Environmental Microbiology (2003) **5**(11), 1133–1143 doi:10.1046/j.1462-2920.2003.00520.x

Microsite-dependent changes in methanogenic populations in a boreal oligotrophic fen

Pierre E. Galand,[1]* Hannu Fritze[2] and Kim Yrjälä[1]
[1]*Department of Biosciences, Division of General Microbiology, PO Box 56, University of Helsinki, 00014 Helsinki, Finland.*
[2]*Finnish Forest Research Institute, Vantaa Research Centre, PO Box 18, 01301 Vantaa, Finland.*

Summary

Wetlands, including peatlands, are the main source of natural methane emission. Well-defined fen microsites have different methane emissions rates, but it is not known whether the methane-producing *Archaea* communities vary at these sites. Possible horizontal variations of communities, in a natural oligotrophic fen, were analysed by characterizing the methanogens from two well-defined microsites: *Eriophorum* lawn and Hummock. Community structures were studied at two different layers of the fen, showing, respectively, high and low methane production. The structure of methanogen populations was determined using molecular techniques targeting the 16SrRNA gene and combined denaturing gradient gel electrophoresis (DGGE) and restriction fragment length polymorphism (RFLP) analysis. Results subjected to non-metric multidimensional scaling (MDS), diversity indices calculation and phylogenetic analysis revealed that upper layer communities changed with site while deeper layer communities remained the same. Phylogenetic analyses revealed six different clusters of sequences grouping with only two known orders of methanogens. Upper layers of Hummock were dominated by sequences clustering with members of *Methanomicrobiales* and sequences dominating the upper part of the *Eriophorum* lawn were related to members of the order *Methanosarcinales*. Novel methanogenic sequences were found at both sites at both depths. Vegetation characterizing the microsites probably influences the microbial communities in the layers of the fen where methane is produced.

Received 10 June, 2003; accepted 7 August, 2003. *For correspondence. E-mail pierre.galand@helsinki.fi; Tel. (+358) 9 19 15 96 55; Fax (+358) 9 19 15 92 62.

Introduction

Methane is one of the main greenhouse gases responsible for global climate change. Wetlands, including peatlands, are the main source of natural methane emission. They emit 100–200 Tg year^{-1} and therefore have a significant impact on global warming (IPCC, 2001). Mires or peatland constitute a third of the land area of Finland; the proportion of land covered by mire is bigger than in any other country (Lappalainen, 1996). With the predicted increase of the average temperature on the globe, the methane production in these ecosystems is expected to increase (Kettunen *et al*., 1996; Saarnio *et al*., 2000). In anoxic wetlands, methanogenesis is the final step of the mineralization of organic substrates. Simple compounds as acetate or H_2 and CO_2 serve as substrates and results in the production of CH_4 gas (Garcia *et al*., 2000). Microbial activity is exclusively responsible for methane production in natural ecosystems (ASM, 2000). The active organisms are methanogens belonging phylogenetically to the domain *Archaea*.

Cultivation-dependent methods have known limitations, and slow-growing anaerobic methanogenic *Archaea* may be especially difficult to recover from environmental samples. Molecular methods, however, have been shown to allow the recovery and identification of, until now, uncultivable microorganisms. Molecular methods, based on the 16S rRNA genes as universal markers, are therefore good alternative tools for community structure analysis (Head *et al*., 1998).

Methanogen populations have previously been studied in different habitats such as rice field soil (Grosskopf *et al*., 1998; Lueders *et al*., 2001), marine and lake sediments (Marchesi *et al*., 2001; Chan *et al*., 2002; Purdy *et al*., 2002) and peat bog (Hales *et al*., 1996; Nercessian *et al*., 1999). We have earlier used functional gene markers to identify methanogens in the Salmisuo fen. The analyses of methyl coenzyme-M reductase (MCR) sequences revealed a variation of communities with depth and new lineage of methanogens at the *Eriophorum* lawn microsite (Galand *et al*., 2002). Even though fens are known to show spatial variation in CH_4 emissions and vegetation cover (Saarnio *et al*., 1997), possible variation of methanogen population with site has never been studied.

Here we searched for possible horizontal heterogeneity in community structure in a natural oligotrophic fen by

Table 1. Sampling sites, water table depth (WT), peat temperature, average pH and average CH_4 production for peat profiles taken at the Salmisuo fen in June 2001.

Microsite and WT	Sample depth (cm)[b]	Peat temperature (°C)	Average pH and SE	Average potential CH_4[c] production ($nmol.g^{-1}.h^{-1}$) and SE
Hummock; WT: −18 cm	+10	11.7	4.36 (0.04)	0.60 (0.60)
	−10	11	4.39 (0.02)	21.88 (5.88)
	−20	10.2	4.25 (0.01)	14.11 (2.66)
	−30	9.2	4.14 (0.06)	3.68 (2.94)
	−40	8.6	4.11 (0.06)	0.03 (0.02)
Eriophorum lawn[a]; WT: −6 cm	−10	11.5	4.38 (0.06)	15.2 (3.6)
	−20	10.8	4.16 (0.09)	17.4 (7)
	−30	10	4.20 (0.05)	8.3 (5.1)
	−40	9	4.24 (0.05)	0.7 (0.4)

a. Results from (Galand *et al*., 2002)
b. Negative values indicate sample taken bellow the water level.
c. Three parallel peat profiles were taken at the microsite

analysing two different, well-defined, microsites: *Eriophorum* lawn and Hummock. Hummock has been shown in an earlier study to emit lower amounts of methane than the *Eriophorum* lawn microsite (Saarnio *et al*., 1997). In order to show possible spatial variations of the community structure we studied two different layers of the peat, showing, respectively, high and low methane production. Several replicate samples were analysed by targeting specific methanogenic 16SrRNA.

Results

Potential CH_4 production

For Hummock microsites, the average potential CH_4 production, the average water table level and peat temperature for all sampling depths are shown in Table 1. CH_4 production was detected in all peat samples taken at 10, 20 and 30 cm below the water table. The production was highest in the submerged upper layers of the peat and decreased with depth. For each peat profile, the maximum production was detected at 10 cm below the water surface (upper layer) and the minimum at 40 cm. Samples taken 40 cm below the water level (deeper layer) showed potential CH_4 production rates close to zero or no production at all. Two out of three samples taken above the water level at Hummock did not show any CH_4 production, the third one had rate close to zero (Fig. 1).

RFLP pattern analysis

Approximately 50 clones from each library were analysed by RFLP. The digestion of the cloned DNA sequences resulted in 14 unique RFLP patterns (Fig. 2). The two samples from the upper layer of the *Eriophorum* lawn (E2-10 and E3-10) showed similar RFLP fingerprinting. Both libraries were dominated by two patterns (C and E) representing more than 70% of the clones. The two samples from the upper layer of the Hummock site were also similar to each other but they differed from the *Eriophorum* samples. These clone libraries were dominated by pattern H (>40% of the clones). The RFLP patterns in the deeper layers of the fen showed homogeneity between microsites. Clone libraries from both sites were dominated by pattern B and G, which represented more than 70% of the clones.

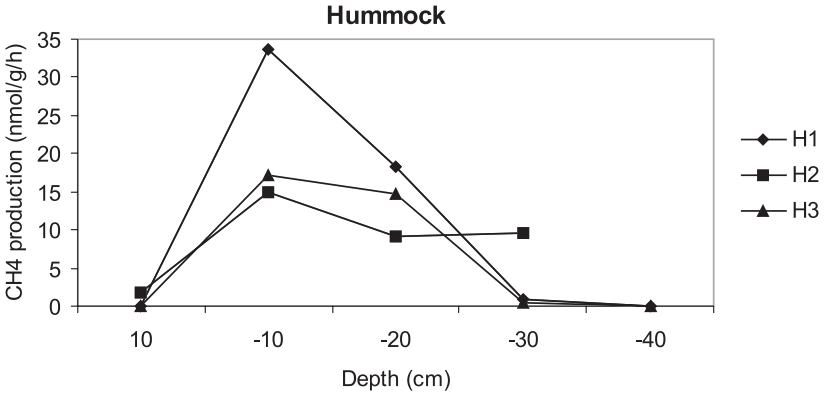

Fig. 1. Potential CH_4 production at different depths for Hummock site at the Salmisuo fen. Three separate peat profiles (H1, H2, H3) were analysed.

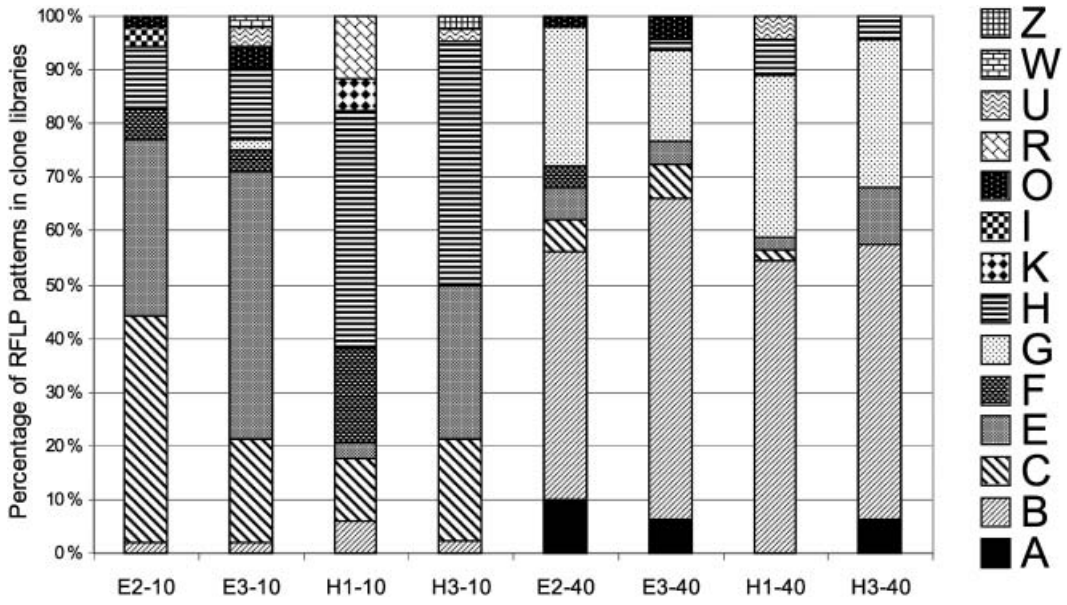

Fig. 2. RFLP analysis of 16SrRNA clone libraries. Sequences were obtained from sample taken 10 and 40 cm below the water level (marked −10 and −40 in the legend). Clone libraries were constructed from two separate peat profiles of the *Eriophorum* lawn (E2 and E3) and two peat profiles from Hummock (H1 and H3). Letters in the legend represent the different RFLP groups.

Pattern A was found exclusively in the deeper layers (Fig. 2). A clear change in *Archaea* 16S rRNA with depth was also seen at both sites; the upper layers of the fen were dominated by patterns C, E, H and F whereas libraries from the deeper part of the fen were dominated by the RFLP patterns B and G.

The MDS plot separated the 16S rRNA libraries in three distinct groups. Upper layer libraries showed heterogeneity, samples from *Eriophorum* lawn and Hummock grouped in two distinct areas. All deeper layer libraries were closely related to each other (Fig. 3). The two replicate samples within each depth and site were very similar in their RFLP pattern composition (Figs 2 and 3) and were therefore grouped together to form four larger distinct clone libraries called E-10, E-40, H-10 and H-40. These pooled libraries contained approximately 100 clones each and were used for diversity analyses.

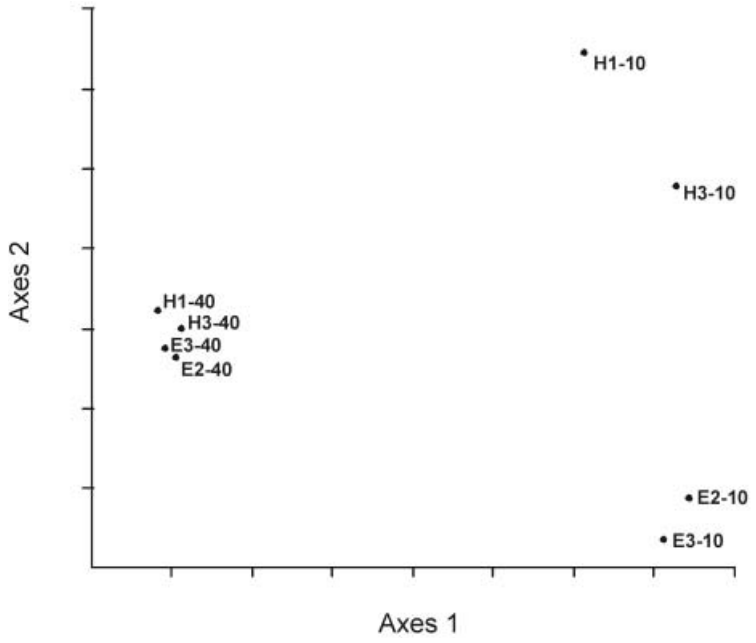

Fig. 3. Representation of non-metric multidimensional scaling plots of 16S rRNA RFLP distances among eight clone libraries calculated with Chord's algorithm. Two depths (–10 and –40) of the *Eriophorum* lawn (E) and Hummock microsite (H) at the Salmisuo fen are represented. See *Experimental procedures* for details.

Table 2. Different diversity indices calculated from RFLP patterns of 16S rRNA methanogen clones. The clone libraries were obtained from two of the fen's microsites: *Eriophorum* lawn (E) and Hummock (H); samples were obtained from 10 and 40 cm below the water level (marked −10 and −40).

	Clone library			
Parameters	E-10	E-40	H-10	H-40
No. of clones analysed	104	97	76	93
Coverage of clone libraries (%)	98	99	97.5	99
Observed no. of RFLP patterns	10	8	9	7
Shannon-Weaver diversity (H′)	1.55	1.43	1.65	1.27
Chao1	10.6 (1.1)	8.5 (1.12)	11 (3)	7.5 (1.1)
RFLP pattern richness (S)[a]	9.23	7.73	9	6.8
Fisher's alpha (α)	2.73	2.1	2.65	1.75
Simpson's evenness (D)	0.71	0.66	0.73	0.63

a. Calculated by rarefaction for a standard sample size of 76 clones.

Diversity indices

Seven different indices representing RFLP richness and evenness were calculated from the pooled clone library data set (Table 2). The percentages of coverage of the clone libraries were superior to 97.5% and the richness curves flattened toward an asymptote (Fig. 4). This indicates that the number of clones analysed covers well the RFLP pattern richness of the libraries. The richness curves for upper layer samples reached higher values than the deeper layer curves, showing higher diversity in the upper layer of the fen. The difference in diversity was confirmed by other calculated indices such as the Shannon-Weaver diversity index, diversity estimators Chao1, Fisher's alpha or Simpson's evenness. They all showed higher values for the upper layers of the fen. No difference in diversity was observed between sites. Some indices showed higher diversity for *Eriophorum* lawns others for Hummock (Table 2).

DGGE fingerprinting

The denaturing gradient used gave a fingerprinting with a high number of bands well spread through out the gel. When PCR products were rerun, each set of samples showed a reproducible DGGE fingerprinting. The MDS analysis of DGGE patterns of the *Eriophorum*/Hummock gel showed that upper layer samples were heterogeneous; the sample plots were separated both within and between sites (Fig. 5). Deeper layer samples were homogenous, grouping close to each other. The two layers of the peat formed two distinct groups of plots (Fig. 5). This variation with depth was also observed when samples for each microsite were run on a separate gel (results not shown). These results are similar to the ones obtained by RFLP analysis; they show more variation in the community structure within the upper layers of the fen than within the deeper ones and a clear difference between communities.

Seven bands were cut from the DGGE gel, re-amplified by PCR, cloned and analysed by RFLP. It revealed that several sequences were present in the same band (up to five); among those one had not been revealed by RFLP analyses (pattern N).

16S rRNA sequences

A total of 33 clones were sequenced, eight chimerical

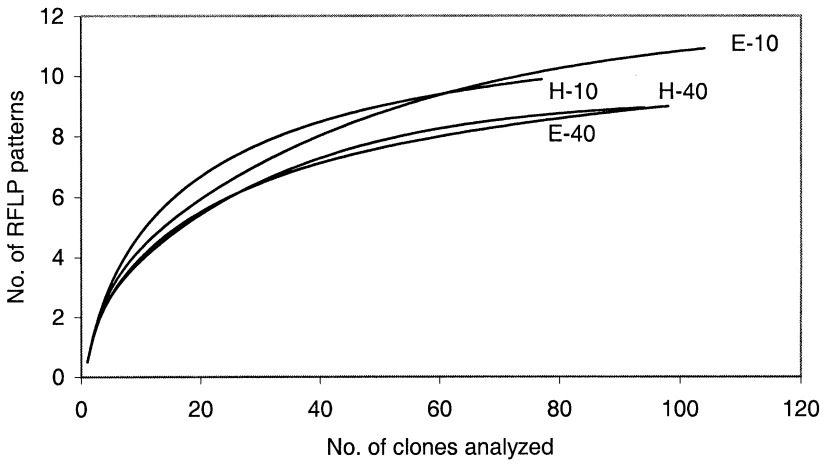

Fig. 4. RFLP pattern richness curve of four 16S rRNA clone libraries. Curves were obtained by rarefaction calculation. See *Experimental procedures* for designations.

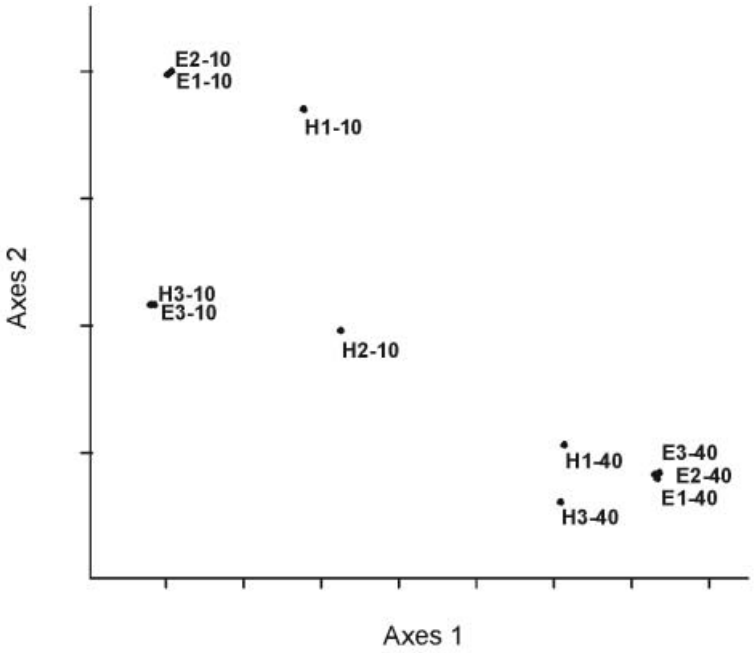

Fig. 5. Representation of non-metric multidimensional scaling plots for two depths (– 10 and –40) of the *Eriophorum* lawn (E) and Hummock microsite (H) at the Salmisuo fen. Data obtained by distance matrix analyses of DGGE fingerprinting. See *Experimental procedures* for details.

sequences were discovered and excluded from the data set. Sequences belonging to a same RFLP group showed high ranges of similarity values (>98%) and clustered together in phylogenetic analyses indicating close relatedness within RFLP groups. Phylogenetic affiliations of the clones retrieved from the boreal fen are shown in Fig. 6. 16S rRNA sequences grouped in six distinct clusters numbered from I to VI. Sequences that grouped in cluster I were closely related to known members of the order *Methanomicrobiales (*similarity ranging values from 93.1 to 93.8%). They were phylogenetically close to environmental sequences retrieved from hydrocarbon-contaminated aquifer (Dojka *et al*., 1998). Sequences grouping in cluster II were closely related to the order *Methanosarcinales*. Sequences E had 97.5–98% similarity to *Methanosarcina siciliae*, *M. acetivorans* and *M. mazei*. It indicates that the sequences belong to the genus *Methanosarcina*. Cluster III and IV both grouped with *Methanosarcinales* and *Methanomicrobiales* (similarity values ≈ 76% for both) but are not directly related to any known methanogens. Sequences from cluster IV grouped with environmental samples retrieved from a forested wetland and sequences from cluster III grouped with clones obtained from rice field soil (RC-III) and oil contaminated soils. Sequences from rice soil were not included in the phylogenetic tree because of their short length. The three sequences contained in cluster V and VI were the ones that showed the largest distance to known methanogens. Sequence F from cluster VI was related to environmental samples retrieved from deep-sea hydrothermal vents (Takai and Horikoshi, 1999) and aquifer contaminated with hydrocarbon- and chlorinated-solvent (Dojka *et al*., 1998). It showed a similarity value as low as 71.3% to its closest recognized species, *Methanococcus infernus*. Sequences O was related to sequences from retrieved environmental samples like wastewater sludge or drainage (Godon *et al*., 1997) and lake sediments (Hershberger *et al*., 1996). The similarity value to closest recognized species was 79.7% to *Methanopyrus kandleri*.

Sequences most commonly found in the upper part of the Eriophorum lawn (pattern C and E) grouped in two different clusters. E pattern sequences were related to *Methanosarcinales* and C patterns to the new cluster III. Organisms that dominated the upper layers of the Hummock site were affiliated to *Methanomicrobiale*s (pattern H). The sequences characteristic for the deeper part of the peat (pattern B and G) were all grouped in cluster IV.

Discussion

A site-related variation of methanogen communities between the upper layers of the Hummock and *Eriophorum* lawn was revealed. This is to the best of our knowledge the first study revealing a horizontal change of communities between well-defined natural microsites. The observed variation is certainly correlated to the vegetation cover and level of the water table characterizing the sites, which again influence nutrient availability in the peat surface.

The lower water table and large aerobic layer defining Hummocks promote oxic decomposition of organic matter and induce a change in the composition of litter entering the anaerobic layers (Svensson and Sundh, 1992). Additionally, the root system of Hummock species concen-

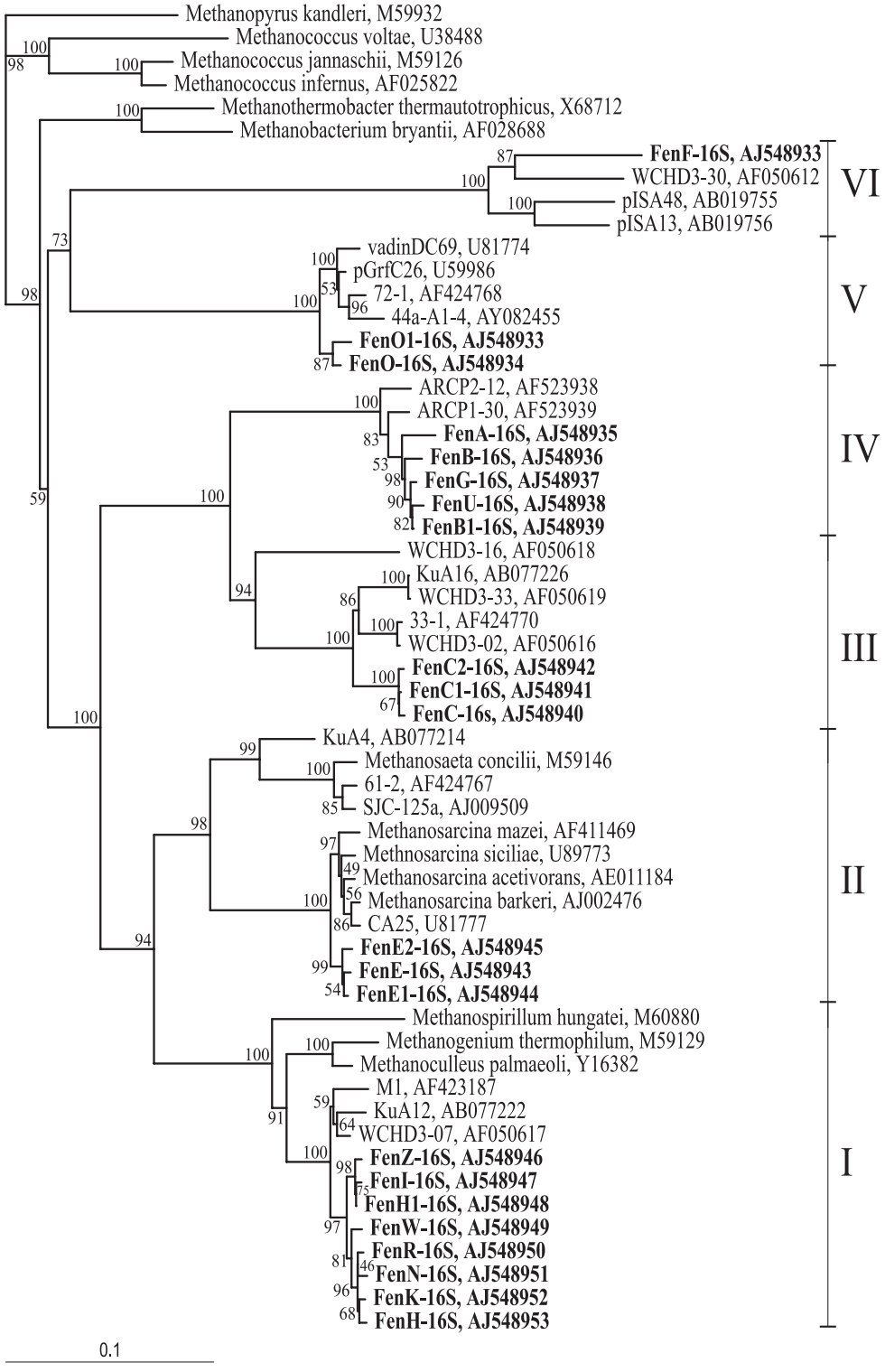

Fig. 6. Phylogenetic dendrogram representing the relationship between 1200 bp long 16SrRNA sequences retrieved from boreal fen and other related methanogenic *Archaea*. Sequences obtained from fen libraries are designated in boldface. The capital letter in the sequence name corresponds to the RFLP pattern; a number beside indicates a second or third representative from the same RFLP pattern. The tree was constructed using FITCH distance matrix analysis. GenBank accession numbers are indicated for all sequences. *Methanopyrus kandleri* was used as outgroup. The scale bar represents 10% sequence divergence. Bootstrap values are shown for each node.

trated in the uppermost aerobic part of the peat affects the transport and availability of carbon compounds in the deeper layers (Saarnio *et al*., 1997). In *Eriophorum* lawn, the nutrient availability is influenced by the presence of vascular plants like *E. vaginatum*. This group of plants provides easily degradable substrate (root exudates and litter) to the anaerobic parts of the fen where decomposition provides nutrient for methanogenesis (Schutz *et al*., 1991).

The phylogenetic analyses showed that the amplified sequences dominating the Hummock site clustered with members of *Methanomicrobiales*, known to include hydrogen utilising methanogens. Several studies have showed hydrogenotrophy to be the dominant nutrition route in peatlands and other wetlands (Lansdown *et al*., 1992; Horn *et al*., 2003). The Hummock's near surface community could be composed of organisms oxidising H_2 and reducing CO_2 to form CH_4. On the other hand, sequences retrieved from the upper part of the *Eriophorum* lawn were related to members of the order *Methanosarcinales* and genus *Methanosarcina*, known to regroup the most versatile methanogens, able to use H_2-CO_2, acetate and methyl compounds (Garcia *et al*., 2000).

In the deeper parts of the fen, no differences in methanogen communities were observed between sites. It indicates that the physicochemical conditions at this depth are stabile horizontally. The influence of the covering vegetation and water table level is low; the nutrient types and amount are probably similar across sites resulting in the presence of similar communities.

The variation in methanogen communities did not have an influence on potential CH_4 production. Comparing the production at Hummock with previously published results for *Eriophorum* lawn (Galand *et al*., 2002), revealed no significant difference in potential CH_4 production between the two microsites at the depth of maximum CH_4 production ($t = 0.96$; $P > 0.19$). Also there were no differences between sites when the sums of all productions for each site were compared ($t = 0.08$; $P > 0.46$). Previous studies have shown a higher emission of CH_4 at *Eriophorum* lawn when compared to Hummock (Saarnio *et al*., 1997). Those results are, however, based on measured flux on site and not on potential production measured in the laboratory.

A clear difference between deeper layer and upper layer communities was shown by RFLP and DGGE fingerprinting and phylogenetic analysis. The depth related variation of methanogen Archaea community has been shown earlier with the functional MCR gene (Galand *et al*., 2002) but never with 16S rRNA. Change of community with depth could reflect an adaptation of microorganisms to changing environmental (temperature, water level) and nutritional conditions. Methods based on DNA extraction and PCR amplification are known to involve bias (Wintzingerode *et al*., 1997). However, as replicate samples in our study were subjected to the same treatment, it is expected that bias operated uniformly allowing proper comparison of results.

Phylogenetic analyses showed that major sequences found at 10 cm bellow the water table where spread through several clusters including some related to *Methanosarcinales*, *Methanomicrobiales* and previously recovered environmental samples. The most common sequences retrieved from a depth of 40 cm were all grouped in a novel cluster, including sequences from environmental samples from coal contaminated forested wetlands. With low similarity values ($\approx$ 76%) to members of the order *Methanosarcinales* and *Methanomicrobiales* those sequences may represent a novel methanogenic lineage. As similarity values to known methanogens are low, it is not possible to confirm that those sequences belong to methanogenic Archaea. Affiliation of this cluster to any physiological group remains difficult.

Our previous study with the functional MCR gene as molecular marker revealed upper layer *Eriophorum* sequences related to *Methanomicrobiales* (Galand *et al*., 2002). Interestingly, 16SrRNA analyses of the upper layer showed sequences related to *Methanomicrobiales*, *Methanosarcinales* and to new groups of environmental samples. The 16S sequences found in this study could, however, also belong to hydrogenotrophs since they are related to members of the genus *Methanosarcina* (possible H_2-CO_2 users). Previous studies have reported a close similarity between 16S and MCR inferred phylogenetic tree topology (Springer *et al*., 1995; Lueders *et al*., 2001; Luton *et al*., 2002). The differences observed here may be a result of the bias inferred by the use of universal primers (Wintzingerode *et al*., 1997; Head *et al*., 1998), but can also be the result of the resolution difference between the 16SrRNA and MCR inferred trees. A lower number of MCR sequences registered in the databases induced a less precise phylogenetic affiliation. Sequences retrieved from the deeper part of the fen, revealed a phylogenetic branching similar to the one obtained with the MCR molecular marker. Study based on the MCR gene also revealed sequences distantly related to *Methanomicrobiales* and *Methanosarcinales*.

The change of community with depth is also reflected by the change in 16S rRNA RFLP diversity. Upper layers characterized by higher amount and diversity of carbon sources could allow the development of a broader range of organisms. The total methanogen diversity in the fen was relatively low (Table 2). Even though the primer pair has been shown to amplify representatives from the four main known methanogen orders (Marchesi *et al*., 2001), it could, when applied to environmental sample, fail to anneal to some groups of sequences. On the other hand, low diversity has been shown earlier in acidic peatland

(Hales *et al*., 1996; Nercessian *et al*., 1999) and the low diversity observed at the Salmisuo fen is probably characteristic for those types of habitats.

Experimental procedures

Environmental site and sample collection

Peat samples were collected in June 2001 from the Salmisuo mire complex in eastern Finland (62°47′N, 30°56′E). The site is a minerogenic, oligotrophic low-sedge Sphagnum papillosum pine fen. Four different types of microsites have been described at the Salmisuo fen: *Eriophorum* lawn, Flark, Hummock and Carex lawn. They are defined by their nutritional statuses and average water table level. Samples were taken from two different microsites: *Eriophorum* lawns and hummocks. The *Eriophorum* lawn is the most common microsite covering 86% of the mire surface. At this site the water level is mainly 5–20 cm below the peat surface and *Sphagnum* mosses, *Eriophorum vaginatum* L., *Vaccinium oxycoccos* and *Andromeda polifolia* dominate the vegetation. Hummocks rise above their surroundings and their average water table is >20 cm below the peat surface. The most typical hummock species is *Spagnum fuscum* (Scimp.) Klinggr. *S. angustifolium* (Russow) C. Jens. *Eriophorum vaginatum* are found in the lower parts of the hummocks (for more details Saarnio et al., 1997; Saarnio et al., 2000). Three parallel peat profiles, distant of approximately 20 cm from each other, were collected from *Eriophorum* lawns (E1-E3) and Hummocks (H1-H3) with a box sampler ($8 \times 8 \times 100$ cm). Soil samples for DNA analyses and potential methane production measurement were taken each 10 cm (±2 cm) of the core (Table 1). All together the sampling resulted in 27 peat samples from which the measurements were taken.

Measurement of methane production and pH

Fifteen ml of peat were added to 100 ml, oxygen free, infusion bottles containing 30 ml of distilled water. The bottles were then flushed with 99.96% nitrogen in order to obtain anoxic conditions (Kettunen *et al*., 1999) and sealed with butyl rubber septa. The glass vials were allowed to stand at 4°C for 5 days to allow the gas present in the peat core section to equilibrate with the headspace. The vessels were then flushed with nitrogen and kept unshaken, at dark, for 170 h. The incubation temperature was +10°C (*in situ* temperature). Four times during the incubation, subsamples were taken from the headspace for analysis of the methane concentration. 100 µl of gas, taken with a Hamilton syringe, were injected to a Perkin Elmer Sigma II gas chromatograph equipped with a flame ionization detector and a 1.5-m column packed with Porapak Q 80 : 100 mesh. Nitrogen was used as a carrier gas at a flow rate of 30 ml min^{-1}. The injector, oven and detector temperatures were set at 35°C, 40°C and 350°C respectively.

The rate of methane production was calculated from the slope of the linear regression given by the CH_4 concentration increase over time. No non-linear ($r^2 < 90\%$) cases were found. After incubation, the peat samples were dried at 60°C and the methane production was normalized to the dry weight.

Ten ml of peat were mixed together with 30 ml H_20 in infusion bottles. After overnight incubation at room temperature, the pH was measured.

DNA extraction

DNA was extracted directly from 0.25 g of peat samples by chemical (detergent) and mechanical (bead beating) cell lysis with the Soil DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA, USA), following the manufacturer's suggested protocol. No further purifications were needed. The DNA quality and yield was assessed in agarose gel. This extraction method has been shown to hold high reproducibility and efficiency when compared with other methods (Niemi *et al*., 2001).

PCR amplification

The 16S rRNA gene was chosen as molecular marker to study the methanogen diversity. The primer pair 146f-1324r (Marchesi *et al*., 2001) was used to amplify specifically an approximately 1200 bp long region of methanogen 16S rRNA gene. A 40-nucleotide long GC-clamp was added to the forward primer to enable DGGE analyses (Muyzer *et al*., 1998). The 50 µl PCR mixture contained 25 pmol of the appropriate primer pairs, 200 µM dNTPs, 1 U Red Hot polymerase (Advanced Biotechnologies, Epsom, UK), PCR reaction buffer and 3 µl of template (concentration determined empirically). Bovine serum albumin (0.1 µg μl^{-1}) was used to prevent PCR inhibition. The reaction conditions were 30 cycles of 94°C for 45 s, 50°C for 45 s, 72°C for 2 min The PCR was performed using a Gene Amp thermal cycler (Perkin Elmer, Wellesley, Massachusetts, USA). Products were analysed on 1% agarose gels with ethidium bromide staining.

Cloning, RLFP and sequencing of PCR product

Eight gene libraries were constructed using DNA extracted and amplified from two depths (−10 and −40 cm) from the two microsites (profiles E2, E3, H1 and H3). Polymerase chain reaction products obtained with 16S rRNA primers (without GC-clamp) were purified with β-agarase and cloned in pGEM-T vector plasmid using JM109 competent cells (Promega, Mannheim, Germany) according to the manufacturer's instructions. Inserted sequences were re-amplified and PCR products were digested with *Hin*fI for restriction fragment length polymorphism (RFLP) analysis. The segments were separated on 3% Synergel (Diversified Biotech, Boston, USA) gel and the resulting RFLP patterns were grouped visually. Colonies showing distinct fingerprint patterns were selected for sequencing. Plasmid DNA was isolated with the Wizard Mini-Preps (Promega) and sequenced using the vector's universal primers SP6 and T7. To assess if the observed RFLP groups contain closely related sequences, two or three representatives from each of the biggest RFLP groups were sequenced. The range of similarity within RFLP groups was determined and the sequences were added to the phylogenetic analyses.

In order to relate the results obtained by DGGE fingerprinting (see below) with those from RFLP analysis of clone libraries, cloned 16S rRNA sequences representing each RFLP groups were re-amplified with GC-clamp and run in DGGE. Positions of the bands representing the cloned sequences were compared with positions of bands from community fingerprinting. Additionally, seven bands from the total community DGGE fingerprint were cut from the gels. They were kept overnight at 4°C in 20 µl H_2O, 5 µl of the suspension were used as template for PCR and the amplified DNA product was cloned as described above. For each cut band, 12 clones containing the insert were analysed by RFLP.

Methanogen 16S rRNA sequences obtained in this study were deposited in the EMBL database, accession numbers AJ548932 to AJ548953.

DGGE fingerprinting

DGGE was performed on all 24 samples from the anaerobic layers with a D-code system (Bio-Rad, Hercules, CA, USA) following the manufacturers instructions. Approximately 400 ng of PCR product were loaded on 6% (wt/vol) polyacrylamide gels in 1 × TAE buffer. The denaturing gradient was determined by empirical trials and vertical DGGE; it ranged from 45 to 57%. One hundred per cent denaturant corresponds to 7 M urea and 40% (v/v) deionized formamide. The gel was run at 60°C, at a constant voltage of 90 V for 18 h. After electrophoresis, the gel was stained for 30 min with SYBR Green I (1 : 10000 dilution; Molecular probes, Eugene, Oreg., USA) and digitized in UV light using Fluor-S multilmager system (Bio-Rad, Hercules, CA, USA). The bands position was marked with gel documentation software Gel Doc 2000 (Bio-Rad). The DGGE analysis was used to compare community between depths within each microsite and to compare community between microsites. All samples for each comparison were run on the same gel.

Phylogenetic analysis

All sequences of approximately 1200 bases were compared to those available in the EMBL database using the BLAST server (Altschul *et al*., 1997) at the European Bioinformatique Institute (EBI, URL http://www.ebi.ac.uk; Hinxton Hall, Cambridge, UK). Sequences were also checked for chimeric artifacts by using CHIMERA_CHECK (Maidak *et al*., 2000) from the Ribosomal Database Project II (RDP) and by analyses of the sequence alignment.

Sequences (≈1200 bp long) were first aligned using CLUSTAL W package (Higgins *et al*., 1994) and then checked manually by using the GENEDOC program [v.2.6.002 (Nicholas *et al.,* 2001)]. A phylogenetic tree was estimated using the PHYLIP package [v.3.57c (Felsenstein, 1993)]. Bootstrap values for 100 replicates were generated with SEQBOOT. DNADIST was used to calculate genetic distances with Kimura-2 model. Calculations were restricted to nucleotide positions that could be unambiguously aligned. The phylogeny was estimated from the distance matrix data by FITCH with global rearrangement of branches and randomized species input order. The dendrogram was verified by maximum parsimony method. Even though full sequences should be used for phylogeny determination (Ludwig and Schleifer, 1994), partial sequences can be used to determine the closest relatives of unknown sequences (Stackebrandt and Rainey, 1995; Ludwig *et al*., 1998).

Statistical analysis

Student *t*-test was used to test possible differences in potential CH_4 production between Hummock and *Eriophorum* lawn microsites.

RFLP patterns data were converted to a distance matrix using Chord algorithm for abundance data and represented in non-metric multidimensional scaling (MDS) plot. DGGE banding patterns were converted to a binary matrix where the presence or absence of a band in each lane at a same position was reported in the matrix with 1 and 0 respectively (van Hannen *et al*., 1999). The binary matrix was transformed to a distance matrix using Jaccard's coefficient for presence-absence data and the resulting similarity values were analysed by MDS plot. Each DGGE lane is represented by one single point in a two dimensional space, the closer the point are to each other, the more similar are the banding patterns. The calculations were made with the PAST software (v.0.97 Hammer and Harper, 2002). The method of Good (Good, 1953; Mullins *et al*., 1995), used to estimate the coverage of the clone libraries, was calculated as: (1-(n/N))*100, where n is the number of unique clones detected in the sample of size N.

Various indices and models were used to analyse the possible variation of the methanogen diversity with depths and sampling sites. Three different categories of measures were used (Magurran, 1988).

Richness indices: Shannon index (*H′*) and OTU richness estimator.

The Shannon-Weaver diversity index (Shannon and Weaver, 1963) was calculated as:

$$H' = -\sum_{i=1}^{s} pi \ln(pi)$$

pi is the proportion of clones belonging to the *i*th OTU and S is the total number of OTUs; RFLP patterns were considered as operational taxonomic units (OTUs) (Moyer *et al*., 1994). OTUs richness estimators were calculated from rarefaction analyses using a standard sample size of 76 clones. The non-parametric estimator Chao 1 was also calculated (Chao, 1984).

Evenness indices: Simpson's index (D; Simpson, 1949).

The Simpson's evenness index was calculated as:

$$D = \sum_{i=1}^{s} pi^2$$

Symbols used are the same as for the Shannon-Weaver formula.

Abundance models: Fisher's Alpha log series richness index (Fisher *et al*., 1943).

Rarefaction and diversity analyses were performed with PAST package (v.0.97 Hammer and Harper, 2002). The online Rarefaction Calculator (http://www2.biology.ualberta.ca/jbrzusto/rarefact.php) was used for Chao1 and species richness estimations for a standardized sample size.

Acknowledgements

This research was supported by Academy of Finland. We thank Veera Kuparinen for technical assistance and Sanna Saarnio for the help provided during the sample taking.

References

Altschul, S., Madden, T., Schaffer, A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25:** 3389–3402.

ASM (American Society for Microbiology) (2000) Environmental change: microbial contributions, microbial solutions. American Society for Microbiology [www document]. URL http://www.asmusa.org/pasrc/reports.htm

Chan, O.C., Wolf, M., Hepperle, D., and Casper, P. (2002) Methanogenic archaeal community in the sediment of an artificially partitioned acidic bog lake. *FEMS Microbiol Ecol* **42:** 119–129.

Chao, A. (1984) Non-parametric estimation of the number of classes in a population. *Scand J Stat* **11:** 265–270.

Dojka, M.A., Hugenholtz, P., Haack, S.K., and Pace, N.R. (1998) Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. *Appl Environ Microbiol* **64:** 3869–3877.

Felsenstein, J. (1993) PHYLIP (Phylogeny Inference Package), Version 3.5c. Department of Genetics, University of Washington, Seattle, USA. Distributed by the author from: http://evolution.genetics.washington.edu/phylip.html

Fisher, R.A., Corber, A.S., and Williams, C.B. (1943) The relation between the number of species and the number of individuals in a random sample of an animal population. *J Anim Ecol* **12:** 42–58.

Galand, P.E., Saarnio, S., Fritze, H., and Yrjala, K. (2002) Depth related diversity of methanogen Archaea in Finnish oligotrophic fen. *FEMS Microbiol Ecol* **42:** 441–449.

Garcia, J.L., Patel, B.K.C., and Ollivier, B. (2000) Taxonomic phylogenetic and ecological diversity of methanogenic Archaea. *Anaerobe* **6:** 205–226.

Godon, J., Zumstein, E., Dabert, P., Habouzit, F., and Moletta, R. (1997) Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. *Appl Environ Microbiol* **63:** 2802–2813.

Good, I.J. (1953) The population frequencies of species and the estimation of the population parameters. *Biometrika* **40:** 237–264.

Grosskopf, R., Janssen, P.H., and Liesack, W. (1998) Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Appl Environ Microbiol* **64:** 960–969.

Hales, B.A., Edwards, C., Ritchie, D.A., Hall, G., Pickup, R.W., and Saunders, J.R. (1996) Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. *Appl Environ Microbiol* **62:** 668–675.

Hammer, O., and Harper, D.A.T. (2002) Data analysis package. Distributed by the author available from: http://folk.uio.no/ohammer/past/.

van Hannen, E.J., Zwart, G., van Agterveld, M.P., Gons, H.J., Ebert, J., and Laanbroek, H.J. (1999) Changes in bacterial and eukaryotic community structure after mass lysis of filamentous cyanobacteria associated with viruses. *Appl Environ Microbiol* **65:** 795–801.

Head, I.M., Saunders, J.R., and Pickup, R.W. (1998) Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. *Microb Ecol* **35:** 1–21.

Hershberger, K.L., Barns, S.M., Reysenbach, A.L., Dawson, S.C., and Pace, N.R. (1996) Wide diversity of Crenarchaeota. *Nature* **384:** 420.

Higgins, D., Thompson, J., Gibson, T., Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting,position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22:** 4673–4680.

Horn, M.A., Matthies, C., Kusel, K., Schramm, A., and Drake, H.L. (2003) Hydrogenotrophic methanogenesis by moderately acid-tolerant methanogens of a methane-emitting acidic peat. *Appl Environ Microbiol* **69:** 74–83.

IPCC (Intergovernmental Panel on Climate Change) (2001) *Climate Change 2001 (the Scientific Basis)*. Cambridge, UK: Cambridge University Press.

Kettunen, A., Kaitala, V., Alm, J., Silvola, J., Nykänen, H., and Martikainen, P.J. (1996) Cross-correlation analysis of the dynamics of methane emissions from a boreal peatland. *Global Biogeoch Cycles* **10:** 457–471.

Kettunen, A., Kaitala, V., Lehtinen, A., Lohila, A., Alm, J., Silvola, J., and Martikainen, P.J. (1999) Methane production and oxidation potentials in relation to water table fluctuations in two boreal mires. *Soil Biol Biochem* **31:** 1741–1749.

Lansdown, J.M., Quay, P.D., and King, S.L. (1992) CH_4 production via CO_2 reduction in a temperate bog: a source of 13-C-depleted CH_4. *Geochim Cosmochim Acta* **56:** 3493–3503.

Lappalainen, E. (1996) Mires of Finland and their use. In *Global Peat Resources*. Lappalainen, E, (ed.). Jyskä, Finland: International Peat Society, pp. 69–74.

Ludwig, W., and Schleifer, K.H. (1994) Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. *FEMS Microbiol Rev* **15:** 155–173.

Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J. *et al.* (1998) Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* **19:** 554–568.

Lueders, T., Chin, K.J., Conrad, R., and Friedrich, M. (2001) Molecular analyses of methyl-coenzyme M reductase alpha-subunit (mcrA) genes in rice field soil and enrichment cultures reveal the methanogenic phenotype of a novel archaeal lineage. *Environ Microbiol* **3:** 194–204.

Luton, P.E., Wayne, J.M., Sharp, R.J., and Riley, P.W. (2002) The mcrA gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill. *Microbiology* **148:** 3521–3530.

Magurran, A.E. (1988) *Ecological Diversity and its Measurement*. London: Chapman & Hall.

Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker, C.T. Jr, Saxman, P.R., Stredwick, J.M. *et al.* (2000) The RDP (Ribo-

somal Database Project) continues. *Nucleic Acids Res* **28:** 173–174.

Marchesi, J., Weightman, A., Cragg, B., Parkes, R., and Fry, J. (2001) Methanogen and bacterial diversity and distribution in deep gas hydrate sediments from the Cascadia Margin as revealed by 16S rRNA molecular analysis. *FEMS Microbiol Ecol* **34:** 221–228.

Moyer, C., Dobbs, F., and Karl, D. (1994) Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S rRNA genes from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. *Appl Environ Microbiol* **60:** 871–879.

Mullins, T.D., Britschgi, T.B., Krest, R.L., and Giovannoni, S.J. (1995) Genetic comparisons reveal the same unknown bacterial lineages in Atlantic and Pacific bacterioplankton communities. *Limnol Oceanogr* **40:** 148–158.

Muyzer, G., Brinkhoff, T., Nubel, U., Santegoeds, C., Schafer, H., and Wawer, C. (1998) Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. In *Molecular Microbial Ecology Manual*. Akkermans, A.D.L., Van Elsas, J.D and De Bruijn, F.J, (eds). Dordrecht, The Netherlands: Kluwer Academic Publishers, pp. 1–27.

Nercessian, D., Upton, M., Lloyd, D., and Edwards, C. (1999) Phylogenetic analysis of peat bog methanogen populations. *FEMS Microbiol Ecol* **173:** 425–429.

Nicholas, K.B., Heiskanen, I., Wallenius, K., and Lindstrom, K. (2001) Extraction and purification of DNA in rhizosphere soil samples for PCR-DGGE analysis of bacterial consortia. *J Microbiol Meth* **45:** 155–165.

Niemi, R.M., Heiskanen, I., Wallenius, K., and Lindstrom, K. (2001) Extraction and purification of DNA in rhizosphere soil samples for PCR-DGGE analysis of bacterial consortia. *J Microbiol Methods* **45:** 155–165.

Purdy, K.J., Munson, M.A., Nedwell, D.B., and Embley, T.M. (2002) Comparison of the molecular diversity of the methanogenic community at the brackish and marine ends of a UK estuary. *FEMS Micobiol Ecol* **39:** 17–21.

Saarnio, S., Alm, J., Silvola, J., Lohila, A., Nykänen, H., and Martikainen, P.J. (1997) Seasonal variation in CH_4 emissions and production and oxidation potentials at microsites on an oligotrophic pine fen. *Oecologia* **110:** 414–422.

Saarnio, S., Saarinen, T., Vasander, H., and Silvola, J. (2000) A moderate increase in the annual CH_4 efflux by raised CO_2 or NH_4NO_3 supply in a boreal oligotrophic mire. *Global Change Biol* **6:** 137–144.

Schutz, H., Schroder, P., and Rennenberg, H. (1991) Role of plants in regulating the methane flux to the atmosphere. In *Trace Gas Emissions by Plants*. Sharkey, T.D.H.E.A and Mooney, H.A., eds. San Diego, USA: Academic Press, pp. 29–63.

Shannon, C.E., and Weaver, W. (1963) *The Mathematical Theory of Communication*. Urbana, IL: University of Illinois Press.

Simpson, E.H. (1949) Measurement of diversity. *Nature* **163:** 688.

Springer, E., Sachs, M.S., Woese, C.R., and Boone, D.R. (1995) Partial gene sequences for the A subunit of methylcoenzyme M reductase (mcrI) as a phylogenetic tool for the family Methanosarcinaceae. *Int J Syst Bacteriol* **45:** 554–559.

Stackebrandt, E., and Rainey, F.A. (1995) Partial and complete 16S rDNA sequences, their use in generation of 16S rDNA phylogenetic trees and their implications in molecular ecological studies. Dordrecht, The Netherlands: Kluwer Academic Publishers.

Svensson, B.H., and Sundh, I. (1992) Factors affecting methane production in peat soils. *Suo* **43:** 183–190.

Takai, K., and Horikoshi, K. (1999) Genetic diversity of Archaea in deep-sea hydrothermal vent environments. *Genetics* **152:** 1285–1297.

Wintzingerode, F.V., Goebel, U.B., and Stackebrandt, E. (1997) Determination of microbial diversity in environmental samples: Pitfalls of PCR-based rRNA analysis. *FEMS Microbiol Rev* **21:** 213–229.