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Identification of novel Crenarchaeota and Euryarchaeota clusters associated with different depth layers of a forest soil

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Abstract

Archaea have been shown to be ubiquitous among soil microbial communities. However, our knowledge on their diversity and spatial distribution in soil ecosystems is still limited. This study was conducted to investigate archaeal community changes along a forest soil depth profile in Unterehrendingen, Switzerland. From four consecutive soil depth layers, bulk soil DNA was extracted. Archaea-specific PCR amplification of small subunit ribosomal RNA genes (rDNA) was performed and combined with restriction fragment length polymorphism (RFLP) analysis with restriction endonuclease *Hae*III [Bundt et al., Soil Biol. Biochem. 33 (2001) 729–738]. Significant changes of the RFLP fingerprints were reproducibly observed from the soil surface to 1 m depth. From the surface soil layer (0–9 cm) and the bottom soil layer (50–100 cm), libraries of PCR-amplified archaeal rDNA fragments were constructed. Screening of the libraries yielded various clones of different *Hae*III RFLP types from the surface and the bottom soil layers, revealing shifts in major archaeal components along the soil depth profile. Clones of all RFLP types were sequenced and phylogenetically affiliated. These analyses revealed even more pronounced Archaea community shifts along the depth gradient. Several novel soil archaeal clusters were identified and some appeared predominantly associated to either the surface or the bottom soil layer. Euryarchaeal rDNA sequences, not yet reported from aerated soils, were found in the surface soil layer and were affiliated to the order *Thermoplasmales* and relatives. Novel crenarchaeal soil clusters were identified that included sequences only retrieved from the bottom soil layer. In this study, a this far unreported variety of archaeal groups was found in a forest soil ecosystem. The distinct depth-related community shift suggested the occurrence of different archaeal types that depend on environmental parameters that change along the soil depth profile.

Keywords: ARDRA; Archaea community changes; Soil depth profile; Small subunit rDNA; Phylogenetic analysis

1. Introduction

For many years it was believed that Archaea are associated with extreme environments only and that they represent archaic life forms adapted to the harsh environmental conditions that existed on earth billions of years ago [1]. Initially, archaeal diversity was based on isolates and has led to the differentiation of two distinct lineages termed Euryarchaeota and Crenarchaeota [2]. Euryarchaeota appeared as a physiologically diverse group, which included extreme halophiles, thermophiles, and methanogens. Crenarchaeota exclusively included sulfur-dependent hyperthermophiles [2]. Only in recent years and by applying cultivation-independent molecular tools, it has become evident that Archaea are ubiquitous and abundant organisms, coexisting with other microorganisms in various environments. Assisted by the use of genetic markers, namely the small subunit (SSU) ribosomal RNA (rDNA) gene, the phylogeny of various isolates and environmental SSU rDNA clones has been inferred [2]; see also the Ribosomal Database Project (RDP-II; [3]). This approach has led to the identification of novel uncultivated archaeal clusters that clearly separate from their cultivated relatives (for a review see DeLong [4]). Within Euryarchaeota, novel groups have been described, which cluster with the diverse order *Thermoplasmales* and relatives. They appear to be important components of marine picoplankton [5,6] and sediment communities [7,8], and were also detected in the anoxic water columns of lakes

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[9,10]. Non-aquatic representatives of *Thermoplasmales* have been retrieved from predominantly anoxic environments such as rice field soils [11,12], and the gut of a soil feeding termite [13]. Many of the environmental sequences cluster with the euryarchaeal orders *Methanomicrobiales*, *Methanobacteriales*, and *Methanosarcinales*, which have previously been isolated and cultured from the environment. Crenarchaeal phylogeny has fundamentally changed by the isolation of novel SSU rDNA clones from mesophilic and psychrophilic habitats. Five new and distinct clusters of uncultivated Crenarchaeota have been derived from various non-thermophilic environments. Clones from marine environments (water column [5,6,14,15]; sediment [16]; the gut of a deep sea cucumber [17]) and lake sediments [18] form a prominent cluster referred to as Group I.1a of uncultured Crenarchaeota [4]. A second cluster, referred to as Group I.1b of uncultured Crenarchaeota [4], is formed by clones that have been retrieved from agricultural and forest soils [19–25] as well as from lake sediments [26] and the gut of a soil feeding termite [13]. Sequences isolated from a boreal forest soil form a novel terrestrial soil cluster referred to as Group I.1c of uncultured Crenarchaeota [4,22]. Group I.2 of uncultured Crenarchaeota has been inferred from few sequences isolated from lake and marine sediments [4,16,26]. Group I.3 of uncultured Crenarchaeota comprises sequences from lake water [10], lake sediments [26,27], rice field soil [11], and deep subsurface paleosol [28]. Despite all the effort undertaken for describing and characterizing the occurrence of Archaea in various environments, no isolates of non-thermophilic Crenarchaeota have been cultivated so far. This has hindered a biochemical and physiological characterization of these still mysterious organisms.

A strategy to learn more about the ecological role of uncultivated Archaea is to study changes of their occurrence in various habitats. This may help to identify Archaea growth promoting environmental factors, and in turn may help to develop new isolation and cultivation strategies. For agricultural and forest soils, few studies have been performed to relate archaeal abundance or population structures and spatial distributions. The interior and the surface of plant roots have been described as a niche with increased abundance and activity of certain crenarchaeal groups [29,30].

In a recent study [31], we applied PCR amplification of an Archaea-specific SSU rDNA fragment followed by restriction fragment length polymorphism (RFLP) analyses with restriction endonuclease *Hae*III to resolve Archaea community structures along a profile to 1 m depth of a forest soil in Switzerland. We reported gradual changes in Archaea community *Hae*III RFLP fingerprints along the depth profile. In the present study the investigations were expanded by cloning these archaeal SSU rDNA fragments and by sequence analyses. The objective of this study was to identify the Archaea community components contributing to the observed archaeal *Hae*III RFLP fingerprint changes between the surface and the bottom soil layers in the depth profile [31]. Our hypothesis was that changes of soil characteristics along the depth profile, as revealed by chemical parameters, promoted growth of phylogenetically different archaeal groups.

2. Materials and methods

2.1. Field site and experimental design

The field site for this study has been described in detail by Bundt et al. [31]. Briefly, the experimental forest plot was located near Unterehrendingen, Switzerland. The stand was planted in 1930 dominantly with Norway spruce (*Picea abies* (L.) Karst.) mixed with beech (*Fagus sylvatica* (L.)) and few other species. The soil type in the area was a typical Haplumbrept [32].

2.2. Soil sampling and analyses

Soil samples for DNA analyses were taken in October 1998 in the context of a study to investigate the effects of preferential water flow on biological characteristics in soil [31]. For this purpose 45 mm water stained with the food dye Brilliant blue (C.I. 42090) (3 g l^{-1}) was sprinkled during 6 h onto a 1×1.5 m wide area. This simulated a rainfall event and stained preferential water flow paths in the soil [33]. One day after application of the dye solution, a trench was opened to 1.2 m depth. A vertical soil profile of 1×1 m was prepared and divided into depth layers of 0-9 cm, 9-20 cm, 20-50 cm, and 50-100 cm. The blue-stained areas were defined as preferential water flow paths, the non-stained areas as soil matrix. Three types of soil samples were collected, i.e. (1) preferential flow path soil from the blue-stained areas; (2) matrix soil from the unstained areas; and (3) bulk soil samples collected from the whole area of each depth layer. The samples used for detailed molecular analyzes in the present study were the bulk soil samples, which represented the regular soil type and included preferential flow path areas and matrix soil. In the trench, a series of five consecutive soil profiles was prepared at 10-cm intervals. Soil samples from corresponding depth layers of all five consecutive soil profiles were pooled and mixed. Chemical and physical soil analyses were described by Bundt et al. [31] (Table 1).

2.3. DNA extraction

DNA was extracted from fresh soil samples using a bead beating procedure. Briefly, fresh soil (approximately 0.5 g dry wt. equivalent) was added to 1.5-ml reaction tubes containing 0.5 g glass beads (0.1 mm diameter). 1 ml extraction buffer (100 mM Tris/HCl, pH 7.4, 10 mM EDTA, 1.5% SDS, 1% deoxycholate, 1% Nonidet P-40, 5 mM thiourea, 10 mM dithiothreitol; [34]) was

Table 1
Physical and chemical soil characteristics of the four forest soil depth layers

Depth [cm]	pH[a]	Sand [%]	Silt [%]	Clay [%]	N_{tot} [%]	C_{org} [%]
0–9	3.4	35.8	45.4	18.8	0.137	2.565
9–20	3.7	35.9	45.6	18.5	0.050	0.855
20–50	3.8	34.5	45.6	19.9	<0.03	0.471
50–100	3.9	29.4	48	22.6	<0.03	0.288

[a]pH in 10 mM $CaCl_2$.

added to each sample. Bead beating was performed for 1 min with a cell homogenizer (MSK Zellhomogenisator, B. Braun Biotech International, Melsungen, Germany) at 4000 oscillations min^{-1}. Slurries were centrifuged (1 min, $16\,000\times g$) and supernatants were saved and extracted once with 1 volume phenol/chloroform/isoamylalcohol (25/24/1) and twice with 1 volume chloroform/isoamylalcohol (24/1). Extracts were mixed with 1 volume precipitation solution (20% polyethylene glycol 6000, 2.5 M NaCl), incubated at 37°C for 1 h and centrifuged at room temperature (15 min, $16\,000\times g$). Pellets were washed once with 0.5 ml 70% ethanol, air dried, and resuspended in TE-buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8) at 1 ml (g dry wt. soil extracted)$^{-1}$ [35]. Extracted DNA was quantified using PicoGreen® (Molecular Probes, Eugene, OR, USA) according to the procedure of Sandaa et al. [36].

2.4. PCR amplification and RFLP analyses

2 ng soil DNA was used for each PCR amplification performed according to Widmer et al. [37]. For PCR, an Archaea-specific forward-primer (ARCH915-for: 5′-AGGAATTGGCGGGGGAGCAC-3′; *Escherichia coli* numbering 915–934 bp [38]) targeting the SSU rRNA genes in conjunction with a universal SSU rRNA gene reverse primer (UNI-b-rev: 5′-GACGGGCGGTGTGT(A/G)CAA-3′; *E. coli* numbering 1390–1407 bp) modified [31] from Amann et al. [38] were used. PCR amplification was performed with 1 U *Taq* DNA polymerase (Amersham, Zürich, Switzerland) and the supplied buffer with 2 mM $MgCl_2$. The annealing temperature was 65°C and PCR was performed with 40 amplification cycles [31]. PCR product quality was analyzed on 1.2% UltraPure agarose gels (Gibco/BRL, Life Technologies AG, Basel, Switzerland). RFLP analysis was performed using restriction endonuclease *Hae*III (Boehringer Mannheim, Rotkreuz, Switzerland) according to Widmer et al. [39]. RFLP patterns were analyzed by electrophoresis in 4% MetaPhor gels (FMC BioProducts, Rockland, USA) and ethidium bromide staining. Gels were photographed using Polaroid 677 films (Polaroid, Uxbridge, UK). Images were scanned (600 dpi Apple ColourOne scanner), and intensities of distinct RFLP bands from each pattern were quantified using NIH Image v.1.61 according to Widmer et al. [40]. For statistical analyses of single bands, total lane intensities (defined as the sum of the intensities of all bands detected in one lane) was set to 100% and each band was expressed as percentage of this standardized sum. Two-sided *t*-tests for pairwise comparisons of standardized band intensities were performed using Excel 98 (Microsoft Corporation, Redmond, WA, USA). Linear correlations between band intensities in community *Hae*III RFLP fingerprints and band occurrences in the gene libraries were calculated with Excel 98 (Microsoft Corporation).

2.5. DNA cloning and sequencing

PCR products were cloned without prior purification using the pGEM®-T Easy cloning kit (Promega, Madison, WI, USA) and *E. coli* JM109 (Promega). Gene libraries were screened by touching white bacterial colonies with a pipette tip and adding cells to 50 μl PCR amplification mixes, prepared as described above for the Archaea-specific PCR. Amplification was performed with 30 amplification cycles using the conditions described above. PCR products were analyzed on 1.2% UltraPure agarose gels and positive products were subjected to RFLP analyses as described above. Clones were classified based on their *Hae*III restriction patterns and labeled 'a', 'b', 'c', etc. Rarefaction analysis of *Hae*III restriction patterns was performed for each library with the program Resampling Rarefaction 1.0 (http://www.uga.edu/~strata/software/) using 10^4 resamplings. Maximal clone richness in each library was estimated by parametric calculation of the asymptote from rarefaction curves using the Michaelis–Menten equation [41]. Plasmid DNA of representative clones was purified using Wizard plasmid miniprep columns (Promega). The sequences of the approximately 495 bp long cloned Archaea SSU rDNA PCR products (location according *E. coli* numbering: 915–1407 bp) were determined from both strands (T7 and SP6 sequencing primer sites) using an ABI310 Genetic Analyzer (Applied Biosystems, Rotkreuz, Switzerland).

2.6. DNA sequence analyses

The RFLP patterns of cloned archaeal SSU rDNA fragments were confirmed using sequence-based calculated restriction fragmentation with MacDNASIS v. 3.6 (Hitachi Software Engineering America, Ltd., San Bruno, CA, USA). Calculated fragmentation patterns were presented as fragment sizes on a logarithmic scale. Twenty new clone

sequences and 118 control sequences retrieved from the public domain databases RDP-II (http://rdp.cme.msu.edu) and GenBank (www.ncbi.nlm.nih.gov/GenBank/) were aligned using the BioEdit version 5.0.9 sequence analysis software [42]. Phylogenetic analyses were performed with BioEdit version 5.0.9 expanded with selected Phylip software package modules [43]. Sequence distance-based phylogenetic tree inference was performed on the whole DNA sequence of the PCR products with Jukes&Cantor distance value calculation [44] and neighbor joining cluster analysis [45]. For maximum likelihood inference of phylogenetic relationships the fastDNAml program [46] was used. Inferred trees were viewed and edited using Tree Explorer version 2.12 from the MEGA2 software package [47].

2.7. Nucleotide sequence accession numbers

Nucleotide sequences of the 20 novel Archaea SSU rDNA clones determined in this study have been deposited in the GenBank database with the accession numbers AF458627–AF458646.

3. Results

3.1. DNA extraction and detection of Archaea populations

Image analysis revealed that the three different soil sample types, i.e. preferential water flow path soil, matrix soil, and bulk soil, all displayed the same depth-dependent changes in the Archaea *Hae*III RFLP fingerprints [31] (Table 2). Therefore the bulk soil samples from each depth layer, representing the actual soil type including preferential flow paths and matrix soil, were chosen for detailed cloning and sequencing analyses of archaeal SSU rDNA PCR products.

The quantities of DNA extracted from fresh bulk soil markedly decreased with increasing depth in the profile. In the surface soil layer (0–9 cm), DNA quantity was highest with 30 μg g^{-1}. Between 9 and 20 cm, 24 μg g^{-1} DNA was extracted. DNA quantity dropped to 13 μg g^{-1} in the soil layer between 20 and 50 cm and was lowest in the bottom soil layer (50–100 cm) with 3 μg g^{-1}. PCR amplification of target rRNA gene fragments was performed on the same quantity (2 ng) of bulk soil DNA to allow for a direct comparison of relative band intensities in the *Hae*III RFLP fingerprints (Fig. 1). High-resolution agarose gel analysis resolved five prominent bands within the Archaea community *Hae*III RFLP fingerprints (Fig. 1, bands I–V). Densitometric quantification and statistical analysis of band intensities from bulk soil, preferential water flow path soil, and matrix soil revealed significant changes in the *Hae*III RFLP fingerprints between the four depth layers ([31]; Table 2). Intensity of Band I decreased from the surface soil layer (0–9 cm) to the bottom soil layer (50–100 cm) to 47% (**$P < 0.01$). Band III displayed the opposite trend as it increased in intensity from the surface to deeper soil layers with a maximum of 170% in the bottom relative to the surface soil layer (*$P < 0.05$). Band II was only detected in the surface, but not in deeper soil layers. Band IV revealed no significant changes along the entire depth profile, whereas band V showed a slight increase to 124% intensity relative to the surface soil layer (*$P < 0.05$). No significant differences were observed when comparing total lane intensities between the four depth layers.

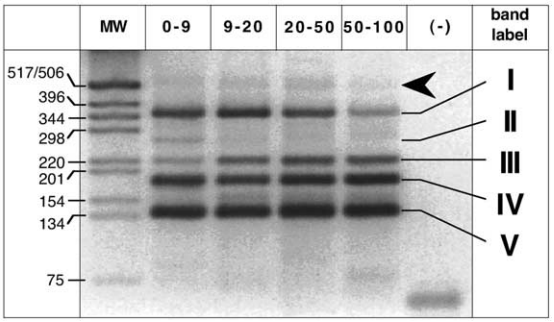

Fig. 1. *Hae*III RFLP patterns of archaeal 16S rDNA fragments amplified from bulk soil DNA extracts from depth layers 0–9, 9–20, 20–50 and 50–100 cm of a Swiss forest soil. MW: 1 kb molecular mass marker (Promega); (−): negative control. The arrowhead indicates the migration position of the undigested Archaea PCR-product at approximately 500 bp.

Table 2
Relative band intensities[a] of Archaea community RFLP fingerprints along the depth gradient

Band label[b]	Depth layer [cm]			
	0–9	9–20	20–50	50–100
I	1.00 ± 0.05	1.17 ± 0.02	0.82 ± 0.01	0.47 ± 0.05
II	1.00 ± 0.25	ND[c]	ND[c]	ND[c]
III	1.00 ± 0.14	1.41 ± 0.07	1.65 ± 0.05	1.70 ± 0.01
IV	1.00 ± 0.07	0.94 ± 0.01	1.05 ± 0.01	1.17 ± 0.03
V	1.00 ± 0.04	1.04 ± 0.01	1.10 ± 0.01	1.24 ± 0.02

[a]Intensities of bands were determined densitometrically and were expressed relative to the value determined for the respective bands detected in the surface soil layer. Standard deviations were calculated using the three soil sample types from each depth layer as replicates.
[b]RFLP bands labeled 'I'–'V' according to labeling indicated in Fig. 1.
[c]ND: not detectable.

3.2. Characterization of changing Archaea populations

In order to gain detailed information on the Archaea populations represented by the changing *Hae*III RFLP fingerprints in the different soil layers, gene libraries of Archaea SSU rDNA amplified from the surface soil layer (0–9 cm) and the bottom soil layer (50–100 cm) were constructed. The two libraries were screened by use of Archaea-specific PCR and *Hae*III RFLP analysis performed on single clones. Among the 104 clones screened (39 from the surface soil library and 65 from the bottom soil library), eight different *Hae*III RFLP patterns were observed (Fig. 2). The abundance of each pattern was quantified in both gene libraries, which allowed for a numeric comparison of the relative clone compositions of the top and the bottom soil libraries (Table 3). Richness estimations indicated that from the surface soil library 91% (5 of 5.5) and from the bottom soil library 89% (5 of 5.6) of the patterns were recovered (data not shown). *Hae*III RFLP pattern 'a' was 1.4-fold more abundant in the bottom soil library while patterns 'b', 'c' and 'e' were exclusively found in the surface soil library. Pattern 'd' was 7.7-fold more abundant in the bottom soil library. Patterns 'f'–'h' were infrequent and restricted to the bottom soil library. Further analysis revealed that each of the five prominent bands observed in the complex Archaea community RFLP fingerprints (Fig. 1) was also identified in the RFLP fingerprint of at least one clone (Fig. 2 and Table 4). Band I was exclusively found in pattern 'b' while band II was detected in pattern 'e' only. Band III was mainly attributed to pattern 'd' with a minor contribution of pattern 'f', while band IV mainly originated from patterns 'a' and 'c' with minor contributions of patterns 'g' and 'h'. Band V was composed by patterns 'a'–'e' with minor contributions of patterns 'f' and 'g'. The R^2-value of the linear correlation between band intensities in the community *Hae*III RFLP fingerprints and the band occurrences in the gene libraries was 0.54. These analyses revealed that each of the five *Hae*III RFLP bands displayed the same trend of abundance in the two clone libraries as indicated by the band intensities of the Archaea community *Hae*III RFLP fingerprints.

Table 3
RFLP pattern frequencies in archaeal SSU rDNA fragment libraries

RFLP pattern labels[a]	Archaea SSU rDNA libraries	
	surface soil[b]	bottom soil[b]
a[c]	48.7 (19)	67.8 (44)
b	17.9 (7)	–
c	23.1 (9)	–
d[c]	2.6 (1)	20.0 (13)
e	7.6 (3)	–
f	–	3.1 (2)
g	–	1.5 (1)
h	–	1.5 (1)
x	–	6.2 (4)
Total	100.0 (39 clones)	100.0 (65 clones)

[a]RFLP pattern labels as defined in Fig. 2; 'x' represents cloning artifacts.
[b]RFLP pattern frequencies are presented in percent for each library. The number of clones identified is given in parentheses.
[c]Sequence and phylogenetic analyses (Fig. 3) revealed that clones from the surface and bottom soil layers, which shared the same *Hae*III RFLP pattern, belonged to different phylotypes.

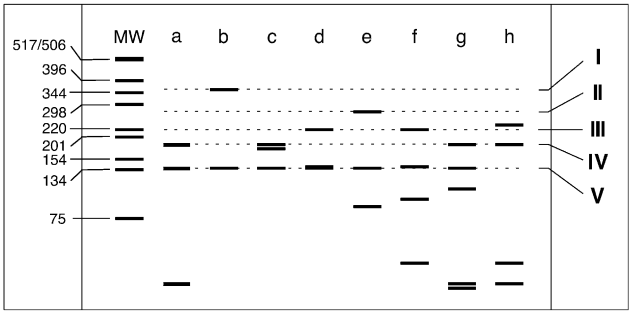

Fig. 2. Calculated *Hae*III RFLP patterns that occurred among 104 cloned archaeal 16S rDNA fragments from surface (0–9 cm) and bottom (50–100 cm) soil layers from a Swiss forest soil. Fragment sizes were inferred from DNA sequences of representative clones. MW: 1 kb molecular mass marker (Promega); RFLP patterns were labeled 'a'–'h'. Bands I–V were detected in the complex RFLP fingerprints (see also Fig. 1a and Table 3).

3.3. Phylogenetic analyses of Archaea clones

The question of which phylotypes were represented in the Archaea community *Hae*III RFLP fingerprints was addressed by sequencing Archaea SSU rDNA clones, which represented the different Hae*III* RFLP types. The 20 new sequences isolated from the surface and the bottom layers of the forest soil depth profile were aligned to 118 defined control sequences derived from public databases. Only control sequences that covered the entire SSU rDNA region defined by the PCR primers used in this study were included in the analysis. The phylogeny of the aligned clone sequences was inferred by using standard distance estimation and cluster analysis routines (data not shown) as well as a maximum likelihood routine (Fig. 3). The resulting phylogenetic trees revealed similar tree topologies and in particular identical clustering of the distinct clusters 'A'–'E' indicated in Fig. 3.

Table 4
Assignment of individual RFLP bands in Archaea community fingerprints to specific RFLP patterns

Band[a]	Size[b]	RFLP pattern[c]
I	355	b
II	269	e
III	218	d (f)[d]
IV	184	a, c, (g, h)[d]
V	138	a, b, c, d, e, (f, g)[d]

[a]Band labels as defined in Fig. 1.
[b]Fragment sizes (in bp) of bands I–V. Sizes were determined from sequence data as shown in Fig. 2.
[c]RFLP pattern labels as defined in Fig. 2.
[d]Patterns shown in parentheses revealed a minor contribution.

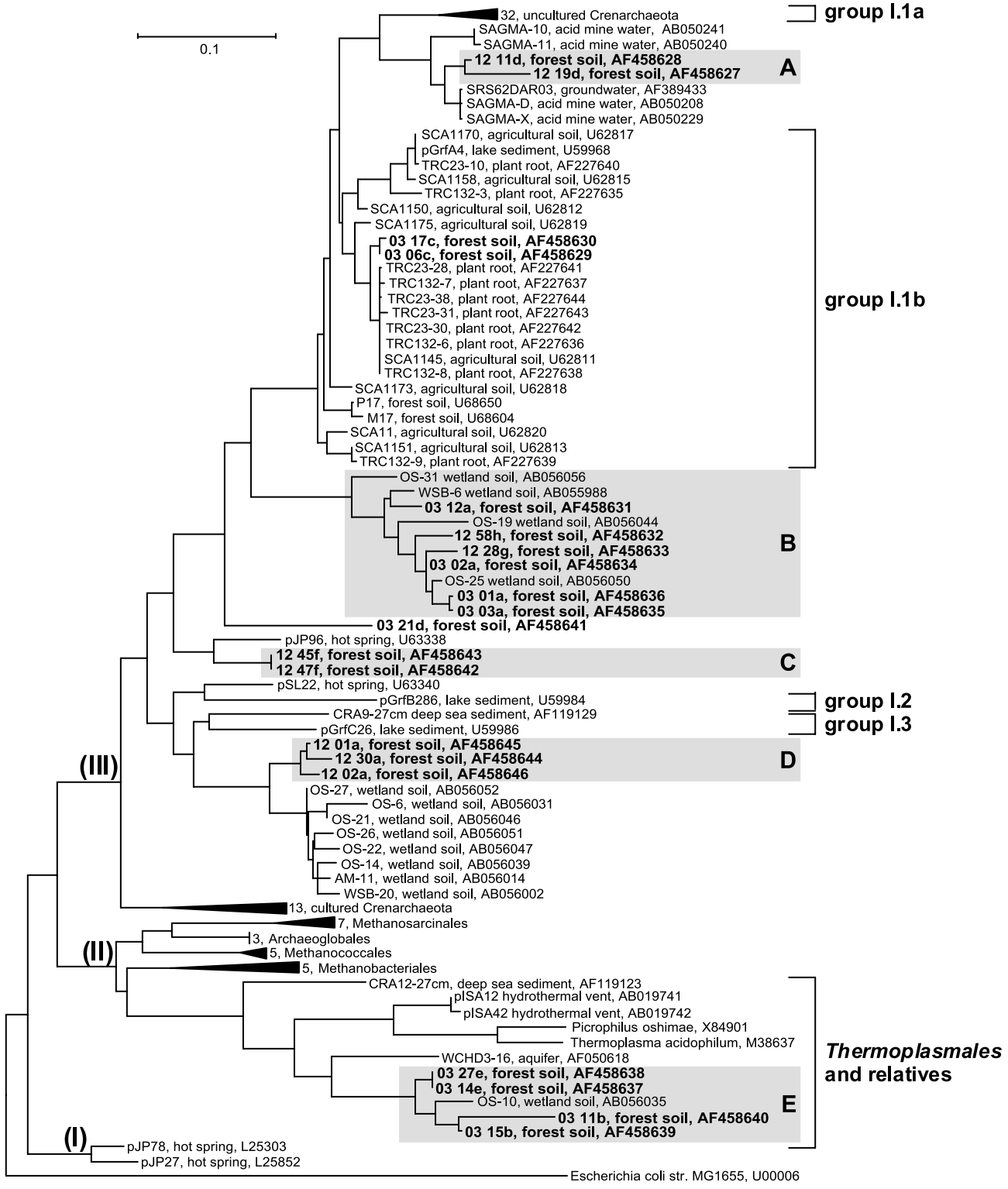

Fig. 3. Phylogenetic tree based on maximum likelihood calculation. Each of the archaeal SSU rDNA sequences is identified by its clone name, by its source, and by its sequence accession number. Sequences isolated in the present study are printed in bold. Clone names indicate the origin of the different clones, i.e. layer (surface soil: 03; bottom soil: 12) followed by the clone number (01–58) and the *Hae*III RFLP type ('a' to 'h'). Cluster (I) defines the archaeal kingdom of Korarchaeota, cluster (II) the kingdom of Euryarchaeota, and cluster (III) the kingdom of Crenarchaeota. Shaded clusters 'A'–'E' mark novel archaeal forest soil clusters. Numbers at condensed clusters indicate the number of sequences included. Brackets at the right margin mark phylogenetic clusters defined in the literature [4]. The scale bar indicates the average substitution rate per nucleotide position.

Of the 20 sequences that were derived from the soil depth profile described in this study, 16 grouped with Crenarchaeota and four with Euryarchaeota. The crenarchaeal sequences were associated with four distinct clusters ('A'–'D'). Cluster 'A' included clones 12 11d and 12 19d from the bottom soil layer and displayed highest similarity to two sequences from deep subsurface acidic mine water (clones SAGMA-D and SAGMA-X; [48]) and to one sequence from groundwater (clone SRS62DAR03). Cluster 'B' included four sequences from the surface soil (clones 03 01a, 03 02a, 03 03a, and 03 12a) and two sequences from the bottom soil layer (clones 12 28g and 12 58h). They grouped with four clones isolated from a wetland soil in Japan (clones OS-19, OS-25, OS-31, and WSB-6). Two sequences from the bottom soil layer (clones 12 45f and 12 47f) formed cluster 'C', which was only weakly associated with clone pJP96 from a Yellowstone hot spring. Three sequences from the bottom soil layer (clones 12 01a, 12 02a, and 12 30a) formed cluster 'D', which branched closely from a group containing eight clones from the Japanese wetland soil mentioned above (clones OS-6, OS-14, OS-21, OS-22, OS-26, OS-27, AM-11, and WSB-20). Two sequences from surface soil (clones 03 06c and 03 17c) were allocated to the terrestrial cluster of uncultured Crenarchaeota (Group I.1b) in close vicinity to sequences cloned from plant roots and agricultural bulk soil (several of the TRC-clones and clone SCA1145). One sequence retrieved from surface soil (clone 03 21d) formed a separate branch without close association to any sequences reported to date. Euryarchaeal sequences derived from this study clustered exclusively with *Thermoplasmales* and relatives (clones 03 11b, 03 15b, 03 14e, and 03 27e) and formed a cluster termed 'E' within this diverse order. One sequence from Japanese wetland soil (clone OS-10) and one sequence from a hydrocarbon-contaminated aquifer (clone WCHD3-16; [49]) supported this cluster.

4. Discussion

Archaea community shifts along the depth profile of a Swiss forest soil were recently reported by Bundt et al. [31] by use of *Hae*III RFLP analysis of SSU rDNA PCR products. Little information is available on associations of specific Archaea populations with changing chemical, physical, or biological conditions in soils. Such information, however, may allow to learn more about physiology and potential functions of uncultivated Archaea [24,25]. The observed archaeal community shifts along a soil depth profile, therefore, represented an interesting model system for assessing differences in Archaea populations in closely connected natural soil habitats. In the present study, detailed molecular analyses were applied for a profound description of the observed changes in archaeal community *Hae*III RFLP fingerprints between the surface soil layer (0–9 cm depth) and the bottom soil layer (50–100 cm depth).

Archaea have repeatedly been described in various agricultural and forest soils [19–25], however, until now no isolates have been cultivated from soil systems. Therefore molecular analyses based on SSU rRNA and its genes can provide important contributions for a detailed assessment of archaeal communities in soil systems. The restrictions to this approach, however, are that only limited information on physiology and potential functions of the organisms can be derived from rRNA sequences. For Archaea this has been particularly limiting, since described archaeal physiologies have tight associations to extreme environments [2]. Therefore, it appears of great interest to assign the occurrence of novel, uncultured Archaea populations to specific environments and to gain knowledge about their preferred habitats and growth conditions.

The occurrence of sequences associated with *Thermoplasmales* and relatives, an euryarchaeal order, was particularly interesting, since no euryarchaeal sequences have yet been described in forest soils. In terrestrial habitats, Euryarchaeota have been found mostly in anaerobic environments such as rice field soils [11,12,50,51] or the methanogenic zone of lake sediments [52]. Therefore, it may be surprising that in the forest soil depth profile, Euryarchaeota were only found in the surface layer, which was certainly better supplied with oxygen than the layer at 50–100 cm depth. RFLP analyses of single clones confirmed this depth-related occurrence. Sequences with *Hae*III restriction patterns 'b' and 'e' (Fig. 2) revealed euryarchaeal origin and displayed a high relative abundance in the surface soil (18% and 7.6%; Table 3). They were not found among the 65 clones isolated from the bottom soil layer. Even though no detailed information on redox conditions were recorded from this experiment, the presence of sequences from mesophilic Euryarchaeota in the surface soil layer of this forest soil suggested that certain groups of *Thermoplasmales* and relatives are not strictly dependent on anoxic environments. Buckley et al., 1998 [23] proposed that some terrestrial Crenarchaeota may be tolerant to oxic conditions. Based on our observations we speculate that also some mesophilic Euryarchaeota may be tolerant to oxic conditions in soil. The recent identification of a euryarchaeal sequence retrieved from maize root [30], presumably a typical oxic habitat, supports this hypothesis.

The novel crenarchaeal clusters 'B' and 'D', both contained clones with *Hae*III RFLP pattern 'a'. Interestingly, cluster 'B' contained only *Hae*III RFLP pattern 'a'-clones from the surface soil, while cluster 'D' contained *Hae*III RFLP pattern 'a'-clones from the bottom soil layer (Fig. 3 and Table 3). The two sequences from the bottom soil layer that displayed *Hae*III RFLP pattern 'd' formed a novel soil cluster between Group I.1a and Group I.1b of uncultivated Crenarchaeota. The first group consists of sequences from marine environments and freshwater lake

sediments [4], whereas the second group is exclusively composed of sequences from terrestrial habitats [4]. Only one clone, which displayed *Hae*III RFLP pattern 'd', was isolated from the surface soil layer (Table 3) and was phylogenetically separated from the same *Hae*III RFLP types retrieved from the bottom soil layer. Thus, the marked increase in relative abundance of pattern 'd' from the surface to the bottom soil layer rather reflected a change of different phylotypes with the same *Hae*III RFLP pattern than an increase of a specific phylotype from the surface to the bottom layer (Table 3). These different phylogenetic associations of clones sharing the same *Hae*III RFLP patterns, i.e. patterns 'a' and 'd', derived from the surface and bottom soil layers (Fig. 3) indicated that the two soil layers harbored highly distinct archaeal populations. Although very powerful for answering our research objectives, these findings clearly indicated limitations of RFLP analyses with one or few restriction enzymes. We demonstrated that organisms of different phylogenetic affiliations shared the same *Hae*III RFLP pattern and only could be distinguished by use of more detailed phylogenetic analyses (Fig. 3).

Crenarchaeal organisms represented by RFLP pattern 'c' were exclusively identified in the surface soil layer (23% abundance) and clustered with Group I.1b of uncultivated terrestrial Crenarchaeota. The relatively minor association of forest soil derived archaeal sequences with this cluster was surprising, since the majority of clones retrieved from aerated soils fall into this group [19–21,23,25,29]. The only exceptions are sequences retrieved from a Finnish forest soil, which form a unique cluster termed FFSB [22]. Unfortunately, it was not possible to relate our sequences to the FFSB cluster since no overlapping sequence portions of the SSU rRNA genes were amplified by the two different PCR protocols. The phylogenetic association of three sequences (clones 03 21d, 12 45f, and 12 47f) within the kingdom of Crenarchaeota remained unclear due to the lack of related sequences in public databases. The closest relatives found in databases were clone SCA1158 with 86% sequence identity to clone 03 21d and clone pJD96 with 91% sequence identity to clones 12 45f and 12 47f. Checking the sequences for chimeric characteristics indicated that they did not represent PCR artifacts of known SSU rRNA genes (data not shown). Therefore, it was not possible in this study to propose a confirmed phylogenetic affiliation for these three clones.

Based on chemical and physical analyses of the four soil depth layers (Table 1), factors that correlated with archaeal community changes could be identified. Organic carbon and total nitrogen showed particularly strong decreases and therefore paralleled the disappearance of Euryarchaeota and Group I.1b Crenarchaeota along the soil depth profile. Fresh organic material has been related to increased archaeal abundance and methane production in anaerobically incubated soil cores [53]. Conversely, texture and pH exhibited less pronounced changes and may be of minor importance for the observed Archaea community shifts.

The approach of isolating and characterizing SSU rRNA gene sequences has been powerful for describing the diversity of the soil microflora. Since no archaeal isolates have been cultivated from soil, we still lack information on their physiology and functions. New approaches are being developed and used for gaining a better understanding of these soil organisms. Information on their functional properties can be gained from metagenome analyses [54,55]. With these approaches, high molecular mass genomic DNA has been cloned directly from environmental habitats. Large inserts containing the rRNA operon allowed first to relate the cloned DNA to its archaeal source and second to explore the genome of the organism. Detailed studies on genome architecture of two marine Crenarchaeota have already been performed and several protein-coding genes have been described [54,55]. It has been possible, for example, to express a DNA polymerase from the native genes of the uncultured psychrophilic Crenarchaeum *C. symbiosum*. Subsequent biochemical characterization demonstrated its heat labile character [56]. This finding was therefore in agreement with the psychrophilic phenotype inferred from the distribution of rRNA genes in the environment. The archaeal SSU rRNA gene sequences described in this study and elsewhere therefore may represent the keys to genomic analyses of uncultivated Archaea in soil.

Data obtained with the PCR/*Hae*III RFLP approach in conjunction with phylogenetic analyses supported the hypothesis that distinct archaeal populations were present in different depth layers of a forest soil. Representatives of Euryarchaeota, associated with the order *Thermoplasmales*, were detected in the surface soil layer, a habitat were they have not been described before. Different archaeal populations were detected in either the surface or the bottom soil layer, indicating dependency on specific environmental factors. Their roles in non-extreme environments still remain unclear, however, growing knowledge on the association of specific Archaea populations with well-characterized eco-niches may be useful to design cultivation-based approaches to retrieve them. In addition to serve as phylogenetic markers, specific SSU rDNA sequences, as described in this study, may be helpful for recovering uncultivated organisms or serve as anchors in metagenome analyses.

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