Microbial Ecology

Methanogen Communities in a Drained Bog: Effect of Ash Fertilization

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Abstract

Forestry practises such has drainage have been shown to decrease emissions of the greenhouse gas methane (CH_4) from peatlands. The aim of the study was to examine the methanogen populations in a drained bog in northern Finland, and to assess the possible effect of ash fertilization on potential methane production and methanogen communities. Peat samples were collected from control and ash fertilized (15,000 kg/ha) plots 5 years after ash application, and potential CH_4 production was measured. The methanogen community structure was studied by DNA isolation, PCR amplification of the methyl coenzyme-M reductase (*mcr*) gene, denaturing gradient gel electrophoresis (DGGE), and restriction fragment length polymorphism (RFLP) analysis. The drained peatland showed low potential methane production and methanogen diversity in both control and ash-fertilized plots. Samples from both upper and deeper layers of peat were dominated by three groups of sequences related to Rice cluster-I hydrogenotroph methanogens. Even though pH was marginally greater in the ash-treated site, the occurrence of those sequences was not affected by ash fertilization. Interestingly, a less common group of sequences, related to the Fen cluster, were found only in the fertilized plots. The study confirmed the depth related change of methanogen populations in peatland.

Introduction

The increasing concentration of atmospheric methane contributes to global warming. Wetlands, including peatlands, are the main source of natural methane emission [18] because anoxic layers of peat provide habitat for methanogenic Archaea (kingdom Euryarchaeota). They produce methane during the final step of the degradation of organic matter.

About one-third of the Finnish land area used to be covered by peatlands, and the proportion of land covered by peatlands is still greater than in any other country [1, 27]. Extensive draining of peatlands in Finland occurred in the 1960s and 1970s to promote tree growth. In addition, ~1.7 million ha of drained peatlands have been fertilized with ash during the past 50 years [2]. Ash adds nutrients, except nitrogen and phosphorus, and transforms vegetation composition and promotes tree growth [33]. It affects soil microbiota [8, 13] and ectomycorrhiza [13, 31]. Drainage has been shown to decrease methane emissions, the main reasons for the decrease being changes of quantity and quality of substrates for methanogenesis and increased methane oxidation [21, 32]. Methanogen communities in peatlands have been described in undisturbed bogs [14, 17, 36] and fens [9, 10], but the effect of forest management such as ash fertilization on the methane-producing Archaea has, however, never been studied.

We previously studied methanogen communities in an undisturbed fen using specific primers for methanogen 16S rDNA [9] and for the A subunit of the methyl coenzyme-M reductase (*mcr*A) gene [10]. *Mcr*, a key enzyme in methanogen metabolism, catalyzes the reduction of methyl coenzyme-M with methane being the final released product [6] and appears to be unique to methanogens [44]. Part of the amino acid sequence is highly conserved [14, 46], and *mcr* sequence data have consequently been used for estimating methanogen diversity in a variety of environments [5, 28–30, 36, 45].

In this study, we examined possible effects of ash fertilisation on methanogen community structure in ash-fertilized (15,000 kg/ha) and control (no ash addition) plots of a drained bog. Potential CH_4 production was measured *in vitro* from replicate peat samples taken at different depths. Methanogen population diversity was

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Table 1. Water table depth (WT), average CH_4 production, and pH for peat profiles from control and fertilized sites taken at Pelso-Resula drained bog in May 2002

Sampling plot, WT	*Sample depth (cm)*[a]	*pH ± SE*	*Average potential CH_4*[b] *production (nmol/g/h) ± SE*
Control (C); WT: −26 cm	0	3.9 (0.1)	0.49 (0.55)
	−10	3.8 (0.1)	1.21 (1.94)
	−20	3.8 (0.1)	2.42 (2.76)
	−30	3.9 (0.2)	0.47 (0.22)
	−40	4.0 (0.2)	1.02 (0.47)
Fertilized (F); WT: −35 cm	0	4.2 (0.5)	0.71 (1.13)
	−10	3.9 (0.2)	5.76 (7.35)
	−20	3.9 (0.1)	2.82 (2.01)
	−30	4.0 (0.1)	1.51 (1.11)
	−40	4.0 (0.2)	1.15 (0.66)

[a]Negative values indicate sample taken below the water level.
[b]Three parallel peat profiles were taken at the sites.

analysed by using molecular methods (PCR-DGGE, cloning and sequencing) targeting the A subunit of the methyl coenzyme-M reductase gene.

Methods

Experimental Site and Sample Collection. The experimental field was located in Muhos, Pelso, Resula (64°30′N, 26°18′E). The site is within the boreal coniferous zone classified as a Cotton grass pine bog [24]. Ditching was done in the 1930's and continued in 1994 and 1997 [33]. The bog is in a transforming state where the typical bog vegetation is changing to forest vegetation as a result of drainage [38]. Peat depth averages 100 cm. Pines (*Pinus sylvestris*) 1–7 m height and 20–60 years of age are growing in the bog. They are intermixed with slowly growing birches (*Betula pendula*).

Three replicate plots (30 m × 30 m) were treated with 15,000 kg/ha of fly ash (F) in June 1997. Another three replicate plots of the same size were kept unfertilized as control (C). One peat core was taken from each replicate plot with a box sampler (4 × 6.8 × 100 cm). In May 2002 after snow melting, peat samples for potential methane production measurements and for DNA extraction were taken from 10 cm (±2 cm) depth intervals in the anoxic parts of the peat cores (Table 1).

Measurement of Potential Methane Production and pH, and DNA Extraction. Fifteen mL of peat was added to 100-mL, oxygen-free, infusion bottles containing 30 mL of distilled water. The bottles were flushed with 99.96% nitrogen in order to obtain anoxic conditions [20] and sealed with butyl rubber septa. Incubation and gas chromatographic analysis of CH_4 were performed as described [10].

pH was measured once a day for 3 days from 10 mL of peat mixed with 20 mL of H_2O, and incubated at room temperature (~23°C).

DNA was extracted from 0.25 g of peat by chemical (detergent) and mechanical (bead beating) cell lysis with the Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA), following the manufacturer's suggested protocol. The DNA quality and yield was assessed by running the extracted DNA in agarose gel. This extraction method has been shown to provide high reproducibility and efficiency when compared with other methods [37].

PCR Amplification. The *mcr*A gene was used as molecular marker to study the methanogen diversity. The primer pair ME1-ME2 [14] was used to amplify specifically a 760-bp-long region of the *mcr*A. A 40-nucleotide-long GC-clamp was added to the forward primer to enable DGGE analyses [35]. 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) was used to prevent PCR inhibition. The reaction conditions were 30 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 2 min. The PCR was performed using a Gene Amp thermal cycler (PerkinElmer, Wellesley, MA, USA). Products were analyzed on 1% agarose gels with ethidium bromide staining.

Cloning, RFLP, Sequencing of PCR Product, and DGGE Fingerprinting. All together eight gene libraries were constructed using DNA extracted and amplified from two depths of the control and fertilized plots. Two replicate libraries were obtained from high (H) potential methane producing layers (10 or 20 cm below water level), and two replicate libraries were obtained from low (L) potential methane producing layers (30 or 40 cm below water level).

PCR products obtained with ME primers without GC-clamp were analyzed by gel electrophoresis, purified with Wizard SV Gel and PCR Clean-Up System (Promega, Mannheim, Germany), and cloned in pGEM-T

vector plasmid using JM109 competent cells (Promega) according to the manufacturer's instructions. Colonies dispersed in H_2O were used as templates to amplify inserted *mcr* sequences with ME primers. PCR products were digested with *Msp*I for RFLP analyses [10].

Colonies showing new distinct fingerprint patterns were selected for sequencing. Plasmid DNA was isolated with the Wizard Mini-Preps (Promega) and sequenced using the vectors universal primers.

The *mcr* sequences obtained in this study were deposited in the EMBL database under accession nos. AJ586241–AJ586250.

DGGE was performed as described earlier [10] on samples from the high and low potential methane-producing layers of the bog with a D-code system (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. The denaturing gradient was determined by empirical trials; it ranged from 45 to 70%. The gel was run at 60°C, at a constant voltage of 250 V for 6 h. The bands position was marked manually with gel documentation software Gel Doc 2000 (Bio-Rad). In order to relate the results obtained by DGGE fingerprinting with those from RFLP analysis of clone libraries, bands from DGGE fingerprinting were cut out from the gel, reamplified, and subjected to RFLP analysis. Additionally, cloned *mcr* sequences representing each RFLP groups were reamplified with GC-clamp and run in DGGE.

Statistical Analysis. Two-way ANOVAs were used to test differences in pH, potential CH_4 production, and diversity indices as a function of fertilization treatment and depth. P values were considered significant at the $P < 0.1$ level.

RFLP data were converted to a distance matrix using the Chord algorithm for abundance data and represented in a dendrogram. DGGE banding patterns were converted to a binary matrix where the presence or absence of a band in each lane at a same height was reported in the matrix with 1 and 0, respectively [9]. The binary matrix was transformed to a distance matrix using Jaccard's coefficient for presence–absence data, and the resulting similarity values were analyzed by cluster analysis. The percentage of coverage of the clone libraries was estimated, following Good's methods [11], as $(1 - (n/N)) \times 100$, where n is the number of unique clones detected in a sample of size N.

The Shannon–Weaver diversity index [40] was calculated as:

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

p_i is the proportion of clones belonging to the ith OTU and s is the total number of OTUs; RFLP patterns were considered as operational taxonomic units (OTUs) [34]. Additionally, Fisher's Alpha log series richness index [7] was calculated. Cluster analysis, rarefaction, and diversity analysis were performed with the PAST package (v.0.97, [15]).

Phylogenetic Analysis. Sequences were compared to those in the EMBL database using the BLAST server [3] at the European Bioinformatics Institute (EBI) (URL http://www.ebi.ac.uk; Hinxton Hall, Cambridge, UK). Amino acid sequences were inferred from the *mcr* nucleotide sequences. Part of the restriction groups detected in the Pelso-Resula drained bog had been detected earlier in a natural fen [10]; they were designated with capital letters whereas new sequences were designated with numbers. Sequences of approximately 250 amino acids were aligned using the CLUSTAL W package [16] and checked manually. Chimeras were excluded from analysis. A phylogenetic tree was estimated using the PHYLIP package (v.3.57c; J. Felsenstein, University of Washington, http://evolution.genetics.washington.edu/phylip.html). Bootstrap values for 100 replicates were generated with SEQBOOT. PROTDIST was used to compute a distance matrix with Dayhoff PAM 001 as amino acid replacement model. The distance matrix was estimated by FITCH with global rearrangement of branches and randomized species input order. The dendrogram was verified by neighbor-joining and maximum parsimony methods.

Results

pH and Water Table. Peat pH varied between 3.8 and 4.0 among depths in control plots and between 3.9 and 4.2 in ash fertilized plots (Table 1) and was marginally greater in the ash treated site than in the controls ($P = 0.105$). No differences in pH were observed between depths. The water table was 9 cm deeper in the fertilized plots than in the controls (Table 1).

Potential CH_4 Production. The peat samples taken at the beginning of the growing season in May showed low potential rates of methane production, but CH_4 production was detected at all depths (Table 1). At both sites, rates were greater 10 and 20 cm below the water table (upper layer) than 30 and 40 cm below water table (deeper layer). No significant difference in CH_4 production was observed between control and fertilized plots.

RFLP of Cloned mcr Sequences. Restriction fragments resulting from the digestion of the *mcr* gene with *MspI* were grouped depending on their restriction pattern, and each group was considered as an operational taxonomic unit (OTU). It has previously been shown that sequences grouping in the same OTU have high

Table 2. Different diversity indices calculated from RFLP patterns of *mcr* gene clones[a]

	Clone library							
Parameters	*Ca(H)*	*Cc(H)*	*Ca(L)*	*Cc(L)*	*Fa(H)*	*Fb(H)*	*Fa(L)*	*Fb(L)*
No. of clones analysed	66	60	60	60	54	59	61	65
Coverage of clone libraries (%)	95	97	97	93	96	96	97	98
Observed no. of RFLP patterns	5	5	5	9	6	5	7	3
Shannon-Weaver diversity (*H'*)	0.89	1.12	0.63	1.32	1.17	0.57	1.10	0.48
Fisher's alpha (α)	1.26	1.30	1.30	2.94	1.73	1.30	2.04	0.65

[a]The clone libraries were obtained from control plots (C) and fertilized plots (F) from the drained bog. Samples were obtained from high (H) and low (L) potential CH_4 producing layers.

similarity [10]. Eighteen different OTUs were observed. The percentages of coverage of the clone libraries ranged from 93% to 98% (Table 2), and the richness curves flattened toward an asymptote (Fig. 1). Therefore the number of clones analyzed covered RFLP pattern richness of the libraries. Upper-layer peat from both fertilized and control clone libraries was dominated by sequences L and B, representing between 65% and 95% of all clones. At both sites, libraries from the deeper layers were dominated by one single sequence (E), which constituted 60 to 80% of the deep-layer clones. Some *mcr* sequences (T, II, III) were practically only found in the fertilized plots (Fig. 2). Cluster analyses of RFLP patterns revealed no ash treatment effect. The libraries did not group by fertilization treatment (Fig. 3a). A depth-related variation of the sequences could clearly be seen. Restriction groups characterizing the upper layers (L, B) were different from the ones characterizing the deeper layers (E) (Fig. 3), and the cluster analysis showed grouping by depth (Fig. 3a). Sample Fb(H) differed from other upper layer samples; it was dominated by pattern E and grouped consequently with deeper layer samples.

DGGE Fingerprinting. DGGE analysis was performed on replicate samples from control and fertilized sites. A low number of *mcr* DNA fragments was present in the gels. Cluster analysis of the banding patterns showed no difference in methanogen *mcr* sequences between control and fertilized plots (Fig. 3b). However, the DGGE fingerprinting revealed a depth-related occurrence of *mcr* sequences; cluster analysis showed that samples grouped according to depth (Fig. 3b).

RFLP analyses of DGGE bands excised from the gel showed that sequences dominating the clone libraries (B, L, and E) were also the ones giving strong signal in the DGGE fingerprinting. Sequences typically found in fertilized plots (T, II, III) were not detected in any of the DGGE fingerprints for control plots.

Diversity Indices. The different indices calculated for diversity estimation are presented in Table 2. Ash treatment did not affect the diversity indices of clone libraries, and the depth-related change in population was not reflected in a change of *mcr* diversity ($P > 0.1$ for *H'* and α).

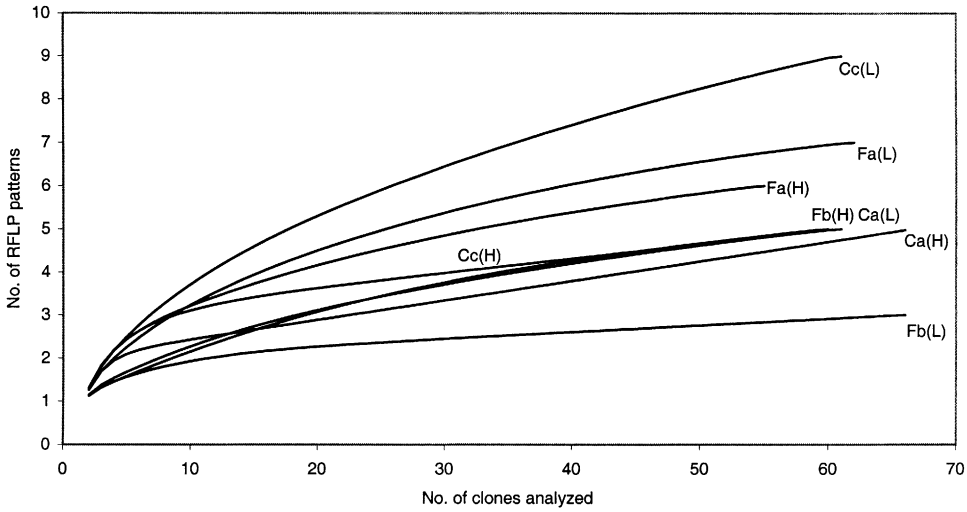

Figure 1. RFLP pattern richness curve of eight *mcr* clone libraries. Curves were obtained by rarefaction calculation. See Methods for designations.

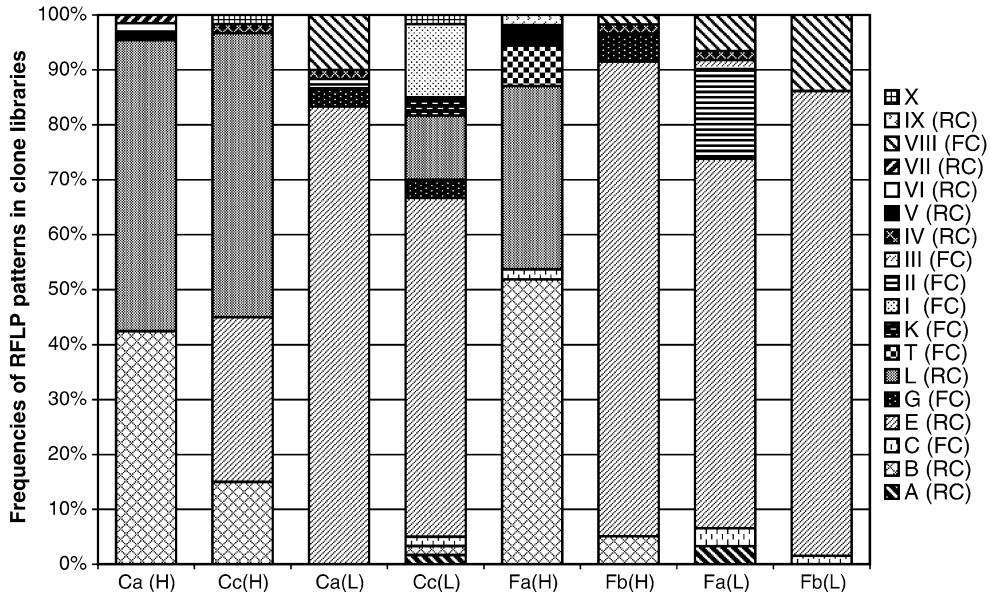

Figure 2. RFLP analysis of *mcr* clone libraries. Sequences were obtained from control plots (C) and fertilized plots (F), from high (H) and low (L) potential CH_4-producing layers of the drained bog. Clone libraries were constructed from replicate peat profiles (a, b, or c). Letters in the legend represent the different RFLP groups. Their phylogenetic affiliation to fen cluster (FC) or rice cluster-I (RC) is indicated in parentheses.

Phylogenetic Analysis. Inferred amino acid sequences were used to assess the phylogenetic relationships between all detected restriction groups. Sequences were separated in two broad clusters, previously defined as Rice cluster-I [12] and Fen-cluster [10] (Fig. 4). Sequences representing the most frequent restriction groups in both upper layer (B and L) and deeper layer (E) all clustered closely together (>94% identity). They were related to sequences belonging to Rice cluster-I. Sequences unique or most abundant in fertilized plots (T, II, III) all grouped together (>96% identity) within Fen cluster. It contains a novel group of *mcr* sequences from uncultured methanogens retrieved from a boreal fen. One sequence represented by a small number of clones (X) clustered with sequences previously retrieved from rice roots and was closely related to known members of the order Methanosarcinales (90% identity).

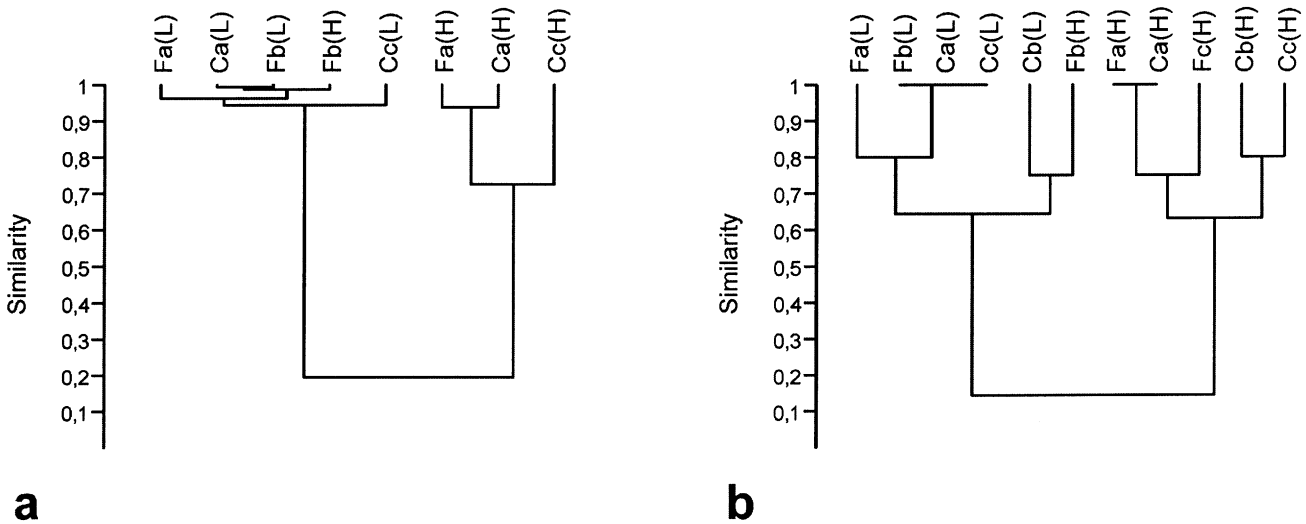

Figure 3. Dendrogram representing the relation between different *mcr* sequences analyzed by RFLP (a) and DGGE (b). Distances were calculated with Chord's algorithm for the clone libraries and with Jaccard's coefficient for DGGE fingerprinting. Samples are from control plots (C) and fertilized plots (F), from high (H) and low (L) potential CH_4-producing layers of the drained bog. Different replicate peat profiles (a, b, and c) are represented.

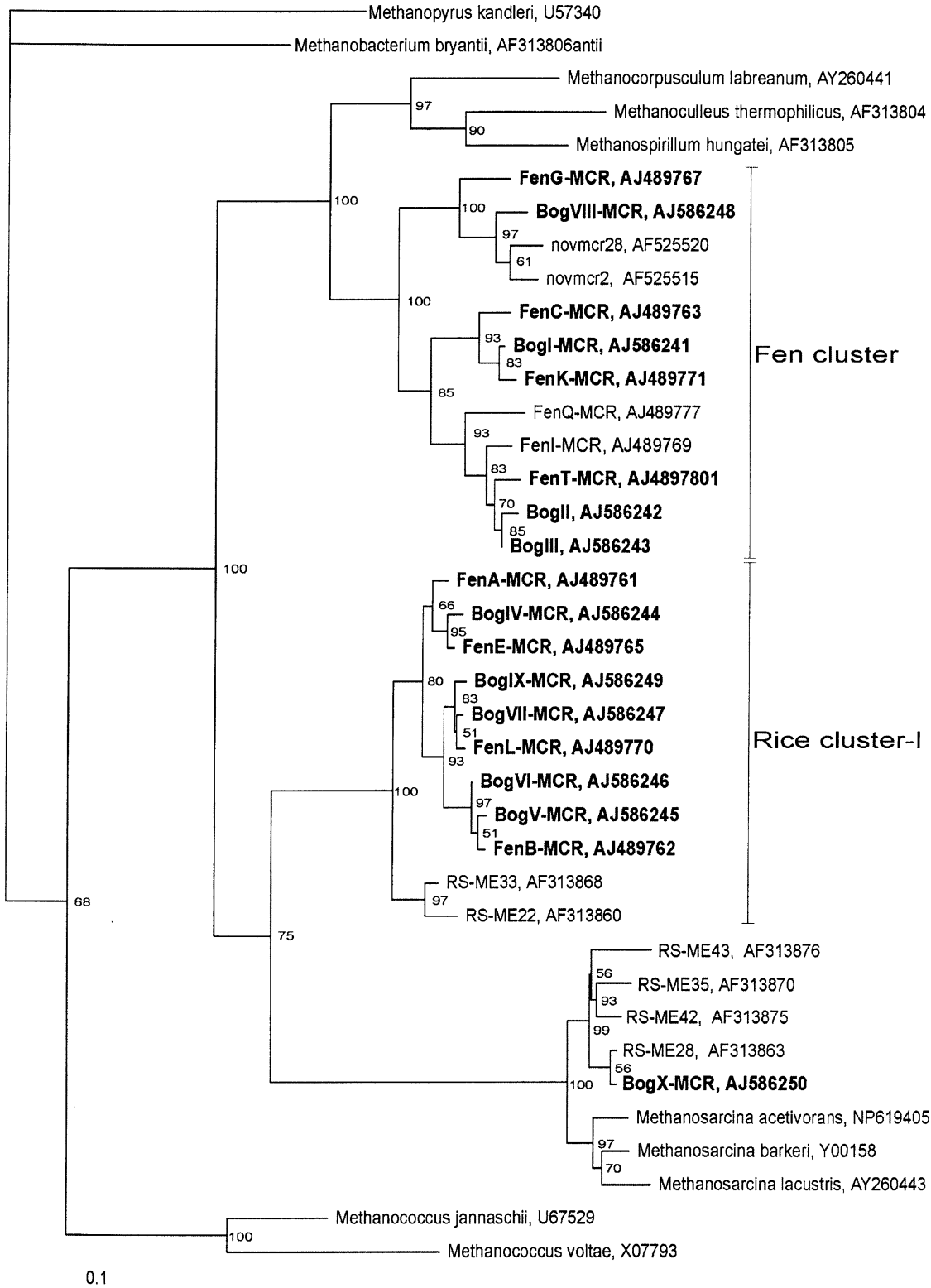

Figure 4. Phylogenetic dendrogram representing the relationship between *mcr* amino acid sequences (250 aa long) retrieved from drained bog and other related methanogenic Archaea. Sequences obtained from bog libraries are designated in boldface. The capital letter or number in the sequence name corresponds to the 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.

Discussion

Maximum potential CH_4 production in upper anaerobic layers and decrease of the production with depth, as detected in the Pelso-Resula drained bog, is typical for boreal peatlands and has been observed in different studies [10, 20, 47]. However, the rates of potential methane production were lower than the values reported for an undisturbed fen in Eastern Finland, even though sampling was done at the same time of the growing season [10]. The low potential CH_4 production suggests a lack of adequate substrate for methanogenesis. Drainage causes typical mire vegetation to change toward forest vegetation [25]. The Pelso-Resula bog has been drained by ditching, and small pines together with some poorly growing birches grow on the site, reflecting the transformation stage of the bog. In natural peatlands, sedges have been shown to transport carbon as exudates to layers of maximum root density [19, 42]. After drainage, sedges decline and are replaced by emerging trees. Primary production increases fast above ground, while the major rooting zone concentrates organic matter in the first 30 cm below the peat surface and less carbon is allocated to anaerobic layers [22, 23]. Additionally, with lowered water table, recently produced organic matter will be efficiently degraded in the increased aerobic layers, limiting potential substrate for methanogenesis to reach anaerobic layers of the peat (e.g., [43]). The composition of the methanogen community reflects the nutrient condition of the drained bog. *Mcr* sequences (B, L, E) dominating the libraries were all previously found to be characteristic for deeper layers of peatland [10]. They grouped phylogenetically with members of Rice cluster-I. *Mcr* sequences belonging to Rice cluster-I were originally retrieved from rice roots [12] and have recently been shown to originate from hydrogenotroph methanogens [41]. This indicates that the methanogens in the drained bog, corresponding to deep-layer community of natural peatlands, are utilizing CO_2:H_2 for methane production. The result is in accordance with studies that have shown hydrogenotrophy to be the major methanogenic pathways in the deeper layers of peatlands [17, 26]. Acetate, which typically originates from root exudates or as an end product of fermentation, is probably not an important precursor for methanogenesis in the drained bog.

Ash fertilization affected the peat by increasing the pH marginally in the submerged, anaerobic layers of the bog. Previous results have shown ash fertilization to have a liming effect, raising the pH and increasing the amount of nutrients (P, K, Ca, and trace elements) in the aerobic layers of peatlands [33, 39]. The little change in pH observed here did not have any major influence on the methanogen community. The three restriction groups dominating the different peat layers were the same in both control and fertilized sites. However, some groups of sequences (II, III, T) were only retrieved from the fertilized plots. Those sequences were closely related pylogenetically and all grouped within the same clad of the Fen cluster. This cluster represents a new group of *mcr* sequences recently retrieved from a natural boreal fen [10]. Even though the precursors used by members of the Fen cluster for methane production remain unknown, grouping of the sequences with members of Methanomicrobiales may suggest that those originate from hydrogenotrophs.

Methanogen community structure differed significantly as a function of peat depth. As shown by both DGGE and RFLP analyses, *mcr* sequences retrieved from upper layers were different from those originating from deeper layers. Depths-related change in methanogen community has earlier been shown in a natural fen and may indicate an adaptation of the methanogens to changes of physicochemical conditions with depth [9, 10].

In summary, methanogen *mcr* sequences dominating the drained bog were similar to sequences retrieved from the deep layers of a natural fen. The main sequences grouped within the Rice cluster-I, indicating the presence of hydrogenotroph methanogens in the bog. Even though ash fertilization increased the pH of the bog, potential methane production was not influenced by fertilization and the main groups of methanogens did not change. However, some less common sequences were found only in the fertilized plots and may indicate a gradual change of the community. They all grouped within the newly defined Fen-cluster and may originate from methanogens responding to physicochemical changes induced by fertilization.

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