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ORIGINAL ARTICLE

Seasonality of rDNA- and rRNA-derived archaeal communities and methanogenic potential in a boreal mire

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Methane (CH_4) emissions from boreal wetlands show considerable seasonal variation, including small winter emissions. We addressed the seasonality of CH_4-producing microbes by comparing archaeal communities and the rates and temperature response of CH_4 production in a boreal fen at three key phases of growing season and in winter. Archaeal community analysis by terminal restriction fragment length polymorphism and cloning of 16S ribosomal DNA and reverse-transcribed RNA revealed slight community shifts with season. The main archaeal groups remained the same throughout the year and were Methanosarcinaceae, Rice cluster II and Methanomicrobiales-associated Fen cluster. These methanogens and the crenarchaeal groups 1.1c and 1.3 were detected from DNA and RNA, but the family Methanosaetaceae was detected only from RNA. Differences between DNA- and RNA-based results suggested higher stability of DNA-derived communities and better representation of the active CH_4 producers in RNA. Methane production potential, measured as formation of CH_4 in anoxic laboratory incubations, showed prominent seasonality. The potential was strikingly highest in winter, possibly due to accumulation of methanogenic substrates, and maximal CH_4 production was observed at ca. 30 °C. Archaeal community size, determined by quantitative PCR, remained similar from winter to summer. Low production potential in late summer after a water level draw-down suggested diminished activity due to oxygen exposure. Our results indicated that archaeal community composition and size in the boreal fen varied only slightly despite the large fluctuations of methanogenic potential. Detection of mRNA of the methanogenic *mcrA* gene confirmed activity of methanogens in winter, accounting for previously reported winter CH_4 emissions.
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Introduction

In boreal regions, cold and wet conditions have promoted the development of peat-forming wetlands (mires) storing large amounts of carbon (Turunen *et al.*, 2002). Together with other wetlands, boreal mires are among the most important natural sources of atmospheric methane (CH_4), a powerful greenhouse gas (Denman *et al.*, 2007). Having short growing seasons and long snow-covered winters, the mires undergo strong seasonal changes. In contrast to temperate and tropical wetlands with less severe annual temperature shifts, CH_4 emissions from northern wetlands show pronounced temperature-driven seasonality (Whalen, 2005).

Methane is produced as a terminal step of anaerobic decomposition in water-saturated peat by a group of anaerobic Archaea, the methanogens. Temperature is one of the main factors controlling CH_4 production in mires, together with substrate availability, water level and peat pH (Svensson and Sundh, 1992; Valentine *et al.*, 1994; Bergman *et al.*, 1998). Temperature influences microbial activity, but it may also alter the course of anaerobic decomposition, thus affecting the methanogenic pathway and substrate availability (Schulz *et al.*, 1997). Seasonal temperature variation could therefore influence mire archaeal community either through direct temperature adaptation or indirectly through temperature-induced changes in substrate supply. Reported shifts in proportion of H_2/CO_2-dependent (hydrogenotrophic) and acetate-dependent (acetoclastic) CH_4 production with incubation

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temperature have been accompanied by changes of archaeal communities in rice field soil (Chin *et al.*, 1999; Fey and Conrad, 2000) but not in lake sediments and peat (Glissman *et al.*, 2004; Metje and Frenzel, 2005, 2007). A recent study, in which arctic peat was incubated for several weeks, reported that some methanogen populations responded to temperature, whereas others were unaffected (Høj *et al.*, 2008). The study also described dominance of non-methanogenic archaea at incubation temperatures below 10 °C but increased contribution of methanogens above 10 °C.

Methane production potential in mires varies temporally (Yavitt *et al.*, 1987; Duddleston *et al.*, 2002), similarly to CH_4 emissions. The pattern of variation, however, depends on vegetation and surface topography (Saarnio *et al.*, 1997; Kettunen *et al.*, 1999; Bergman *et al.*, 2000). In mires where acetate was an important methanogenic precursor, the contributions of hydrogenotrophic and acetoclastic CH_4 production have been observed to shift with season (Avery *et al.*, 1999; Chasar *et al.*, 2000). Methanogen community variation between mires has been related to vegetation, pH and hydrology (Galand *et al.*, 2003; Juottonen *et al.*, 2005; Cadillo-Quiroz *et al.*, 2006; Rooney-Varga *et al.*, 2007), but seasonality of wetland archaeal communities has scarcely been explored (Høj *et al.*, 2005, 2006).

Although northern mires emit most CH_4 during the warmest months, emissions in winter can contribute 4–33% of the annual CH_4 flux (Dise, 1992; Alm *et al.*, 1999). Because of their longer winters, boreal wetlands presumably have greater winter emissions than temperate wetlands (Melloh and Crill, 1996; Alm *et al.*, 1999). Whether the emissions result from release of CH_4 produced during warmer seasons or from continual methanogenic activity in cold remains unclear (Panikov and Dedysh, 2000).

Our first objective was to follow the methanogenic archaeal community composition in a boreal fen through seasonal temperature shifts. The communities were assessed by RNA/DNA extraction and terminal restriction fragment length polymorphism (T-RFLP) analysis of archaeal 16S rRNA genes, thus including also other groups of Euryarchaeota and Crenarchaeota. The study site, Siikaneva fen in southern Finland, is a focus of carbon cycling research, and its CH_4 emissions have been continuously monitored throughout the year (Rinne *et al.*, 2007; Riutta *et al.*, 2007). Recent results showed that 20% of the annual carbon (CO_2) uptake was emitted as CH_4, and winter emissions accounted for 5–8% of the flux (Rinne *et al.*, 2007). Our second aim was to compare the rates and temperature response of CH_4 production between seasons, with particular interest on winter potential. The third aim was to determine whether methanogenic archaea are active during winter.

Materials and methods

Site description and sample collection

The study site, Siikaneva, is a boreal fen in southern Finland (61°50′N, 24°12′E). The site is nutrient poor (oligotrophic) and acidic with peat pH of 3.9–4.3. Vegetation in the sampled hollow and lawn areas consists of *Scheuchzeria palustris*, *Carex rostrata*, *C. limosa*, *Andromeda polifolia*, *Vaccinium oxycoccus* and *Betula nana* in the field layer, and *Sphagnum majus*, *S. papillosum* and *S. balticum* in the moss layer (Riutta *et al.*, 2007). Samples were collected on 26 October 2005 (end of growing season before snow fall), 8 February 2006 (mid-winter), 23 May 2006 (after snowmelt) and 14 August 2006 (late summer). Table 1 shows average peat temperatures for the week preceding sampling and water levels at sampling dates. During the year studied, the minimum peat temperature at the depth of 20 cm was −0.7 °C in April and the maximum was 14.6 °C in July (Rinne *et al.*, 2007, unpublished data). Surface peat was frozen from early December to late April, but temperature at 20 cm remained between 0 and 0.7 °C until mid-April and only went below 0 °C in late April. In February, peat was frozen to a depth of 8–15 cm, and snow cover was 35 cm.

At each sampling, a peat profile from three marked locations (overall $n = 3$) several meters apart was collected with a box sampler (8 cm × 8 cm × 90 cm). The samples were transported to laboratory for immediate processing, with storage at 5 °C for no more than a few hours. Slices of 4 cm from each profile were taken for nucleic acid and CH_4 analysis

Table 1 Environmental conditions, potential CH_4 production and quantification of archaea

Sampling	*Peat temperature at 20 cm (°C)*	*Water level (cm)*	*CH_4 production (nmol gdw^{-1} h^{-1})*		*Archaeal 16S rRNA genes (10^9 copies gdw^{-1})*
			6 °C	*14 °C*	
October 2005	4.5	4 ± 4	0.4 ± 0.6	0.4 ± 0.4	0.8 ± 1.0
February 2006	0.1	Frozen	2.2 ± 2.7	11.9 ± 6.1	3.8 ± 1.6
May 2006	8.1	−4 ± 3	0.1 ± 0.0	7.4 ± 4.5	3.4 ± 1.2
August 2006	13.5	−23 ± 4	0.1 ± 0.1	1.5 ± 0.6	4.2 ± 0.3

Abbrevation: gdw, grams dry weight.
Mean ± s.d., $n = 3$. Water level is negative below peat surface.

at 20 (±2) cm below the peat surface. This layer is generally water-submerged and showed higher CH_4 production than the depths of 10 and 50 cm (unpublished data). Subsamples for nucleic acid analysis were frozen within few hours from sampling and kept frozen until extraction.

Nucleic acid extraction
Total DNA and RNA were extracted from 0.4 g of frozen peat as in Korkama-Rajala *et al.* (2007). Briefly, peat was homogenized in a FastPrep cell disrupter (Qbiogene, Illkirch, France) at a speed setting 4.5 m s^{-1} for 30 s (adjusted from the original protocol). Phenol–chloroform-extracted nucleic acids were purified in a polyvinylpolypyrrolidone column and by polyethylene glycol precipitation, dissolved in TE buffer, and stored at −70 °C.

Reverse transcription
RNA was prepared by treating 4 μl of nucleic acid extracts with RQ1 DNase (Promega, Madison, WI, USA). To denature RNA secondary structure, 2 μl of RNA and 200 ng of random hexamer primers (Roche, Mannheim, Germany) in 9 μl of diethylpyrocarbonate-treated H_2O were heated at 70 °C for 5 min and chilled on ice. Reverse transcriptase buffer (1×, supplied with the enzyme) and 2 μl of 10 mM dNTPs were added before incubation at 25 °C for 5 min. After addition of RevertAid M-MuLV reverse transcriptase (200 U; Fermentas, Vilnius, Lithuania), reverse transcription (RT) was performed at 25 °C for 10 min and 42 °C for 60 min, followed by inactivation at 70 °C for 10 min. Total volume was 20 μl. To check for DNA contamination, DNase-treated extracts and control RT reactions without reverse transcriptase were used as PCR template.

PCR
Archaeal 16S rRNA gene fragments (ca. 800 bp) were amplified using primers Ar109f and Ar912rt (Grosskopf *et al.*, 1998a; Lueders and Friedrich, 2002). Reactions (50 μl) contained 1× DNA polymerase buffer, 0.2 mM dNTPs, 15 pmol of primers, 1 U of DNA polymerase (Biotools, Madrid, Spain) and 1 μl of total DNA (1:10 dilution) or RT products (1:5 dilution) as template. In PCR for T-RFLP, the reverse primer Ar912rt was 5′-labelled with 6-carboxyfluorescein (FAM), and the amount of primers was 10 pmol. Reaction conditions in a GeneAmp 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA) were an initial denaturation (94 °C, 3 min), 28 (DNA) or 32 (RT products) cycles of 94 °C for 45 s, 52 °C for 1 min and 72 °C for 1.5 min; and a final extension (72 °C, 7 min). Products were checked in 1% agarose gels with ethidium bromide staining. Fen cluster (FC) methanogens were detected with primers A-gE372 and A-gE540aR, which target a subgroup of the order Methanomicrobiales (Cadillo-Quiroz *et al.*, 2006), in 25-μl reactions with 10 pmol of primers and the following reaction conditions: 94 °C for 1.5 min, 35 cycles of 94 °C for 45 s, 54 °C for 1 min and 72 °C for 45 s; and a final extension (72 °C, 5 min). Primer specificity was tested using FC and Rice cluster II (RCII) clones as template. *mcrA* mRNA was detected with primers of Luton *et al.* (2002) in 25-μl reactions, with 10 pmol of primers, and RT products (undiluted or 1:5 dilution) of winter samples as template. Reaction conditions were as in 16S rRNA gene PCR but with 35 cycles and 1-min extensions.

T-RFLP analysis
Archaeal PCR products were quantified with Qubit fluorometer (Invitrogen, Eugene, OR, USA) and by visual inspection of agarose gels. Product DNA (5–15 ng) was digested with restriction endonuclease *Taq*I (3 U; Fermentas) at 65 °C for 4 h in a total volume of 20 μl. Digested DNA was ethanol precipitated and dissolved in a mixture of 15 μl of Hi-Di formamide (Applied Biosystems) and 0.4 μl of GeneScan-500 TAMRA size standard (Applied Biosystems). Fragments were separated by capillary electrophoresis in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Each sample was electrophoresed twice. Fragments from 50 to 500 bp in the electropherograms were analyzed with GeneScan software (v. 3.7; Applied Biosystems) using a minimum peak height threshold of 100 relative fluorescence units. To eliminate variation in DNA amount between samples, we estimated the total amount of each profile by summing peak areas. The lowest total area among the profiles divided by the total area of a given profile served as a correction factor for its peak heights (Dunbar *et al.*, 2001). Peaks with corrected heights below 100 fluorescence units were excluded. Terminal restriction fragments (T-RFs) were assigned to phylogenetic groups by comparing *in silico* terminal fragments of clone sequences and by T-RFLP analysis of clones. Relative peak areas were used in data presentation and analysis.

Community data analysis
DNA- and RNA-derived T-RFLP data were compared by analysis of similarity using Bray-Curtis distances with PAST software v. 1.73 (O Hammer, DAT Harper, http://folk.uio.no/ohammer/past/). Analysis of similarity returns *R*-values; value of 0 indicates as large variation within the groups being compared as between them, and value of 1 indicates that variation between groups is always higher.

Detrended canonical correspondence analysis of T-RFLP data where detrending was done by segments revealed a short main gradient; length of the first axis was <2 s.d. units for DNA and RNA separately. Following this, we selected linear methods for further analysis (ter Braak and Prentice, 1988). We applied principal component analysis

(PCA) to explore compositional variation in T-RFLP data. To determine factors that best explained variation in rDNA and rRNA communities, we used partial redundancy analysis (RDA) where spatial variation was levelled using the three sampling locations as categorical covariables. The factors tested were CH_4 production potential (continuous variable) and the four different sampling times (categorical variables) together forming a variable season. Significance of the factors was tested using Monte Carlo permutations (499 permutations). All the analyses were carried out with Canoco for Windows 4.52 (ter Braak and Šmilauer, 2002). Level of significance in all statistical analyses was $P \leqslant 0.05$. The effect of variables with higher risk level is mentioned when considered close enough to 0.05 to be relevant.

Cloning and DNA sequencing
Five clone libraries were constructed from selected rDNA- and rRNA-derived archaeal PCR products representing all four samplings. After gel purification with Wizard SV Gel and PCR Clean-Up kit (Promega), PCR products were ligated into pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* DH5α competent cells. Inserts from ca. 30 blue–white-screened clones from each library were amplified with primers Ar109f and Ar912rt and digested with *Taq*I at 65 °C for RFLP screening. Fragments were separated by agarose gel electrophoresis in 3% Synergel (Diversified Biotech, Boston, MA, USA). On the basis of RFLP banding patterns, clones were divided into restriction groups. Coverage of clone libraries was calculated as in Galand *et al*. (2003).

Two to four representatives of each RFLP group were amplified with vector-specific RP (5′-TTTCAC ACAGGAAACAGCTATGAC-3′) and UP (5′-CGACGT TGTAAAACGACGGCCAGT-3′) primers and sequenced with vector primer T7 or SP6. The sequences have been submitted to the EMBL database under accession nos. AM905390-AM905421.

Phylogenetic analysis
Partial 16S rRNA gene sequences (ca. 800 bp) were compared to database sequences by BLAST searches (http://ncbi.nlm.nih.gov/BLAST). The presence of chimeric sequences was checked with the Bellerophon server (http://foo.maths.uq.edu.au/~huber/bellerophon.pl) (Huber *et al*., 2004). Sequences were aligned with NAST alignment tool on the Greengenes server (http://greengenes.lbl.gov) (DeSantis *et al*., 2006). The alignment was edited manually to remove ambiguous positions. A maximum likelihood tree was constructed with PhyML (http://atgc.lirmm.fr/phyml/) (Guindon and Gascuel, 2003) using a general time reversible nucleotide substitution model with estimated proportion of invariable sites and gamma distribution parameter. The model was selected with the FindModel server (http://hcv.lanl.gov/content/hcv-db/findmodel/findmodel.html). Bootstrap values were generated from 100 samplings in PhyML.

Measurement of potential methane production
Rates of potential CH_4 production were determined without substrate addition in short-term incubations. Within few hours from sampling, 15 ml of peat was transferred to 120-ml infusion bottles containing 30 ml of N_2-flushed distilled H_2O. Five bottles were prepared from each peat slice (one for each incubation temperature). The bottles were flushed with N_2 and closed with butyl rubber stoppers. After 4–6 days at 5 °C, N_2 flushing was repeated twice to remove residual CH_4 before the start of the temperature experiments. The bottles were incubated at 5.7 (±0.7), 14.2 (±0.9), 24.2 (±0.3), 32.8 (±1.9) or 40.2 (±3.3) °C (mean ± s.d. for the four experiments) in water baths inside incubators in dark for 90–95 h. In October, incubation time was 70 h and no samples were incubated at the highest temperature. Methane concentration was determined daily by gas chromatography as in Jaatinen *et al*. (2005). Rates of potential CH_4 production were calculated for the period of linear or closest to linear increase in CH_4 concentration after the 1-day lag phase that some samples exhibited.

Real-time quantitative PCR
Archaeal 16S rRNA genes were quantified by real-time quantitative PCR with primers Arch967F (5′-AATTGGCGGGGGAGCAC-3′) and Arch1060R (5′-GGCCATGCACCWCCTCTC-3′) (Cadillo-Quiroz *et al*., 2006 and references therein). Reactions (20 µl) contained LightCycler 480 SYBR Green I Master reaction mixture (Roche), 6 pmol of primers and 1 µl of total DNA (dilutions 1:10 and 1:20). Reactions were carried out in triplicate. Reaction conditions in a LightCycler 480 instrument (Roche) were denaturation at 95 °C for 5 min and 45 cycles of 95 °C for 10 s, 60 °C for 15 s and 72 °C for 10 s. Data were collected during the extension step. For creating a standard curve, 16S rRNA genes were amplified from total DNA with methanogen primers 146f and 1324r (Marchesi *et al*., 2001; Galand *et al*., 2003). The amplicons were gel purified as above, quantified with Qubit fluorometer, and diluted to contain 10^1–10^8 copies of the amplicon. The standard curve was created from duplicate reactions of each standard. Inspection of melting curves and analysis in 3% agarose gel ensured specificity of standard and sample products. Results are expressed per gram dry weight of peat (density of peat ~0.1 g (dry weight) cm^{-3}).

Results

T-RFLP fingerprinting of archaeal communities
Archaeal T-RFLP profiles from four seasons displayed altogether 11 T-RFs, with individual profiles

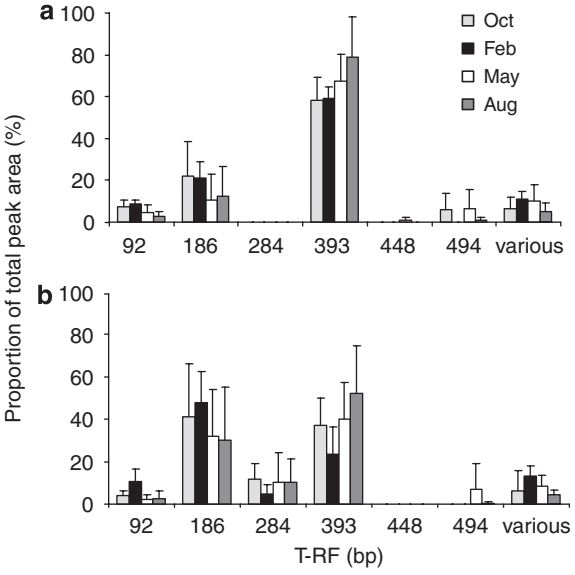

Figure 1 Terminal restriction fragment (T-RF) sizes and relative proportions in archaeal 16S rDNA- (**a**) and rRNA-derived (**b**) terminal restriction fragment length polymorphism (T-RFLP) profiles from the boreal fen at four times of the year. Bars represent mean + s.d. of relative peak areas in profiles from three peat cores per season. 'Various' includes unidentified minor T-RFs (62, 84, 115, 119 and 175 bp).

showing 4–7. DNA- and RNA-derived communities overlapped but also clearly differed (analysis of similarity $R = 0.40$, $P < 0.001$). The most explicit difference was the occurrence of a 284-bp T-RF in rRNA profiles only (Figure 1). In rDNA-derived profiles the 393-bp T-RF predominated, but in rRNA profiles the dominant T-RF was either 393 or 186 bp (Figure 1).

The 186- and 393-bp T-RFs were the most prominent ones all year round (Figure 1) and nearly ubiquitous; only in one August profile the 186 bp peak was absent. The main peaks' relative proportions suggested shifts between winter and summer. The 186-bp peak had on average 13% (range 2–36%) larger proportion and the 393-bp peak 24% (range 5–37%) smaller proportion in February than in August (Figure 1). These shifts occurred in both rDNA and rRNA profiles. Additionally, winter rRNA profiles showed a lower proportion of the 284-bp T-RF and higher proportion of the 92-bp T-RF. These community shifts and their significance were further investigated by PCA and RDA of T-RFLP data. The first PCA axis, which is related to the main compositional variation, separated rDNA-derived communities of February and October from May and August (Figure 2a). The main variation was associated with differences in the relative abundance of the 186- and 393-bp T-RFs. For rRNA data, the clearest separation of seasons was between February and August (Figure 2b). Although none of the explaining factors was statistically significant in strict terms for rDNA data, partial RDA revealed that temporal variation was the best predictor for compositional variation:

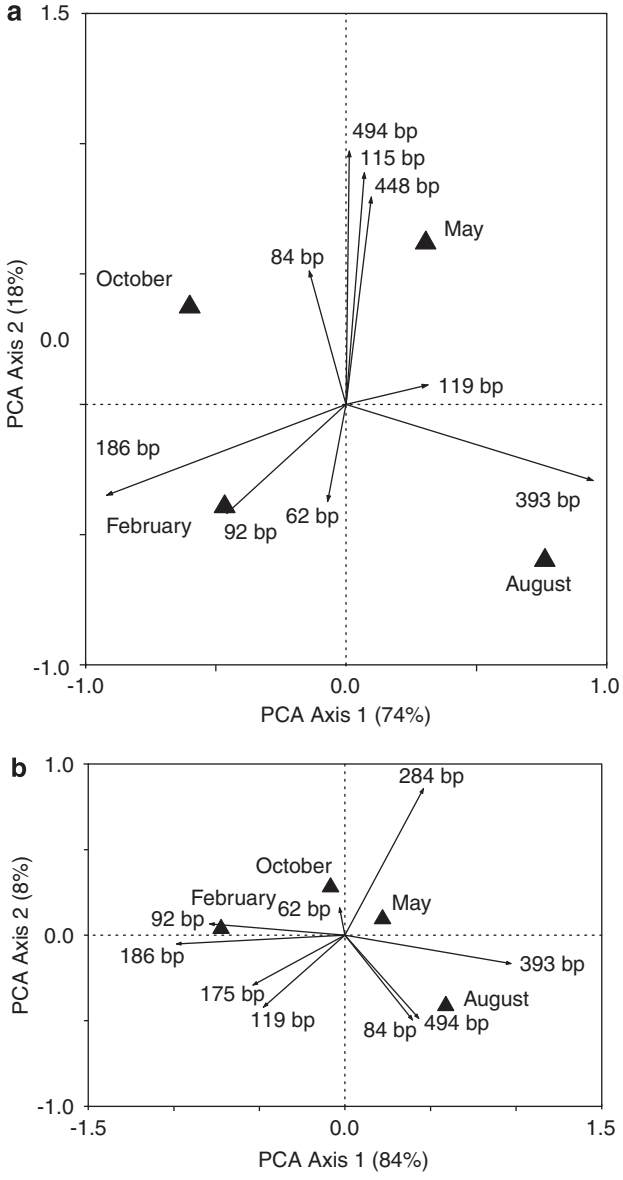

Figure 2 Principal component analysis (PCA) of archaeal rDNA- (**a**) and rRNA-derived (**b**) T-RFLP community data from peat collected at four times of the year. The values in parenthesis indicate the percentage of community variation explained by the axes.

the sampling times explained 25% of DNA community variation ($P = 0.088$). For rRNA data, however, a better and stronger explanatory variable turned out to be CH_4 production potential, which explained 38% of RNA community variation ($P = 0.020$).

Phylogenetic analysis and identification of T-RFs

Construction of 16S rDNA- and rRNA-derived clone libraries allowed identification of all major T-RFs (Table 2). Coverage values of the libraries ranged from 83% to 100%. Library compositions were in good agreement with the corresponding T-RFLP profiles (data not shown). The libraries supported

Table 2 Identification of T-RFs based on the phylogenetic distribution of RFLP-screened archaeal clones in rDNA- and rRNA-derived libraries

T-RF (bp)	*Phylogenetic affiliation*	*No. of clones*
92	Methanobacteriaceae/LDS cluster[a]	6
186	Methanosarcinaceae	42
186	Group 1.1c Crenarchaeota	7
284	Methanosaetaceae	7
393	Fen cluster (Methanomicrobiales)	22
393	Rice cluster II	36
448	Thermoplasmatales-related	2
494	Rice cluster II	4
>700	Group 1.3 Crenarchaeota	9
>800	Group 1.3 Crenarchaeota/ARMAN[a]	7

Abbrevations: RFLP, restriction fragment length polymorphism; T-RF, terminal restriction fragment.
[a]No differentiation of groups in RFLP.

the detection of the 284-bp T-RF exclusively from RNA. All three RNA-derived libraries contained clones with this T-RF, but the two DNA-derived libraries revealed none. Phylogenetic analysis assigned clone sequences with the 284-bp T-RF to Methanosaetaceae (Figure 3). Sequences with the 186-bp T-RF grouped with *Methanosarcina lacustris* and with Methanosarcinaceae sequences from peat, but also with group 1.1c Crenarchaeota (Figure 3). On the basis of RFLP patterns, Methanosarcinaceae clones were consistently more numerous in clone libraries ($30 \pm 16\%$, mean ± s.d. of five libraries) than 1.1c crenarchaea clones ($5 \pm 4\%$). In total, 86% of the clones with the 186-bp T-RF belonged to Methanosarcinaceae (Table 2), and therefore the T-RF most likely represented mainly this group. The other main T-RF of 393 bp occurred in sequences of Methanomicrobiales-associated methanogenic FC (Galand *et al.*, 2002; Bräuer *et al.*, 2006) and the uncultured but putatively methanogenic RCII (Grosskopf *et al.*, 1998b) (Figure 3). RFLP analysis differentiated FC and RCII in clone library data, but because different libraries revealed only FC, only RCII, or 40% FC and 60% RCII, it was not possible to estimate the relative contributions of these groups. To gain further insight into the identity of the 393-bp T-RF, the occurrence of FC was investigated with Methanomicrobiales-specific 16S rRNA gene primers, which amplify FC (Cadillo-Quiroz *et al.*, 2006) but, according to sequence analysis and PCR of Siikaneva clones, do not amplify RCII. Both total DNA and reverse-transcribed RNA from 11 out of 12 samples yielded good PCR products (data not shown), indicating the presence of FC throughout the year.

Other sequenced clones, representing minor T-RFs, were affiliated with Methanobacteriaceae, with euryarchaea belonging to an uncultured Lake Dagow Sediment cluster (LDS, Glissman *et al.*, 2004) or distantly related to Thermoplasmatales, and with group 1.3 Crenarchaeota (Table 2 and Figure 3). Two sequences (SnDF3 and SnDO5) showed the highest similarity (90%) to an uncultured euryarchaeote ARMAN-2 from acid mine drainage (Baker *et al.*, 2006). Because group 1.3 and ARMAN-2-related T-RFs were outside the size range of the T-RFLP standard, they were not included in Figure 1. No chimeric sequences were detected. Five minor T-RFs (62, 84, 115, 119 and 175 bp) remained unidentified with no matching sequences.

Seasonality and temperature response of potential CH_4 production

In parallel with the RNA/DNA analyses, we determined the endogenous methanogenic potential of the peat samples at field temperatures (Table 1). The potential was also measured at higher temperatures to determine the temperature of maximal production (Figure 4). Rates at 6 and 14 °C were small, varying from 0 to 18.7 nmol g^{-1} h^{-1} (Table 1). The potential at 14 °C varied significantly with season ($P = 0.024$, Kruskal–Wallis test). Production potential increased from October to February, and the highest CH_4 production was unexpectedly observed in winter (Table 1). The potential remained fairly high in the following spring but decreased clearly by August.

Methane production rates were markedly higher above 14 °C, with maximal potential between 27 and 33 °C (Figure 4). In May and August, however, the rates of two samples out of three showed no clear difference between 24 and 33 °C (Figure 4). Seasonal production rates differed at 33 °C ($P = 0.030$, one-way analysis of variance). The high production potential in winter was even more striking at 33 °C, with rates from 263 to 475 nmol g^{-1} h^{-1}. Compared to the rates of the other seasons at 33 °C, ranging from 17 to 277 nmol g^{-1} h^{-1}, the winter rates were on average 3.5 and up to 28 times as large.

Quantification of archaea

To elucidate factors behind the variation of CH_4 production potential, we determined the size of the archaeal community by quantitative PCR of 16S rDNA. Archaeal numbers differed with season ($P = 0.025$, one-way analysis of variance), with October showing the lowest numbers (Table 1). In contrast to the differing CH_4 production potentials of February and August, the archaeal community size in winter and summer was similar (Table 1).

Detection of mcrA *RNA in winter peat*

To confirm the presence of active methanogens in winter, we attempted to detect the mRNA of *mcrA*, a methanogen-specific marker gene coding for methyl-coenzyme M reductase and indispensable for CH_4 production (Friedrich, 2005). Amplification of *mcrA* cDNA was successful from all winter samples (Figure 5).

Discussion

Our results demonstrated strong seasonality of CH_4 production in a boreal fen, with surprisingly high

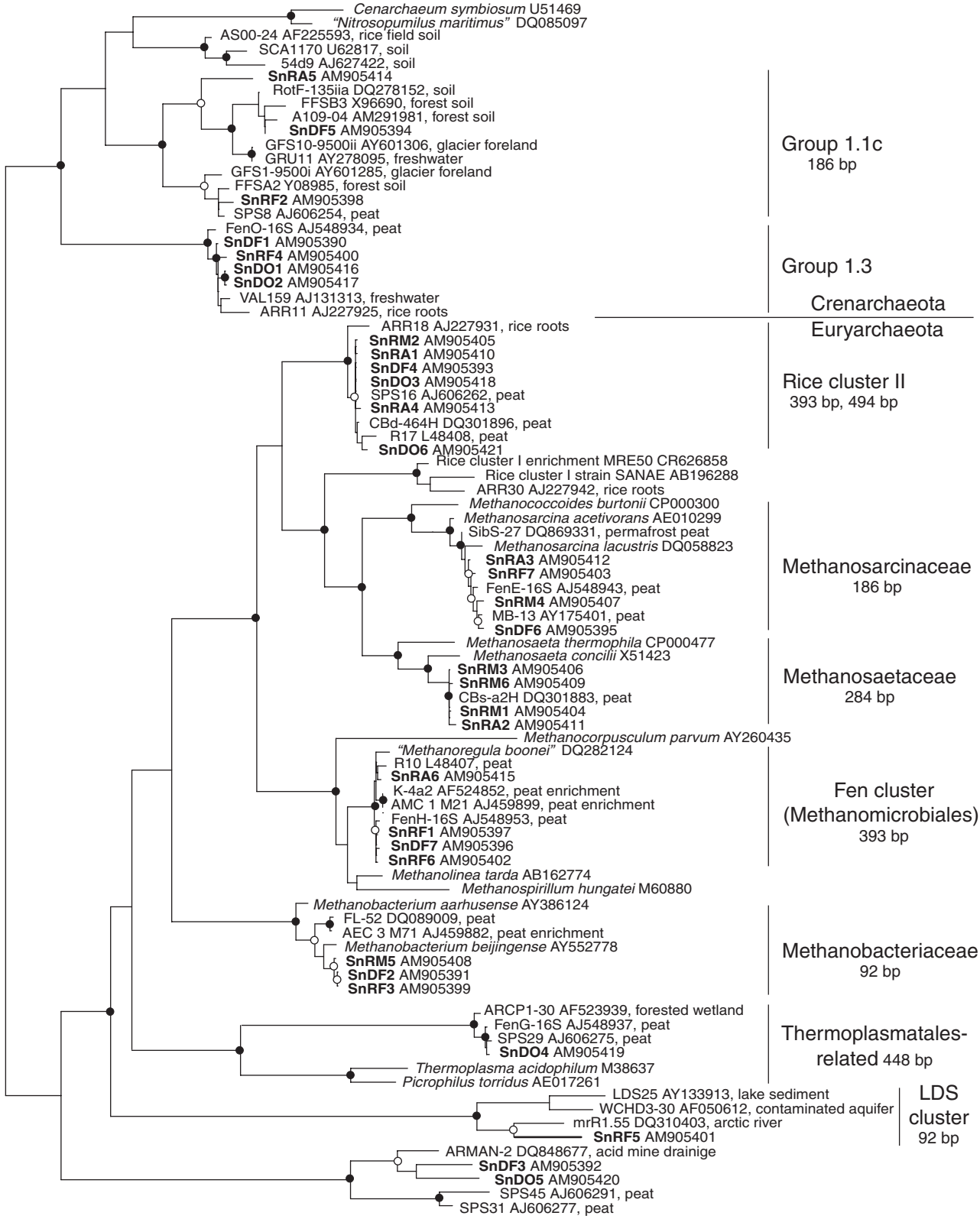

Figure 3 Maximum likelihood tree representing partial archaeal 16S rRNA gene sequences (ca. 650 bp) from the boreal fen (in bold) and reference sequences. Size of terminal restriction fragment is indicated for the groups detected by terminal restriction fragment length polymorphism (T-RFLP). Scale indicates 10% sequence divergence. Nodes with bootstrap values >95% are indicated with filled circles and >75% with unfilled circles. The tree was rooted between Crenarchaeota and Euryarchaeota. In bold sequence names, D denotes sequences from rDNA and R from reverse-transcribed rRNA. O, October; F, February; M, May and A, August stand for sampling dates. Group 1.1c Crenarchaeota also contains a sequence cluster known as 1.1c associated or group 1.1d (Kemnitz *et al.*, 2007).

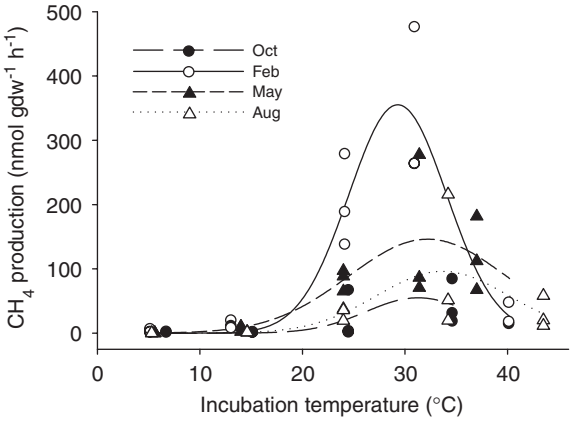

Figure 4 Temperature response of potential CH_4 production at four times of the year. Peat samples ($n=3$) were incubated without added substrates at temperatures from 5 to 43 °C for 4 days. Lines are Gaussian fits for visual aid. gdw, grams dry weight.

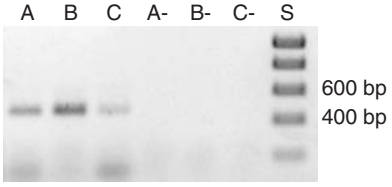

Figure 5 Amplification of the methanogenic *mcrA* gene fragment (470 bp) from reverse-transcribed RNA extracted from winter peat of three replicate peat profiles (A, B and C). A−, B− and C− are control reactions with DNase-treated nucleic acid extracts as PCR template. Size standard (S) on the right.

methanogenic potential in winter, and less conspicuous shifts in archaeal community structure. To the best of our knowledge, this is the first report of temporal comparison of archaeal communities together with methanogenic potential in a boreal wetland. Moreover, the detection of methanogenic *mcrA* mRNA revealed the presence of active methanogens in winter.

High CH_4 production potential in winter peat has not been reported before. The very few reports on winter CH_4 production have observed, in temperate wetlands, the lowest potentials in winter (Yavitt *et al.*, 1987; Avery *et al.*, 1999). Our opposite observation in boreal peat could be due to the lower autumn temperatures. In a boreal fen, potential CH_4 production increased towards autumn (Saarnio *et al.*, 1997; Kettunen *et al.*, 1999). The authors suggested that when temperature and thus methanogenic activity dropped, substrates accumulated. High levels of substrates accumulating as a result of bacterial activity in autumn and winter would, together with sufficient methanogenic biomass, allow the high CH_4 production potential in February.

We propose that methanogens in the unfrozen peat beneath the snow cover exhibit low activity. The high CH_4 production potential and the archaeal community size comparable to spring and summer indicated that methanogenic biomass in winter was substantial. Analysis of rRNA revealed the same archaeal groups as in warmer seasons, and the detection of *mcrA* mRNA confirmed active methanogenesis. Methane production has been detected at 1 °C and even below (Simankova *et al.*, 2003; Wagner *et al.*, 2007); at our site peat temperature at the depth of 20 cm remained above 0 °C all winter except a few days before snowmelt. Furthermore, anaerobic conditions beneath the snow and ice cover are highly stable, which may be beneficial for methanogenesis in cold (Kotsyurbenko, 2005). The small winter CH_4 emission and the CH_4 pulse after snowmelt, which have been detected in this fen (Rinne *et al.*, 2007; Riutta *et al.*, 2007), could therefore result from active but severely temperature-limited methanogenesis during winter. The high temperature of maximal potential CH_4 production and the stark contrast between production at 14 and 24 °C (Figure 4) showed that field temperatures limited methanogenesis not only in winter but also throughout the year.

The summer of 2006 was exceptionally dry, and the water level dropped below the sampled depth of 20 cm. Consequently, inactivation of the anaerobic microbial community by exposure to oxygen may have resulted in the low methanogenic potential in late summer, despite undiminished archaeal numbers. Yet because of the higher field temperatures, the archaeal community in late summer during low methanogenic potential most likely produced more CH_4 *in situ* than the community in winter when the potential was markedly higher.

Comparison of rDNA and rRNA data has commonly revealed overlapping microbial communities with DNA- and RNA-exclusive members (Duineveld *et al.*, 2001; Mengoni *et al.*, 2005; Gentile *et al.*, 2006). Here, the similarity of rDNA- and rRNA-derived phylogenetic groups demonstrated an expected level of agreement, whereas the observed community structures differed. Ribosomal RNA content is generally highest in actively growing cells although exceptions are known (Kerkhof and Ward, 1993; Kerkhof and Kemp, 1999). Owing to the lability of RNA, analysis of rRNA can nevertheless be considered to emphasize the metabolically active population and rDNA the numerically abundant. The prominence of Methanosarcinaceae (186 bp T-RF) in rRNA compared to rDNA (Figure 1) might indicate high cellular rRNA content and thus potentially higher methanogenic activity. The detection of Methanosaetaceae exclusively from RNA could signify the high rRNA content of a small population, which in DNA analysis was below the detection limit. Because members of Methanosaetaceae have slow growth rates (Jetten *et al.*, 1992), their environmental responses may primarily appear in rRNA instead of rDNA, as has been observed in an anaerobic digester and rice field soil (Delbes *et al.*, 2001; Lueders and Friedrich, 2002). The high variability of rRNA-derived communities (Figure 1b)

implied small-scale spatial or temporal fluctuations in archaeal activity, predominating over the more stable patterns of rDNA-derived populations. Considering the rRNA community variation and its connection with CH_4 production as indicated by the partial RDA, it is noteworthy that a recent study suggested CH_4 fluxes to be controlled more by cellular activity of methanogens than by cell numbers (Röling, 2007).

In agreement with the methanogenic potential of the studied peat layer, methanogens dominated the archaeal communities. The main groups Methanosarcinaceae, FC and RCII have been prominent in northern wetlands, and many of the 16S rRNA gene sequences of these groups and of Methanosaetaceae and Methanobacteriaceae grouped with sequences from boreal, temperate and arctic peat (Basiliko *et al.*, 2003; Galand *et al.*, 2003; Horn *et al.*, 2003; Kotsyurbenko *et al.*, 2004; Høj *et al.*, 2005; Metje and Frenzel, 2005, 2007; Cadillo-Quiroz *et al.*, 2006). The results hint at the occurrence of both hydrogenotrophic (Methanomicrobiales-associated FC and Methanobacteriaceae) and acetoclastic (Methanosaetaceae) methanogenesis. Members of Methanosarcinaceae could be acetoclastic, hydrogenotrophic or methylotrophic, because many strains in this versatile family have all three pathways (Garcia *et al.*, 2000). FC has been a dominant group in highly acidic mires (Juottonen *et al.*, 2005; Cadillo-Quiroz *et al.*, 2006), and its detection is in accordance with the low pH of this site. The FC sequences showed 97–98% sequence similarity to '*Candidatus* Methanoregula boonei', the first cultured FC member, which was isolated from an acidic temperate bog and has a pH optimum of ca. 5, the lowest known for a methanogen (Bräuer *et al.*, 2006).

Crenarchaea of groups 1.1c and 1.3 were less prevalent but present in peat at different seasons. Group 1.1c has been detected in other low pH soil environments such as forest soils (Jurgens *et al.*, 1997; Kemnitz *et al.*, 2007; Nicol *et al.*, 2007). Group 1.3 (or Rice cluster IV) appears to be widespread in northern wetlands (Galand *et al.*, 2003; Utsumi *et al.*, 2003; Kotsyurbenko *et al.*, 2004; Høj *et al.*, 2006; Rooney-Varga *et al.*, 2007). Function of these crenarchaeal groups remains unknown, but their detection in water-saturated peat suggests they might survive in anoxic conditions or thrive in oxic microenvironments, for example, in the vicinity of vascular plant roots (Simon *et al.*, 2000).

It has been suggested that root exudate-utilizing microbes thrive in summer, but when plant activity ceases in winter, substrate use shifts towards recalcitrant dead plant material (Lipson *et al.*, 2002). Bergman *et al.* (2000) reported that boreal mire sites with distinct plant communities showed different seasonal patterns of CH_4 production and that a notable source of temporal variation was substrate supply. Here, substrate quality or quantity may have induced the seasonal differences in CH_4 production potential and also the small fluctuation of archaeal communities between summer and winter. For instance, members of Methanosarcinaceae have been prominent at high substrate levels (Fey and Conrad, 2000; Lu *et al.*, 2005), whereas hydrogenotrophic methanogenesis, the only known pathway for Methanomicrobiales and thus FC, has been associated with low substrate availability of peat (Hornibrook *et al.*, 1997). Hydrogenotrophic Rice cluster I methanogens have also been associated with low H_2 availability (Sakai *et al.*, 2007). Whether the observed shifts of relative T-RF abundances represent substrate-dependent changes in size or activity of particular methanogen populations warrants further study. Considering the variable contribution of FC and RCII in clone libraries, the abundance of these poorly known groups would be particularly interesting to monitor in relation to peat chemistry and vegetation.

In conclusion, with their distinct seasons, boreal mires are excellent ecosystems for elucidating the microbial activity behind the annual cycles of wetland greenhouse gas emissions in the changing climate. Season affected particularly the methanogenic potential but also, to a lesser extent, the archaeal communities and quantities in the studied boreal fen. Assessing not only rDNA but also rRNA provided a more comprehensive description of community composition over the seasons. Analysis of rDNA revealed archaeal communities with relatively stable composition and small temporal variation, whereas the rRNA-derived communities appeared more dynamic. The detected methanogenic activity in winter calls attention to wetland microbial processes in cold. Further studies addressing the seasonal dynamics of both methanogens and the bacterial substrate producers in different types of mires have potential to advance understanding of community responses to peat chemistry and vegetation.

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