*, 2005). It is also possible, that completely new metabolic lifestyles, obtained by e.g. LGT, can be detected in the mycorrhizospheric archaea, or that these organisms with similar 16S rRNA gene sequences have no metabolic similarities to other halobacteria.

Archaeal 16S rRNA gene sequences belonging to *Halobacterium* have not been obtained from other non-saline environments than the boreal forest soil and mycorrhizosphere, although other genera of the order Halobacteriales have been found. Elshahed *et al*. (2004) were able to isolate aerobic strains of the genus *Haloferax* from a freshwater spring, which grew on salt concentrations as low as 6%. Using culture independent approaches, they were also able to detect 16S rRNA gene sequences from other archaeal halophilic genera, except for *Halobacterium*. Purdy *et al*. (2004) also isolated strains strains belonging to *Haloferax* as well as *Halogeometricum* and *Halorubrum* and novel moderately halophilic archaea able to grow on NaCl concentrations as low as 2.5% (level of salt in sea water). Many of these new isolates (HA Group 1) in Purdy *et al*. (2004) were also able to grow anaerobically. However, in the phylogenetic analyses, the mycorrhizospheric *Halobacterium* sequences did not affiliate with these novel moderately halophilic archaea.

In our experiments, methanogenic archaea do not appear to be numerous in the mycorrhizosphere, and were not found by direct DNA extraction followed by PCR. This is in accordance with other archaeal studies on forest soil (e.g. Jurgens and Saano, 1999; Pesaro and Widmer, 2002; Nicol *et al.*, 2007) where no methanogenic sequences were detected. However, methane emissions have been reported from forest soils (Kusel and Drake, 1994; Yavitt *et al.*, 1995; Kusel *et al.*, 1999). Extensive methane production was detected in the anaerobic enrichment cultures, although only few OTUs of methanogenic taxa could be detected by PCR and DGGE. Only two sequences belonging to genus *Methanolobus* (Order Methanosarcinales) were detected from the anaerobic enrichment cultures on yeast extract media amended with H_2+CO_2. Nevertheless, in a more resent experiment, a relatively extensive appearance of 16S rRNA gene sequences belonging to the genera *Methanosarcina*, *Methanosaeta* and *Methanolobus* was detected in the roots and mycorrhizosphere

of Scots pine and silver birch growing in Finnish boreal forest humus (Bomberg *et al*., in preparation).

Boreal forest soils are relatively aerobic, although the oxygen conditions in microhabitats may fluctuate. The interior of soil aggregates are often anaerobic due to microbial activity (Lavelle and Spain, 2003). We have hypothesised that the uneven surfaces of ECM mantles and the fungal strands may contain niches, which are also rendered microaerobic or anaerobic due to microbial respiration. These pockets with their protecting surrounding microbial community may present ideal environments for these methanogens. It may be that the anaerobic halobacteria-like archaea and the Crenarchaeota present the link between the anaerobic and aerobic environments in the boreal forest soil habitats. RC-I methanogens, which form a phylogenetically distinct group related to the Methanosarcinales and Methanomicrobiales, are aerotolerant (Erkel *et al.*, 2006). They possess unique antioxidant enzymes, which protect them from the harmful effects of oxygen. If the mycorrhizospheric *Methanolobus*-type methanogens posses the same enzymes, they are very likely to survive pulses of oxygen. It is also likely that these methanogens are readily activated when favourable conditions arise. This hypothesis is supported by the fact that in the enrichment cultures, the CH_4 production started immediately after the samples were injected into the anaerobic enrichment media.

5. Conclusions

Archaea were found to inhabit the mycorrhizospheric habitats of the boreal forest soil. They were detected from only a few of the bulk soil samples, which were collected from areas of the microcosms without mycorrhizal fungal hyphae. However, this may bias the results compared to native samples, where both the saprotrophic and mycorrhizal hyphal networks are intact. Both the type of ECM fungus and the species of tree host had an effect on the population of archaea in the rhizosphere and mycorrhizosphere. The diversity and detection frequency of archaea were higher in the mycorrhizal roots than in non-mycorrhizal roots. Most of the tested ECM fungi, with the exception of *P. involutus*, supported Euryarchaeota belonging to the genus *Halobacterium*, a group of archaea that is generally found in saline environments. *P. involutus* revealed more crenarchaeotal 16S rRNA sequences than the other types of ECM fungi studied. In general, the mycorrhizas of all tested tree species revealed a higher archaeal detection frequency than the non-mycorrhizal tree roots. Alder was an exception and displayed a unique and diverse crenarchaeotal population, compared to the other trees. The colonisation of the tree roots by an ECM fungus decreased the impact of the tree. In the phylogenetic analyses the 16S rRNA gene sequences of the root and mycorrhizal Crenarchaeota appeared in different clusters. Mycorrhizal 1.1c Crenarchaeota grew both anaerobically and aerobically in C-1 cycling microbial communities, indicating a role for these Crenarchaeota in both anaerobic and aerobic carbon cycling. Halobacterial and *Methanolobus* sequences were detected only in anaerobic enrichments. Auto-fluorescent methanogenic archaea were detected in enrichment cultures of mycorrhizal samples and high amount of CH_4 was produced by the mycorrhizal methanogenic community. The corresponding CH_4 amounts produced in humus was 10 times lower.

The involvement of archaea in the carbon cycling in moderate environments is mostly unstudied. Our results have shown that the archaea constitute a defined part of the microbial communities in forest soil ecosystems. Although nothing is yet known about the functions of the uncultivated boreal forest soil archaea they may have a considerable effect on the carbon budget of this ecosystem. These archaea have been shown to associate with boreal forest trees and mycorrhizal fungi and a great potential for CH_4 production has been demonstrated. However, the impact of forest management and exploitation on the archaeal communities has not been thoroughly investigated.

More information and a better understanding of the carbon cycling microbial communities in the forest soil is required to prevent CH_4 emissions from boreal forest soils. The microbial communities' capacity to bind C-1 carbon into biomass may also be of great importance for future forest management. The functions of the mycorrhizospheric Crenarchaeota and halobacteria in the boreal forest soil ecosystem are, however, yet to be discovered.

6. Future prospects

The world of non-extremophilic archaea is still largely unstudied. Pure cultured strains have been obtained from only a few groups. These cultured archaea have revealed new functions for this group of organisms, and metabolic pathways novel to the whole microbial community. Uncultured archaea have also been studied by sequencing large genome fragments and the results from several studies imply that they are responsible for major ecological processes.

In the work presented above, new insights into the importance of both Crenarchaeota and Euryarchaeota in the boreal forest tree mycorrhizospheres and soil have been suggested. The archaea detected in the mycorrhizosphere may have important functions in the carbon cycling. As trees allocate a considerable part of their produced photosynthates for the benefit of the rhizospheric and mycorrhizospheric microbial flora, it would be possible to provide the tree with ^{13}C labelled CO_2 and track the ^{13}C in the nucleic acids of the rhizosphere community. This would show which microorganisms are directly sustained by the tree host. Since it has been shown that both Cren- and Euryarchaeota can be enriched in cultures, it would be possible to study which carbon substrates they consume by stable isotope probing. More knowledge about the carbon cycling microbial communities is crucial for the prediction of emissions of green house gases from forest soil ecosystems as a result of forest management and global climate change.

The use of non-extremophilic archaea in biotechnological applications requires cultivation of the microorganisms in pure cultures, or enriched in functional consortia. Based on the information gained from this work, this possibility is more likely than before as possible growth conditions have been indicated. This can be used for further isolation of the microorganisms. It may also be easier to extract large genome fragments from enrichment cultures than from environmental samples directly. By characterisation of these large genome fragments, information of the putative functions of the unique boreal forest mycorrhiza and soil inhabiting archaeal communities may finally be obtained. This study has shown a preference of archaea to certain parts of the mycorrhizosphere. By combining this knowledge to metagenomics and proteomics, it may be possible to detect new metabolic pathways and functions *in situ*. To obtain insight to the metabolic potential and functions of defined microbial communities in the forest soil and mycorrhizosphere *in situ*, the employment of polyphasic approaches is of outmost importance.

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