Environmental Microbiology (2005) **7**(3), 337–347 doi:10.1111/j.1462-2920.2004.00698.x

Primary succession of soil *Crenarchaeota* across a receding glacier foreland

Graeme W. Nicol,[1] Dagmar Tscherko,[2] T. Martin Embley[3] and James I. Prosser[1]*

[1]*School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK.*

[2]*Institute of Soil Science and Land Evaluation, University of Hohenheim, Emil-Wolff-Straße 27, 70599 Stuttgart, Germany.*

[3]*School of Biology, University of Newcastle, King George VI Building, Newcastle NE1 7RU, UK.*

Summary

The development of soil archaeal community structures in relation to primary succession in bulk and rhizosphere soil was examined across the forefield of the receding Rotmoosferner glacier in Austria. Using cloning and denaturing gradient gel electrophoresis (DGGE) analysis of reverse transcription polymerase chain reaction (RT-PCR) products of extracted 16S rRNA, archaeal community structure was compared over a chronosequence representing approximately 150 years of soil development and to reference sites outside the glacier forefield, representing soil exposed for approximately 9500 years. Archaeal community composition was found to be dominated by members of the non-thermophilic or Group 1 *Crenarchaeota*, where a dramatic yet highly structured successional sequence was observed. Succession over the 150 years sequence could be identified as occurring in three stages, each of which had a phylogenetically distinct 1.1b crenarchaea community with those organisms present in pioneering and intermediate stages belonging to a lineage distinct from those in developed soils. Climax communities also contained organisms belonging to three other major non-thermophilic crenarchaeal lineages. Comparison of archaeal communities in the rhizosphere indicated that plant species composition was not the major driver of specific crenarchaeal populations. These results indicate the potential role of soil crenarchaea in the development of soil substrates, as well as ecological diversity within and between major Group 1 lineages.

Received 27 April, 2004; accepted 21 July, 2004. *For correspondence. E-mail j.prosser@abdn.ac.uk; Tel. (+44) 1224 555848; Fax (+44) 1224 555844.

Introduction

Glacier forefields are ideal environments for studying primary succession, with receding ice cover presenting a chronosequence of development from bare substrate to complex plant communities. Primary succession in recently deglaciated areas is initiated by the deposition of organic detritus and pioneering microbial and invertebrate communities, which facilitate the conditioning of soil substrate and provision of nutrients essential for future plant growth (Hodkinson *et al*., 2002). Increases in organic matter and nitrogen content are essential for establishment of pioneering plant species (Chapin *et al*., 1994), often with associated mycorrhizae (Jumpponen *et al*., 1998; Hodkinson *et al*., 2003), and plant community development can be rapid with shrub or tree species present within 100 years (Hodkinson *et al*., 2003).

Soil microbial communities are recognized as a major influence on plant communities (Bever *et al*., 1997) and are dynamic across successional gradients, which show increases in biomass and changes in community structure, functional diversity and resource utilization efficiency (Ohtonen *et al*., 1999; Sigler *et al*., 2002; Tscherko *et al*., 2003). For example, using polymerase chain reaction (PCR) and phylogenetic analysis of fungal 18S rRNA gene sequences, Jumpponen (2003) suggested that sequences obtained from young substrates devoid of vegetation (which included sequences affiliated with obligate plant parasitic fungi) represented an allochthonous spore bank, while those from older substrates were representative of an active fungal community. Bacterial communities increase in total numbers and activity with succession (Sigler and Zeyer, 2002; Tscherko *et al*., 2003), though the evenness and richness of the dominant members of the bacterial community may decrease (Sigler *et al*., 2002).

Cultivation-independent surveys of microbial communities in the environment have greatly increased our understanding of prokaryotic diversity and have led to the discovery of considerable sequence diversity within the domain *Archaea*. These prokaryotic organisms are evolutionarily distinct from *Bacteria* and represent one of three primary domains of life (Woese *et al*., 1990). It is now

© 2005 Society for Applied Microbiology and Blackwell Publishing Ltd

recognised that they are not restricted to environments considered to be extreme with respect to temperature, salinity or anoxia, as indicated by cultivation-based methods (DeLong, 1998), but are also distributed in a wide range of mesophilic terrestrial and aquatic environments. Organisms belonging to the largely non-thermophilic 'Group 1' *Crenarchaeota* lineage appear to be ubiquitous in soil systems including grassland, forest, agricultural and rice field soils (e.g. Bintrim *et al*., 1997; Jurgens *et al*., 1997; Buckley *et al*., 1998; Großkopf *et al*., 1998; Nicol *et al*., 2003a) and may be dominant over euryarchaeal populations in grassland soils (Nicol *et al*., 2003b). Despite an increasing understanding of crenarchaeal diversity in the environment, little is known about the drivers of crenarchaeal diversity and their ecological functioning in soil systems (Nicol *et al*., 2004). However, the dynamism of soil crenarchaeal communities in response to different environmental factors (e.g. Sandaa *et al*., 1999; Nicol *et al*., 2003a) and their apparent ubiquity but reduced complexity in comparison with bacterial communities make crenarchaea a useful target group when making comparisons between different environmental samples. Crenarchaea have been reported to constitute a significant proportion of soil prokaryotes ranging from 0.16% to 3% of 16S rRNA genes (Ochsenreiter *et al*., 2003) and approximately 1% of hybridized whole cells (Sandaa *et al*., 1999) or extracted 16S rRNA (Buckley *et al*., 1998). Several studies have indicated that soil crenarchaea may have a specific association with plant roots (Großkopf *et al*., 1998; Simon *et al*., 2000; Chelius and Triplett, 2001) and crenarchaea may therefore play an important role in the rhizosphere.

The aim of this study was to examine the development of soil archaeal community structures in relation to primary succession using cloning and denaturing gradient gel electrophoresis (DGGE) analysis of reverse transcription polymerase chain reaction (RT-PCR) products of 16S rRNA extracted from soil. The study of archaeal communities in both bulk and rhizosphere soils at different stages of development (i.e. from recently exposed substrate to soils of developed acidic alpine grassland) may provide further insights into the ecology of organisms belonging to different lineages within the Group 1 *Crenarchaeota*.

Results

Nucleic acid extraction and RT-PCR amplification of archaeal 16S rRNA

Soil samples were collected from the forefront of the receding Rotmoosferner glacier in the Ötz valley at Tyrol, Austria. Total nucleic acids were extracted from 0.5 g of soil from three replicate samples of bulk soil representative of seven successional ages (4, 14, 20, 48, 75, 135 and approximately 9500 years) and duplicate samples of rhizosphere soil from representative plant species from four successional ages (20, 75, 135 and approximately 9500 years). After extraction, 5 μl of extract was subjected to agarose gel electrophoresis to determine yields. DNA or RNA bands from extracts of 4-, 14- and 20-year-old soil samples were not visible on gels. However, RT-PCR products were obtained from all samples except for two of three 4-year-old bulk soil samples and one of three 14-year-old bulk soil samples.

Denaturing gradient gel electrophoresis analysis of 16S rRNA RT-PCR products from bulk soil samples

The primers used in this study exhibit good coverage of sequences placed within all major Group 1 crenarchaeal lineages. Ar3F/Ar9R amplicons were nested with rSAf/PARCH519r primers to generate products suitable for DGGE analysis of archaeal communities (Table 1). Primer rSAf is the previously described SAf primer (Nicol *et al*., 2003a) but with omission of the final 3′ nucleotide to remove potential bias against some group 1.3 crenarchaeal sequences. Because of necessary degeneracies in the primers, DGGE analysis of rSAf/PARCH519r PCR products of individual sequences generates DGGE band 'doublets'. After initial DGGE analysis, selected first-round PCR products approximately 900 bp in length were cloned and screened by DGGE against a community profile of the same first-round PCR product, to obtain clones representative of the major bands in the community profiles. Cloned sequences were prefixed with GFS1–10 (Glacier forefield soil) reflecting the DGGE migration position of each clone after nested amplification. GFS1 and GFS10 migrated the shortest and greatest distances, respectively.

Denaturing gradient gel electrophoresis analysis revealed a large and continuous shift in archaeal community structure across the successional gradient (Fig. 1). There appeared to be three successional stages within the *Archaea* community characterized by changes in the dominating sequence and an increase in overall diversity. Denaturing gradient gel electrophoresis profiles of recently deglaciated or 'primary' substrate (4- to 48-year-old) had a stable community structure consisting of one doublet representing a single 16S rRNA sequence, corresponding to GFS2 sequences in the marker lane. Profiles of soils exposed for 75 to approximately 9500 years showed a shift in archaeal community structure, with GFS2 bands being replaced by an 'intermediate' community structure largely represented by GFS3 bands. GFS4 bands then became dominant in the profiles of 135- and approximately 9500-year samples representative of 'mature' alpine grassland. The 135-year-old samples showed an increasingly complex community structure. These profiles were most similar to those of mature alpine

Table 1. Summary of primers and PCR conditions used.

Primer	Positions[a]	Sequence 5′-3′	Reference	PCR round	PCR thermal cycling conditions
Ar3F	2-21	TTCCGGTTGATCCTGCCGGA	Giovannoni *et al*. (1988)	1st	95°C for 5 min; followed by five cycles of 94°C at 30 s, 55°C at 30 s, 72°C at 1 min; followed by 30 cycles of 92°C at 30 s, 55°C at 30 s, 72°C at 1 min; followed by 72°C at 10 min
Ar9R	906-927	CCCGCCAATTCCTTTAAGTTTC	Jurgens *et al*. (1997)		
rSAf(i)[b]	341-357	CGCCCGCCGCGCGCGGCGGGC GGGGCGGGGGCACGGGGGGC CTAYGGGGCGCAGCAG[c]	This study	2nd	95°C for 5 min; followed by five cycles of 94°C at 30 s, 63°C at 30 s, 72°C at 1 min; followed by 30 cycles of 92°C at 30 s, 63°C at 30 s, 72°C at 1 min; followed by 72°C at 10 min
rSAf(ii)[b]	341-357	CGCCCGCCGCGCGCGGCGGGC GGGGCGGGGGCACGGGGGGC CTACGGGGCGCAGAGG[c]			
PARCH519r	519-533	TTACCGCGGCKGCTG	Øvreås *et al*. (1997)		

a. *Escherichia coli* numbering.
b. Primer rSAf is a mixture of primers rSAf(i) and rSAf(ii) at a molar ratio of 2:1 (rSAF(i) contains one degeneracy). This primer is a revised version of previously published SAf primer (Nicol *et al*., 2003a).
c. Sequence underlined corresponds to GC-clamp (Muyzer *et al*., 1993) and is not homologous to 16S rRNA sequence.
K = G + T; Y = C or T.

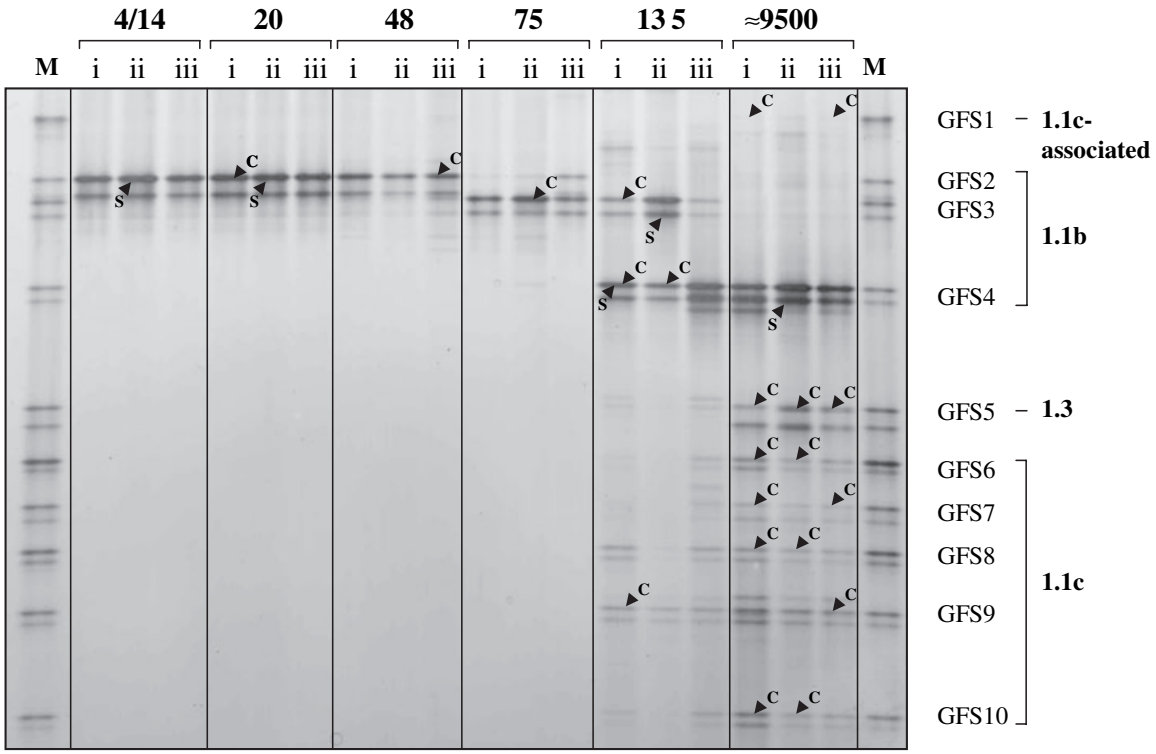


Fig. 1. Denaturing gradient gel electrophoresis profiles of archaeal 16S rRNA RT-PCR products amplified from soil sampled along a successional chronosequence. Triplicate soil samples were analysed at each successional age (4, 14, 48, 75, 135 and approximately 9500 years) with each lane representing an individual soil sample. Only one of three replicate extractions from 4-year samples and two of three 14-year samples yielded RT-PCR products. Lanes labelled M represent a marker consisting of nested PCR fragments from 10 clones, which are placed into one of four Group 1 phylogenetic clusters (1.1b, 1.1c, 1.1c-associated and 1.3) and have migration patterns representative of 10 major band positions in the environmental profiles. Band positions highlighted with a 'c' indicate that an Ar3F/Ar9R-derived clone was obtained with an identical DGGE migration position from a PCR product of the same environmental sample profiled. Band positions highlighted with 's' indicate bands that were successfully amplified and sequenced.

Table 2. Group 1 cluster affiliation of retrieved sequences present in soils of different succession ages.

Group 1 cluster	4/14 years	20 years	48 years	75 years	135 years	9500 years
1.1b	+	+	+	+	+	+
1.1c					+	+
1.1c-associated						+
1.3						+

grassland, with additional bands appearing including those that comigrate with GFS1 and GFS5 to GFS10 band positions.

To determine whether differences in DGGE profiles resulted from bias associated with varying amounts of nucleic acid extracted along the transect, serial dilutions of nucleic acid extracts from samples generating simple and complex DGGE profiles (20ii and 9500i respectively) were subjected to a further round of amplification by RT-PCR. Polymerase chain reaction products were obtained from 10^0 to 10^{-3} and 10^{-5} dilutions and DGGE profiles from each dilution from one individual extract were identical, indicating that differences in profiles were not associated with any template concentration bias (data not shown).

Phylogenetic analysis of GFS clones

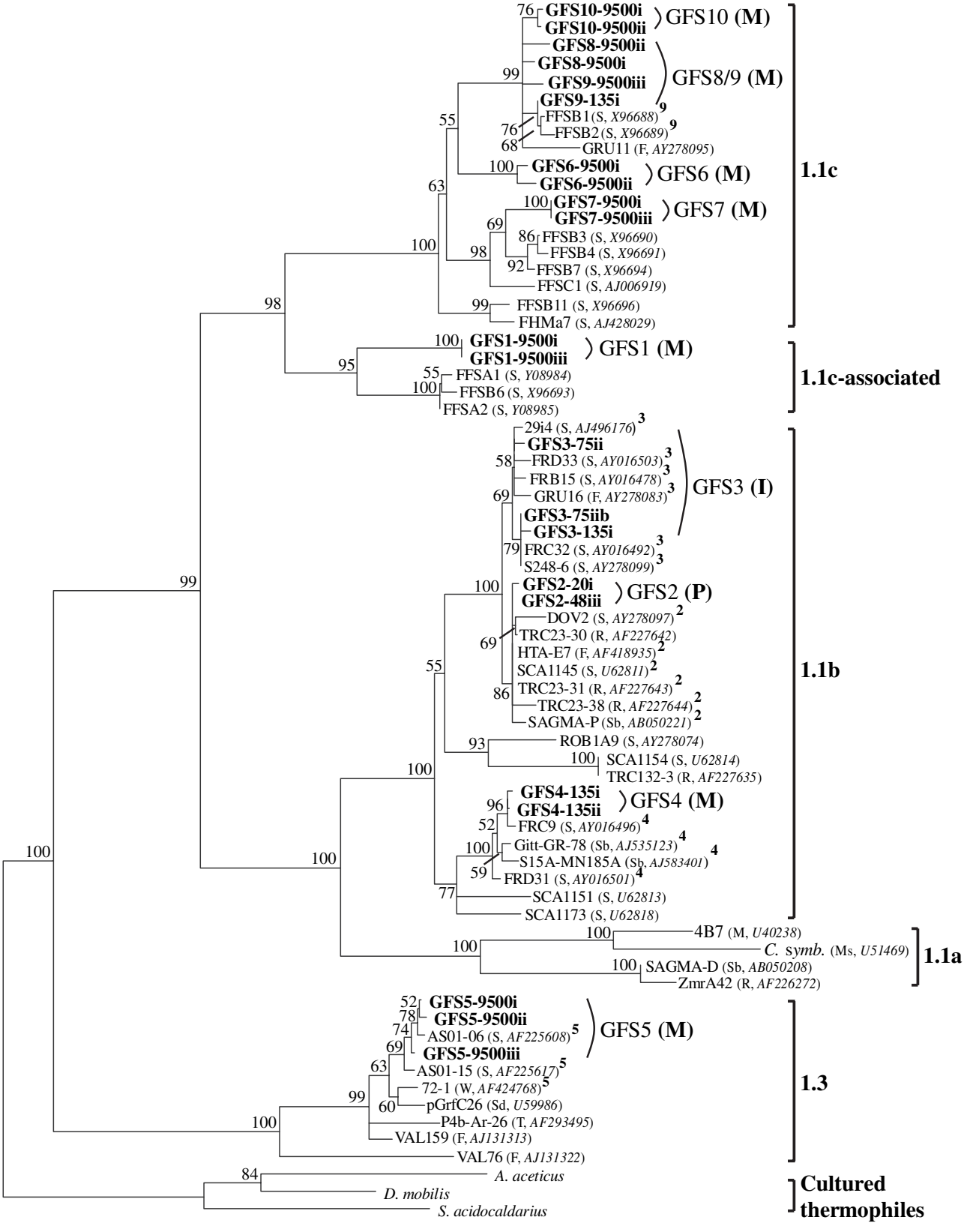
First-round PCR products derived from eight soils samples (20i, 48iii, 75ii, 135i, 135ii, 9500i, 9500ii and 9500iii) were cloned and screened to obtain clones representative of 10 band positions represented in the environmental DGGE profiles. The composition of the clone libraries generally reflected the environmental profiles. For example, only six clones from the library of sample 20ii were screened by DGGE with all clones migrating to the same position as the dominant band in the environmental profile. However, 58 clones from sample 9500i were screened to obtain one clone with a representative migration position identical to that of GFS7, which represented a minor component of the DGGE profile (Fig. 1).

A minimum of two clones from two different libraries were fully sequenced for each of 10 DGGE band migration positions. Sequences were compared with GenBank database sequences using BLASTn searches and all were highly similar to crenarchaeal sequences from mesophilic environments (97.8–100%). Sequences of all clones migrating to the same position were identical over the region amplified by nested PCR for DGGE analysis. A selection of dominant DGGE bands (of GFS2, 3 and 4 positions) were also successfully excised, re-amplified and sequenced (Fig. 1) and were identical over the comparable region of sequence to the cloned sequences with identical migration patterns.

The phylogenetic placement of 22 crenarchaeal sequences initially grouped by DGGE migration position is shown in Fig. 2. All sequences could be placed into one of four lineages of Group 1 Crenarchaeota (DeLong, 1998); 1.1b, 1.1c, 1.3, and a lineage generally associated with 1.1c sequences. The Group 1 cluster affiliation of sequences present in soils along the chronosequence is summarized in Table 2. The two clones with a GFS1 migration position were placed within the group 1.1c-associated lineage, GFS2, 3 and 4 clones within group 1.1b, three GFS5 clones within group 1.3 and GFS6–GFS10 clones within group 1.1c (Fig. 2).

Bands associated with the group 1.1b lineage, i.e. either GFS2, 3 or 4, dominated all profiles. GFS2 and GFS3 DGGE fragments differed by only one nucleotide (out of 150) with all five sequences (two GFS2 and three GFS3) exhibiting ≥98.5% identity over 820 bp. The differentiation of these two groups by DGGE was also reflected in phylogenetic analysis, GFS2 and GFS3 sequences forming two separate clusters with environmental sequences that possessed the same 150-bp sequence (respectively highlighted with superscript '2' or '3' in Fig. 2). GFS2- (pioneer) and GFS3-like (intermediate) organisms also grouped together in a cluster distinct from GFS4-like (mature) sequences. GFS2, 3 and 4 sequences clustered with

Fig. 2. Phylogenetic tree constructed by neighbour-joining of LogDet/Paralinear distances of estimated variable sites (53% of 711 nucleotides) showing the placement of 22 cloned 16S rRNA sequences from glacier forefield soil within major non-thermophilic *Crenarchaeota* groups. Clones are described by their DGGE migration pattern after nested PCR with rSAf/PARCH519r (GFS1–GFS10) and the sample from which they were obtained (20i, 48iii, 75ii, 135i, 135ii, 9500i, 9500ii or 9500iii). P, I or M indicates whether the sequence is representative of a pioneer, intermediate or mature archaeal community structure respectively. Group 1 clusters (1.1a/b/c and 1.3) are based on those described by DeLong (1998) and environmental 16S rRNA clone sequences are presented as 'name (environment of retrieval, *accession number*)' with S = soil, R = root, F = freshwater, M = marine water, Ms = marine sponge, Sd = sediment, Sb = subsurface, T = termite gut and W = wastewater sludge. Sequence names highlighted with a 2, 3, 4, 5 or 9 in superscript denote sequences from other studies, which possess an identical sequence over the 150-bp DGGE fragment to those of GFS2, 3, 4, 5 or 9 sequences respectively. Multifurcation indicates branches where the relative branching order could not be determined in the majority of bootstrap re-samplings (1000 replicates). The scale bar represents an estimated 0.05 changes per nucleotide position. The tree was rooted with thermophilic crenarchaeal sequences *Sulfolobus acidocaldarius*, *Desulphurococcus mobilis* and *Acidilobus aceticus*.


GFS10-9500i
GFS10-9500ii
GFS10 (M)
GFS8-9500ii
GFS8-9500i
GFS9-9500iii
GFS9-135i
GFS8/9 (M)
FFSB1 (S, X96688)
FFSB2 (S, X96689)
GRU11 (F, AY278095)
GFS6-9500i
GFS6-9500ii
GFS6 (M)
GFS7-9500i
GFS7-9500iii
GFS7 (M)
FFSB3 (S, X96690)
FFSB4 (S, X96691)
FFSB7 (S, X96694)
FFSC1 (S, AJ006919)
FFSB11 (S, X96696)
FHMa7 (S, AJ428029)
1.1c
GFS1-9500i
GFS1-9500iii
GFS1 (M)
FFSA1 (S, Y08984)
FFSB6 (S, X96693)
FFSA2 (S, Y08985)
1.1c-associated
29i4 (S, AJ496176)
GFS3-75ii
FRD33 (S, AY016503)
FRB15 (S, AY016478)
GRU16 (F, AY278083)
GFS3 (I)
GFS3-75iib
GFS3-135i
FRC32 (S, AY016492)
S248-6 (S, AY278099)
GFS2-20i
GFS2-48iii
GFS2 (P)
DOV2 (S, AY278097)
TRC23-30 (R, AF227642)
HTA-E7 (F, AF418935)
SCA1145 (S, U62811)
TRC23-31 (R, AF227643)
TRC23-38 (R, AF227644)
SAGMA-P (Sb, AB050221)
ROB1A9 (S, AY278074)
SCA1154 (S, U62814)
TRC132-3 (R, AF227635)
GFS4-135i
GFS4-135ii
GFS4 (M)
FRC9 (S, AY016496)
Gitt-GR-78 (Sb, AJ535123)
S15A-MN185A (Sb, AJ583401)
FRD31 (S, AY016501)
SCA1151 (S, U62813)
SCA1173 (S, U62818)
1.1b
4B7 (M, U40238)
C. symb. (Ms, U51469)
SAGMA-D (Sb, AB050208)
ZmrA42 (R, AF226272)
1.1a
GFS5-9500i
GFS5-9500ii
AS01-06 (S, AF225608)
GFS5-9500iii
GFS5 (M)
AS01-15 (S, AF225617)
72-1 (W, AF424768)
pGrfC26 (Sd, U59986)
P4b-Ar-26 (T, AF293495)
VAL159 (F, AJ131313)
VAL76 (F, AJ131322)
1.3
A. aceticus
D. mobilis
S. acidocaldarius
Cultured thermophiles
0.05

those retrieved from various soil types, including forest and agricultural soils, freshwater and subsurface samples.

Group 1.1c-associated (GFS1), group 1.3 (GFS5) and group 1.1c sequences (GFS6–GFS10) only appeared in developed soils, indicating restricted distribution of these organisms across the successional gradient in comparison with group 1.1b organisms. The three GFS5 sequences possessed ≥98.2% identity over 852 nucleotides and were placed in a group containing sequences from diverse environments including freshwater sediment, a wastewater digester and rice field soil. The two GFS1 sequences fell within a group described here as 'group 1.1c-associated', which contains only three sequences retrieved from boreal forest soil in Finland. All group 1.1c sequences (GFS6–10) also exhibited closest sequence similarity to sequences obtained from Finnish and Colorado forest soils.

GFS8 and GFS9 DGGE fragments differed by only one nucleotide (out of 150) with all four sequences (two GFS8 and two GFS9) exhibiting ≥97% identity over 851 bp. Variability in similarity within GFS8 sequences was similar to that between GFS8 and the two GFS9 sequences analysed. The four sequences could not be separated into GFS8 and GFS9 groups in phylogenetic analysis, either in Fig. 2 or in analyses with those sequences in the highlighted GFS8/9 cluster only (data not shown). Sequences retrieved from grassland soils placed within group 1.1c have been found previously (Nicol *et al.*, 2003a), but were not included in this analysis because of their comparatively short length.

Denaturing gradient gel electrophoresis analysis of 16S rRNA RT-PCR products from rhizosphere soil samples

Rhizosphere soil associated with different plant species was collected from individual plants (two per species) at four successional stages (20, 75, 135 and 9500 years). The dominant plant species at each of the four successional points analysed were different, except that *Poa alpina* plants were a major component at 20, 75 and 9500 years. Denaturing gradient gel electrophoresis profiles of rhizosphere archaeal communities generally reflected the structure and successional sequence observed for the bulk soil samples (Fig. 3A). Pioneering GFS2-type sequences dominated early profiles, showing greatest relative abundance at 20 years before being replaced by GFS3 sequences at intermediate stages (75–135 years), while GFS4 sequences dominated the mature alpine grassland profiles (9500 years). Group 1.1c sequences were also only prevalent within the DGGE profiles of rhizosphere samples from mature soils. Rhizosphere-associated archaeal community structures at any one successional stage appeared to be very similar with little evidence of a plant species effect. *Poa alpina* plants were present in the plant communities of 20-, 75- and 135-year successional stages. Comparison of the replicate profiles (Fig. 3B) at these three successional stages indicates that individual plant species did not select for particular archaeal communities in either bulk or rhizosphere soils.

Discussion

The structure of archaeal communities in bulk and rhizosphere soil was examined in relation to primary succession across a receding glacier forefront. Denaturing gradient gel electrophoresis and phylogenetic analysis of 16S rRNA sequences demonstrated a structured change. Pioneering and intermediate archaeal communities were dominated by a restricted group of 1.1b crenarchaea, before an increase in the relative abundance of sequences placed within other major mesophilic crenarchaeal lineages.

To correlate cloned 16S rRNA sequences with the sequences of the dominant bands in environmental DGGE profiles, clone libraries were screened by DGGE and clones with identical migration positions analysed. It was presumed that clones and DGGE fragments migrating to the same position would be identical in sequence over the rSAf/PARCH519r-amplified region, and would represent monophyletic groups excluding sequences with different migration positions. These assumptions proved largely correct except for GFS8 and GFS9 sequences, which differed by only 1 bp over the DGGE fragment, and could not be resolved into separate groups. Most cloned sequences were unique with only the two GFS1 and two GFS7 sequences displaying 100% identity over 848 and 851 nucleotides respectively. It is therefore likely that DGGE bands of 150 nucleotides represent a number of different (but closely related) 16S rRNA gene sequences.

DGGE profiles of bulk soil communities showed that initial, pioneering archaeal communities (4–48 years) were dominated by organisms with GFS2-like sequences before replacement by those with GFS3-like sequences (75–135 years). These different sequence groups reflected different ecotypes that were differentiated within the 1.1b lineage in phylogenetic analysis. Although only a limited number of clones were analysed for each sequence type identified by DGGE, GFS2-, GFS3- and GFS4-type sequences could be clearly resolved with other environmental sequences, which possessed the same sequence over the 150 nucleotide region amplified in this study for DGGE analysis. GFS2 and GFS3 sequence-types that were found in young and intermediate soil substrates formed a cluster distinct from that of 'mature soil' GFS4 sequence-types within the 1.1b group in phylogenetic analysis. The gradual replacement of GFS2 sequence-types by GFS3-type organisms and their

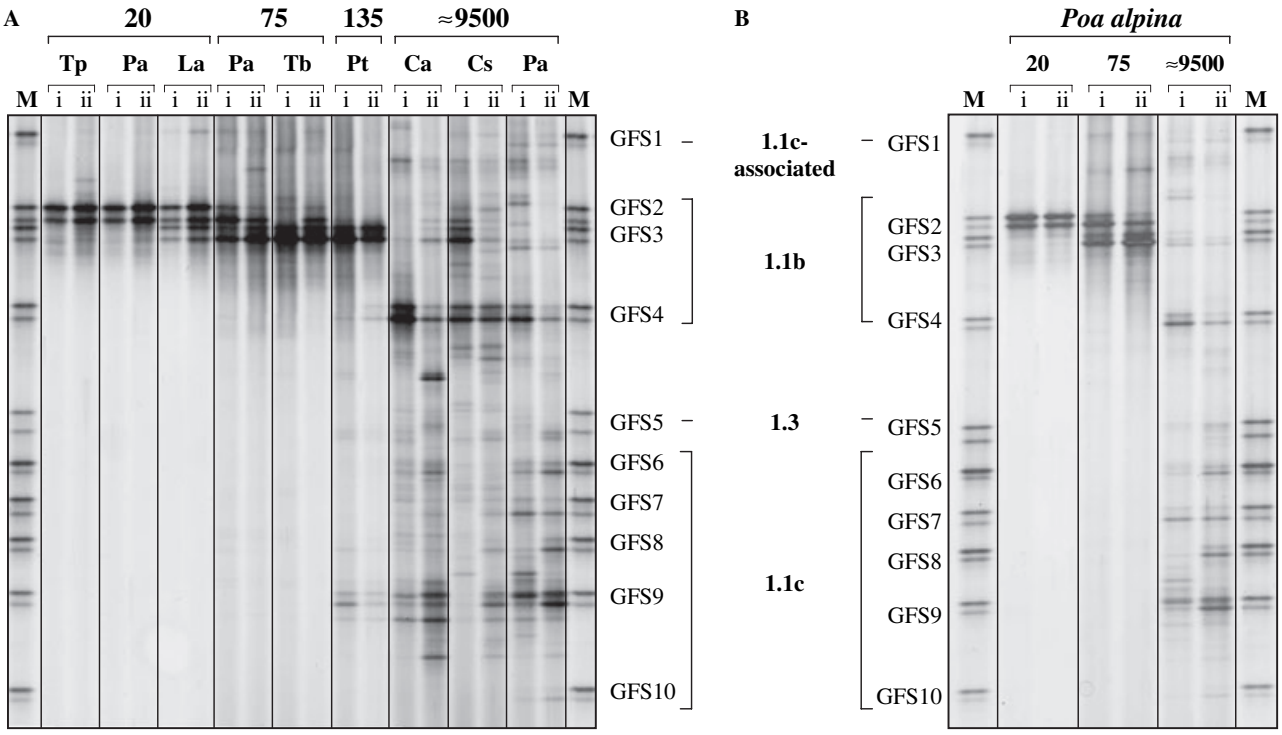


Fig. 3. Denaturing gradient gel electrophoresis profiles of archaeal 16S rRNA RT-PCR products amplified from rhizosphere soil associated with different plant species in soils at different successional ages (20, 75, 135 and approximately 9500 years). Two rhizosphere samples were examined for each plant species at a particular successional stage with each lane representing soil associated with the roots of an individual plant. Lanes labelled M represent a marker consisting of nested PCR fragments from 10 clones, which are placed into one of four Group 1 phylogenetic clusters (1.1b, 1.1c, 1.1c-associated and 1.3) and have migration patterns representative of 10 major band positions in the environmental profiles.
A. Abbreviations represent the following plant species: Tp, *Trifolium pallescens*; Pa, *Poa alpina* var. *vivipara*; La, *Linaria alpina*; Tb, *Trifolium badium*; Pt, *Potentilla aurea*; Ca, *Crepis aurea*; Cs, *Carex sempervirens*.
B. Denaturing gradient gel electrophoresis profiles of archaeal communities present in the rhizosphere soil of *Poa alpina* var. *vivipara* plants at three successional stages.

close relationship within the 1.1b crenarchaeal community may indicate that the ecological function of these organisms is the same but they occupy different niches (e.g. pH-defined) as soils develop.

GFS2-type sequences dominated profiles from soil aged 4–48 years, that were mostly devoid of vegetation (≤30%), while those from later years (>75 years) were covered with vegetation (70–80%). However, sequences GFS2-20i and GFS2-48iii, from organisms which may be active in soils with little vegetation, possessed >99% identity to three sequences (TRC23-28, TRC23-31 and TRC23-38) from crenarchaea, which colonize tomato plant roots. Taking into account the distribution of group 1.1b crenarchaea throughout the successional sequence, and the recognition that crenarchaea within the 1.1b lineage appear to be more common in soil systems than other Group1 lineages (Nicol *et al*., 2003a; Ochsenreiter *et al*., 2003), the 1.1b lineage may contain greater functional diversity than the other major Group 1 soil crenarchaeal lineages.

Soil succession resulted in an increase in archaeal diversity, associated with an increase in different sequence types and the number of major Group 1 lineages present. The detection of group 1.1c, 1.1c-associated and 1.3 crenarchaea only in more developed soils, which had been ice-free for more than 75 years, indicates substantial ecological differentiation between the major soil crenarchaeal groups. The developed soils contrasted with the young substrates in characteristics such as organic carbon content, pH and total nitrogen content [e.g. 20-, 75- and 9500-year-old soils had organic carbon contents of 7.0, 16.0 and 48.8 mg g^{-1}, pH values of 7.51, 5.95 and 4.23, and total nitrogen contents of 0.28, 1.35 and 3.91 mg g^{-1} respectively (D. Tscherko, U. Hammesfahr, G. Zeltner, E. Kandeler and R. Böcker, submitted)]. These factors, associated with more developed alpine grassland soil, may be required for establishment of these groups. The substantial sequence divergence between these lineages may also reflect differences in physiologies and ecosystem function. Sequences belonging to the 1.1c cluster were major components of the archaeal community only in the developed soils. Sequences of this lineage have been found in fewer studies than the 1.1b cluster, and are largely (though not

exclusively) found in coniferous forest soils. This study supports evidence from an earlier study (Nicol *et al*., 2003a) that they are also prevalent in grassland systems.

Denaturing gradient gel electrophoresis analysis demonstrated a homogeneous archaeal community structure over the first 48 years. A stochastic community structure over recently exposed substrates would perhaps indicate an allochthonous archaeal community structure, as reported for the fungal community by Jumpponen (2003). However, the GFS2-dominated community structure during the first 50 years of succession would indicate that these organisms are active 'pioneers' and not dormant members of an aerially deposited archaeal community. All PCR products were generated by RT-PCR of 16S rRNA. Although it is dangerous to conclude that these organisms are active from RNA-DGGE profiles alone, it has been suggested that RT-PCR targets predominantly active organisms (Felske and Akkermans, 1998). Targeting 16S rRNA genes might have led to a more stochastic community structure if communities were dominated by allochthonous, dormant *Archaea*.

The Rotmoosferner glacier forefield has been extensively studied in terms of soil development, plant succession, microbial activity and faunal succession. Kaufmann and colleagues (2002) reported that within 50 years of deglaciation, macrofaunal biomass and mesofaunal abundance were at levels similar to those of older stages. Tscherko and colleagues (2003) found microbial functional diversity (Shannon diversity index and evenness) to be stabilized after the same length of time. In this study, archaeal community structure remained very stable during the first 50 years, after which it developed into that typical of mature alpine grassland. The fact that archaeal community structure changed dramatically only through soil successional stages in which functional diversity was largely stable is intriguing, but not necessarily contradictory. Little is known of the functional contribution of soil crenarchaea and different groups may contribute towards one particular functional process across the chronosequence with other factors (e.g. organic matter content, pH, etc.) selecting for particular groups.

Several studies have indicated a specific association of soil crenarchaea with plant roots (Sliwinski and Goodman, 2004) and it has been suggested that plant communities may be a major driver for crenarchaeal community structure in grasslands subject to different management regimens (Nicol *et al*., 2004). Plant community structures shifted across the successional gradient with each successional stage having a distinct plant species composition. A plant species effect on the total rhizosphere microbial community has been observed in late successional soils of the Rotmoosferner glacier (D. Tscherko, U. Hammesfahr, G. Zeltner, E. Kandeler and R. Böcker, submitted). However, these results indicate that the rhizospheres of individual plant species did not directly select for particular crenarchaeal community structures in either early or late successional soils, and that the rhizosphere-associated communities did not appear to differ greatly from those within the bulk soils. However, it may not necessarily follow that plant species composition has no effect on crenarchaeal community structure. The abundance of annuals decreased and the abundance of perennials increased with succession (D. Tscherko, U. Hammesfahr, G. Zeltner, E. Kandeler and R. Böcker, submitted). Annuals release much less C than perennials (Harris *et al*., 1980) and quantitative differences in exudates may be a factor. Rhizodeposition from individual plant species may produce particular soil characteristics or metabolites (via other soil organisms), which select for specific crenarchaeal populations in the bulk soil. Other organisms, selected by conditions across the gradient may in turn influence crenarchaeal community structure. For example, Bomberg and colleagues (2003) reported a specific association of group 1.1c crenarchaea with the ectomycorrhizal fungi associated with Scots pine plants. Alternatively, the soil that was defined as 'rhizosphere' soil in this study may not have been under the direct influence of the root system and was, in part, 'bulk' soil. A more discriminative sampling approach (e.g. the rhizoplane only) may have shown plant species specific selection.

Examining archaeal community structure over a chronosequence, which represents substantial gradients in soil characteristics and plant species composition, has indicated substantial ecological diversity both within and between major group1 *Crenarchaeota* lineages. Successional gradients should provide an ideal environment for assessing plant–soil–crenarchaea interactions and for increasing understanding of the functional roles of specific lineages within the Group 1 *Crenarchaeota*.

Experimental procedures

Study site

The study site was located on the foreland of the Rotmoosferner glacier (46°50′N, 11°03′E) in the Ötz valley (Tyrol, Austria) at an altitude of 2280–2450 m above sea level. The glacier foreland starts with the youngest site (4 years) which was 10 m away from the glacier terminus and ends at the terminal moraine dated 1850. The area in front of the glacier foreland has been ice-free for approximately 9500 years (Patzelt and Bortenschlager, 1979) and served as an undisturbed reference site. Glacial retreat has resulted in a gradient of successional plant communities, which develops towards acidic alpine grassland. The dominant plant species composition at each successional stage was different with the exception of *P. alpina*, which was largely found in all successional stages (D. Tscherko, U. Hammesfahr, G. Zeltner, E. Kandeler and R. Böcker, submitted).

The parent material of the soil is mainly neoglacial moraine till and fluvio-glacial sands with soils classified as Leptic and Eutric Regosols. The chemical properties of these soils have been described previously (D. Tscherko, U. Hammesfahr, G. Zeltner, E. Kandeler and R. Böcker, submitted). However, decreases in pH, carbonate content and increases in available phosphorous and nitrogen were the general trends observed with soil development.

Soil sampling

Soil samples were collected in September 2001 along the glacial forefield representing seven time points since deglaciation [4, 14, 20, 48, 75, 135 and approximately 9500 years (reference site outside the glacier)]. Three replicates of non-rhizosphere soil (bulk soil) collected from the surface (0–10 cm) were analysed for each time point. Rhizosphere soil from representative plant species at four time points (20, 75, 135 and 9500 years) was analysed (20 years – *Linaria alpina*, *Trifolium pallescens*, *Poa alpina* var. *vivipara*; 75 years – *Trifolium badium*, *Poa alpina* var. *vivipara*; 135 years – *Potentilla aurea*; approximately 9500 years – *Carex sempervirens, Crepis aurea*, *Poa alpina* var. *vivipara*). Rhizosphere soil was extracted by removing the plant from the soil and separating adhering soil from roots with a pair of tweezers. The soil from each plant was bulked. Two individuals of each plant species taken from the study site, representing two replicate samples were analysed. Bulk soil and rhizosphere soil were stored in plastic bags at −20°C.

Nucleic acid extraction and RT-PCR amplification of archaeal 16S rRNA genes

Nucleic acids were extracted using a method based on that of the study by Griffiths and colleagues (2000). Briefly, 0.5 g soil was placed in a 2-ml screw-cap Blue Matrix tube containing a silica and ceramic bead mixture (Hybaid, Ashford, Middlesex, UK) with 0.5 ml extraction buffer [0.25 ml 240 mM potassium phosphate buffer (pH 7.8), 0.25 ml 10% (w/v) hexadecyltrimethylammonium bromide (CTAB) in 0.7 M NaCl] and 0.5 ml phenol:chloroform:isoamyl alcohol (25:24:1). Cells were lysed in a Hybaid Ribolyser (Hybaid) for 30 s at speed 4.0. After centrifugation at 16 000 *g* for 5 min, the aqueous phase was removed and extracted with 0.25 ml chloroform:isoamyl alcohol (24:1) followed by further centrifugation at 16 000 *g* for 5 min. The aqueous phase was removed and total nucleic acids were precipitated by adding 2 volumes 30% (w/v) PEG 6000 in 1.6 M NaCl and leaving on ice for 2 h. Nucleic acids were pelleted by centrifugation at 16 000 *g* for 10 min and washed in 1 ml ice-cold 70% (v/v) ethanol before further centrifugation at 16 000 *g* for 5 min. After decanting the ethanol wash and removing residual liquid, the pellets were dried by warming for approximately 1 min at 55°C in a hot-block. Pellets were re-suspended in sterile 50 μl dH_2O.

Reverse transcription was used to produce cDNA of 16S rRNA for PCR amplification. Total nucleic acids were treated with DNase to remove DNA using RQ1 DNase (Promega, Southampton, UK) in a 10-μl reaction according to manufacturer's instructions. Reverse transcription was then performed using Superscript RNase H^- reverse transcriptase (Invitrogen, Paisley, UK) in a 20-μl reaction according to manufacturer's instructions, using primer Ar9R (Jurgens *et al*., 1997) to select for archaeal rRNA. Two negative controls were performed with all reactions [no template (water only) and template but no RT enzyme].

Polymerase chain reaction products were obtained using primers Ar3F (Giovannoni *et al*., 1988) and Ar9R (Jurgens *et al*., 1997) (Table 1) generating a PCR product approximately 900 bp in length. For DGGE analysis, these PCR products were used as template in a nested PCR reaction using primers rSAf/PARCH519r (Table 1) generating a PCR product 118 bp in length (excluding primers). Primer sequences and thermal cycling conditions are described in Table 1. All PCR amplifications were performed in 50-μl reactions on a Hybaid Express thermal cycler (Hybaid) using Biotaq DNA polymerase (Bioline, London, UK) at 1 unit per reaction, $MgCl_2$ at 2 mM, dNTPs at 1 mM and each primer at 0.4 μM with 1 μl of template.

Denaturing gradient gel electrophoresis analysis

Denaturing gradient gel electrophoresis was performed using a DCode Universal Mutation Detection System (Bio-Rad, Hertfordshire, UK) according to the manufacturer's instructions. Gels were prepared as described previously (McCaig *et al*., 2001) and contained a linear gradient of 45–70% denaturant [with 100% denaturing solution defined as 40% formamide (v/v) and 7 M urea (42% w/v)]. Gels were electrophoresed in 7 l of 1× TAE buffer at a constant temperature of 60°C for 900 min at 100 V. Gels were silver-stained as described by the study by McCaig and colleagues (2001) with modifications. Gels were covered in 200 ml of fixing solution [10% (v/v) ethanol, 0.5% (v/v) glacial acetic acid] for a minimum of 30 min. The fixing solution was removed and the gel covered in 200 ml of staining solution [0.1% (w/v) $AgNO_3$] for 20 min. The staining solution was removed and the gel briefly rinsed in dH_2O before covering in dH_2O for 5 min. The water was drained and the gel covered in 200 ml of developing solution [3% sodium hydroxide (w/v), 3 ml formalin (40% formaldehyde solution)] until the gel was deemed sufficiently developed. The gel was rinsed briefly in dH_2O before covering in 200 ml of fixing solution for a minimum of 15 min before scanning using an Epson GT9600 scanner with transparency unit (Epson Ltd, Hemel Hempstead, Hertfordshire, UK).

Cloning and sequence analysis

Purified PCR products were ligated into pGEM-T vector (Promega, Southampton, UK) and transformed into XL1-Blue supercompetent *Escherichia coli* cells (Stratagene, Cambridge, UK). Transformants were transferred onto fresh Luria–Bertani-ampicillin agar plates and screened for inserts of the correct size by colony PCR using vector primers M13f and M13r. To obtain clones representative of the major DGGE bands in a community profile, clones obtained from a library of the same Ar3F/Ar9R PCR product were nested with rSAf/PARCH519r and compared with the community DGGE profile.

Selected M13f/M13r PCR products were sequenced using primers Ar3R, PARCH519r, PARCH533f (reverse-complement of PARCH519r) and Ar9R (Table 1) to ensure coverage in both directions and assembled using Sequencer 4.1 (Genes Codes Corp., Ann Arbor, Michigan, USA). For phylogenetic analysis, closely related sequences in the GenBank Database at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) were identified using the BLASTn search tool (Altschul *et al*., 1990). All sequences were aligned manually against a secondary structure alignment of archaeal sequences downloaded from the Ribosomal Database project II (Cole *et al*., 2003) using BioEdit Sequence Alignment Editor (Hall, 1999) before removing regions of ambiguous alignment leaving 711 positions. Pairwise distance relationships were calculated using LogDet/Paralinear analysis (Lake, 1994) of variable sites (Lockhart *et al*., 1996) using PAUP [PAUP v4.01 (Swofford, 1998)] with bootstrap support calculated 1000 times. The proportion of invariable sites (47%) was estimated by maximum-likelihood analysis of the most parsimonious trees constructed from a subset of the sequence data representing the major phylogenetic groups. Phylogenetic trees were constructed by the neighbour-joining method (Saitou and Nei, 1987).

Denaturing gradient gel electrophoresis bands were recovered for sequence analysis as previously described (Nicol *et al*., 2003a) and sequenced using primer PARCH519r.

Accession numbers

Sequences of all cloned GFS sequences have been deposited in the GenBank database with accession numbers AY601285 to AY601306.

Acknowledgements

This study was supported by the UK Natural Environment Research Council (Award NER/A/S/2000/01128 to J.I.P. and T.M.E.).

References

Altschul, S.F., Gish, W., Miller, E., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215:** 403–410.

Bever, J.D., Westover, K.M., and Antonovics, J. (1997) Incorporating the soil community into plant population dynamics: the utility of the feedback approach. *J Ecol* **85:** 561–573.

Bintrim, S.B., Donohue, T.J., Handelsman, J., Roberts, G.P., and Goodman, R.M. (1997) Molecular phylogeny of archaea from soil. *Proc Natl Acad Sci USA* **94:** 277–282.

Bomberg, M., Jurgens, G., Saano, A., Sen, R., and Timonen, S. (2003) Nested PCR detection of *Archaea* in defined compartments of pine mycorrhizospheres developed in boreal forest humus microcosms. *FEMS Microb Ecol* **43:** 163–171.

Buckley, D.H., Graber, J.R., and Schmidt, T.M. (1998) Phylogenetic analysis of nonthermophilic members of the kingdom *Crenarchaeota* and their diversity and abundance in soil. *Appl Environ Microbiol* **64:** 4333–4339.

Chapin, F.S., Walker, L.R., Fastie, C.L., and Sharman, L.C. (1994) Mechanisms of primary succession following degalciation at Glacier Bay, Alaska. *Ecol Monogr* **64:** 149–175.

Chelius, M.K., and Triplett, E.W. (2001) The diversity of *Archaea* and *Bacteria* in association with the roots of *Zea mays* L. *Microb Ecol* **41:** 252–263.

Cole, J.R., Chai, B., Marsh, T.L., Farris, R.J., Wang, Q., Kulam, S.A., *et al.* (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res* **31:** 442–443.

DeLong, E.F. (1998) Everything in moderation: *Archaea* as 'non-extremophiles'. *Curr Opin Genet Dev* **8:** 649–654.

Felske, A., and Akkermans, A.D.L. (1998) Spatial homogeneity of abundant bacterial 16S rRNA molecules in grassland soil. *Microb Ecol* **36:** 31–36.

Giovannoni, S.J., Delong, E.F., Olsen, G.J., and Pace, N.R. (1988) Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial-cells. *J Bacteriol* **170:** 720–726.

Griffiths, R.I., Whiteley, A., O'Donnell, A.G., and Bailey, M.J. (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Appl Environ Microbiol* **66:** 5488–5491.

Großkopf, R., Stubner, S., and Liesack, W. (1998) Novel euryarchaeotal lineages detected on rice roots and in the anoxic bulk soil of flooded rice microcosms. *Appl Environ Microbiol* **64:** 4983–4989.

Hall, T.A. (1999) BioEdit: a friendly biological sequence alignment editor and analysis program for Window 95/98/NT. *Nucleic Acids Symp Ser* **41:** 95–98.

Harris, W.F., Santantonia, D., and McGinty, D. (1980) The dynamic below-ground ecosystem. In *Forests: Fresh Perspectives from Ecosystem Analysis.* Waring, R.H. (ed.). Corvallis, USA: Oregon State University Press, pp. 119–130.

Hodkinson, I.D., Webb, N.R., and Coulson, S.J. (2002) Primary community assembly on land – the missing stages: why are the heterotrophic organisms always there first? *J Ecol* **90:** 569–577.

Hodkinson, I.D., Coulson, S.J., and Webb, N.R. (2003) Community assembly along proglacial chronosequences in the high Arctic: vegetation and soil development in north-west Svalbard. *J Ecol* **91:** 651–663.

Jumpponen, A. (2003) Soil fungal community assembly in a primary successional glacier forefront ecosystem as inferred from rDNA sequence analyses. *New Phytol* **158:** 569–578.

Jumpponen, A., Mattson, K.G., and Trappe, J.M. (1998) Mycorrhizal functioning of *Phialocephala fortinii* with *Pinus contorta* on glacier forefront soil: interactions with soil nitrogen and organic matter. *Mycorrhiza* **7:** 261–265.

Jurgens, G., Lindstrom, K., and Saano, A. (1997) Novel group within the kingdom *Crenarchaeota* from boreal forest soil. *Appl Environ Microbiol* **63:** 803–805.

Kaufmann, R., Fuchs, M., and Gosterxeier, N. (2002) The soil fauna of an Alpine glacier foreland: colonization and succession. *Arct Antarct Alp Res* **34:** 242–250.

Lake, J.A. (1994) Reconstructing evolutionary trees from DNA and protein sequences: paralinear distances. *Proc Natl Acad Sci USA* **91:** 1455–1459.

Lockhart, P.J., Larkum, A.W.D., Steel, M.A., Waddel, P.J., and Penny, D. (1996) Evolution of chlorophyll and bacteriochlorophyll: the problem of invariant sites in sequence analysis. *Proc Natl Acad Sci USA* **93:** 1930–1934.

McCaig, A.E., Glover, L.A., and Prosser, J.I. (2001) Numerical analysis of Grassland bacterial community structure under different land management regimens by using 16S ribosomal DNA sequence data and denaturing gradient gel electrophoresis banding patterns. *Appl Environ Microbiol* **67:** 4554–4559.

Muyzer, G., de Waal, E.C., and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes encoding for 16S rRNA. *Appl Environ Microbiol* **59:** 695–700.

Nicol, G.W., Glover, L.A., and Prosser, J.I. (2003a) The impact of grassland management on archaeal community structure in upland pasture rhizosphere soil. *Environ Microbiol* **5:** 152–162.

Nicol, G.W., Glover, L.A., and Prosser, J.I. (2003b) Molecular analysis of methanogenic archaeal communities in managed and natural upland pasture soils. *Glob Change Biol* **9:** 1451–1457.

Nicol, G.W., Glover, L.A., and Prosser, J.I. (2004) Differential response of archaeal and bacterial communities to nitrogen inputs and pH changes in upland pasture rhizosphere soil. *Environ Microbiol* **6:** 861–867.

Ochsenreiter, T., Selezi, D., Quaiser, A., Bonch-Osmolovskaya, L., and Schleper, C. (2003) Diversity and abundance of Crenarchaeota in terrestrial habitats studied by 16S RNA surveys and real time PCR. *Environ Microbiol* **5:** 787–797.

Ohtonen, R., Fritze, H., Pennanen, T., Jumpponen, A., and Trappe, J. (1999) Ecosystem properties and microbial community changes in primary succession on a glacier forefront. *Oecologia* **119:** 239–246.

Øvreås, L., Forney, L., Daae, F.L., and Torsvik, V.L. (1997) Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Appl Environ Microbiol* **63:** 3367–3373.

Patzelt, G., and Bortenschlager, S. (1979) Spät- und nacheiszeitliche Gletscher- und Vegetationsentwicklung im inneren Ötztal. In *Gletscherkundliche Forschungen in den Ostalpen und im Großen Pamir.* Patzelt, G. (ed.). Innsbruck, Austria: Universität Innsbruck, pp. 78–79.

Saitou, N., and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4:** 406–425.

Sandaa, R.-A., Enger, O., and Torsvik, V.L. (1999) Abundance and diversity of *Archaea* in heavy-metal-contaminated soils. *Appl Environ Microbiol* **65:** 3293–3297.

Sigler, W.V., and Zeyer, J. (2002) Microbial diversity and activity along the forefields of two receding glaciers. *Microb Ecol* **43:** 397–407.

Sigler, W.V., Crivii, S., and Zeyer, J. (2002) Bacterial succession in glacial forefield soils characterised by community structure, activity and opportunistic growth dynamics. *Microb Ecol* **44:** 306–316.

Simon, H.M., Dodsworth, J.A., and Goodman, R.M. (2000) Crenarchaeota colonise terrestrial plant roots. *Environ Microbiol* **2:** 495–505.

Sliwinski, M.K., and Goodman, R.M. (2004) Comparison of crenarchaeal consortia inhabiting the rhizosphere of diverse terrestrial plants with those in bulk soil in native environments. *Appl Environ Microbiol* **70:** 1821–1826.

Swofford, D.L. (1998) *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods).* Sunderland, MA, USA: Sinauer Associates.

Tscherko, D., Rustemeier, J., Richter, A., Wanek, W., and Kandeler, E. (2003) Functional diversity of the soil microflora in primary succession across two glacier forelands in the Central Alps. *Eur J Soil Sci* **54:** 685–696.

Woese, C.R., Kandler, O., and Wheelis, M.L. (1990) Towards a natural system of organisms: proposal for the domains *Archaea*, *Bacteria*, and *Eucarya*. *Proc Natl Acad Sci USA* **87:** 4576–4579.