 doi:10.1111/j.1462-2920.2005.00838.x

Methanogen communities and *Bacteria* along an ecohydrological gradient in a northern raised bog complex

Heli Juottonen,[1] Pierre E. Galand,[1†] Eeva-Stiina Tuittila,[2] Jukka Laine,[2,3] Hannu Fritze[4] and Kim Yrjälä[1*]

[1]*Department of Biological and Environmental Sciences, General Microbiology, PO Box 56, 00014 University of Helsinki, Finland.*

[2]*Peatland Ecology Group, Department of Forest Ecology, PO Box 27, 00014 University of Helsinki, Helsinki, Finland.*

[3]*Finnish Forest Research Institute, Parkano Research Station, Kaironiementie 54, 39700 Parkano, Finland.*

[4]*Finnish Forest Research Institute, Vantaa Research Centre, PO Box 18, 01301 Vantaa, Finland.*

Summary

Mires forming an ecohydrological gradient from nutrient-rich, groundwater-fed mesotrophic and oligotrophic fens to a nutrient-poor ombrotrophic bog were studied by comparing potential methane (CH_4) production and methanogenic microbial communities. Methane production was measured from different depths of anoxic peat and methanogen communities were detected by detailed restriction fragment length polymorphism (RFLP) analysis of clone libraries, sequencing and phylogenetic analysis. Potential CH_4 production changed along the ecohydrological gradient with the fens displaying much higher production than the ombrotrophic bog. Methanogen diversity also decreased along the gradient. The two fens had very similar diversity of methanogenic methyl-coenzyme M reductase gene (*mcrA*), but in the upper layer of the bog the methanogen diversity was strikingly lower, and only one type of *mcrA* sequence was retrieved. It was related to the Fen cluster, a group of novel methanogenic sequences found earlier in Finnish mires. Bacterial 16S rDNA sequences from the fens fell into at least nine phyla, but only four phyla were retrieved from the bog. The most common bacterial groups were *Deltaproteobacteria*, *Verrucomicrobia* and *Acidobacteria*.

Received 1 December, 2004; accepted 5 April, 2005. *For correspondence. E-mail kim.yrjala@helsinki.fi; Tel. (+358) 9 19 15 92 20; Fax (+358) 9 19 15 92 62. †Present address: Département de Biologie, Université Laval, Québec (Québec), G1K 7P4, Canada.

Introduction

Northern mires are wetland ecosystems that are sustained by humid climate. In the prevailing high water level conditions decomposition is incomplete, and a part of the net production of the ecosystem accumulates as peat (Clymo, 1984). Since the last deglaciation, northern mires have formed a carbon store of 300–450 Pg (Gorham, 1991; Turunen *et al*., 2002). As a result of the activity of methanogenic archaea in the water-saturated peat layers, mires emit an estimated 30–40 Tg of CH_4 annually (Bartlet and Harris, 1993; Laine *et al*., 1996) that contributes to the increasing levels of atmospheric CH_4 and thereby to global warming.

Northern mires have developed and exist as mosaics of hydroseral gradients (Korhola, 1996). The most prominent spatial gradient in raised bog complexes is found in the ecohydrological continuum from the minerotrophic margin fens to the ombrotrophic central bogs (Seppä, 1996). On spatial scale, the relative proportion of the ombrotrophic centre in the complex increases over time (Korhola, 1996). Development of these ecosystems is also shown in the vertical layering of the peat stratigraphy, where minerotrophic fen plant communities have gradually been replaced by ombrotrophic vegetation (Tolonen, 1967; Hughes and Dumayne-Peaty, 2002).

Research on plant community structure and dynamics together with traditional paleoecological studies have increased understanding on the spatial and temporal variation in the function of mires. However, the study of functional microbial communities in these ecosystems has so far almost been neglected. Methane-producing archaea communities have been described in various peat ecosystems (e.g. McDonald *et al*., 1999; Basiliko *et al*., 2003; Galand *et al*., 2003; 2005a) but never in relation to a gradient of different ecohydrological conditions. Few studies have investigated prokaryotes other than methanogens or methanotrophs in mires (Rheims *et al*., 1996; Fisher *et al*., 1998; Horn *et al*., 2003; Mitchell *et al*., 2003; Sizova *et al*., 2003), and the role and occurrence of bacteria in those ecosystems is consequently poorly known.

Nucleic acid-based methods take advantage of molecular markers for the study of microbial communities without having to culture the organisms relevant for microbial activity (Morris *et al*., 2002a). Methane production is the

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unique property of methanogens and *mcrA* is a methanogen-specific gene needed in the last step of CH_4 formation (Thauer, 1998). Primers amplifying *mcrA* gene fragments have been used to describe methanogenic communities in different environments such as rice fields (MCR primer; Lueders *et al*., 2001), peatlands (ME primer; Hales *et al*., 1996; McDonald *et al*., 1999; Nercessian *et al*., 1999; Galand *et al*., 2002; 2005a) and landfills (ML primer; Luton *et al*., 2002). The ML primer pair, shown to detect representatives from all orders of methanogens (Luton *et al*., 2002), was here used to study CH_4-producer communities in Lakkasuo mire complex in Central Finland.

Our aim was to look for possible variation in potential CH_4 production and methanogen communities between mire sites forming an ecohydrological gradient. The gradient starts from a wet nutrient-rich mesotrophic fen and, through a slightly poorer oligotrophic fen, ends with a nutrient-poor ombrotrophic bog as a climax stage. As mire vegetation changes from sedge- and herb-dominated fens towards *Sphagnum*-dominated bog communities (e.g. Liedenpohja, 1981), and as functional composition of microbial communities in mires has been observed to relate to local vegetation (Fisk *et al*., 2003), we postulated that there is a decreasing trend in methanogenic diversity along the gradient.

Results

Methanogenic activity and pH

Potential endogenous CH_4 production decreased significantly ($P < 0.05$) along the ecohydrological gradient from the fens towards the ombrotrophic bog, where the production rates were often close to zero (Table 1). Similarly to potential CH_4 production, the acidity of peat was also related to ecohydrological gradient. Peat pH decreased significantly ($P < 0.001$) along the gradient towards the ombrotrophic bog, where it varied between 4.1 and 4.3 at different depths (Table 1). The two fens showed considerably higher pH with values ranging from 4.9 to 5.3, and there was even a significant difference in pH between the mesotrophic and the oligotrophic fen. With both variables there was a significant interaction between site and depth. Both the ombrotrophic bog and the mesotrophic fen had higher CH_4 production below water table, whereas the oligotrophic fen had the highest CH_4 production at the water table. The mesotrophic fen exhibited the highest pH value at the water table while at the other two sites the pH increased with depth.

Methanogen community structure and diversity

Restriction fragment length polymorphism (RFLP) analysis of 514 clones from nine *mcrA* clone libraries revealed a total of 82 different operational taxonomic units (OTUs). All libraries contained dominating OTUs representing at least a third of library clones (Fig. 1). The two OTUs Lak15 and Lak16 dominating the ombrotrophic bog (site OMB) constituted 95% of the clones in the upper layer and 47% in the deeper layer. These OTUs (belonging to the *Methanomicrobiales*-associated Fen cluster) were practically absent in the libraries from oligotrophic and mesotrophic fens (sites OLI and MES respectively). The dominating OTU Lak2 in both fen sites (related to *Methanosaeta*) constituted 37% of all fen clones. Other typical fen OTUs were Lak1 and Lak11. Lak8 and Lak17 characterized the MES libraries, and Lak10 and Lak19 the OLI libraries (Fig. 1).

Cluster analysis of OTU distributions separated the *mcrA* communities from the sites in different ecohydrological stages (Fig. 2). The bog communities were clearly distinct from the others, while the communities from the two different fen sites were closer to each other (Fig. 2). In the bog site community structure changed drastically with depth in the peat profile (OMB communities in Figs 1

Table 1. Water table (WT) depth, pH and potential endogenous methane production at different depths of peat profiles from three different sites in the Lakkasuo mire complex.

Site and WT	Sample depth (cm)[a]	pH[b]	Potential CH_4 production (nmol g^{-1} h^{-1})[b]
Ombrotrophic bog (OMB); WT: –25 cm	0	4.1 (0.1)	0.34 (0.42)
	–10	4.1 (< 0.1)	3.34 (5.87)
	–20	4.3 (< 0.1)	4.79 (6.28)
	–40	4.3 (0.8)	2.10 (1.30)
Oligotrophic fen (OLI); WT: –16 cm	0	4.9 (< 0.1)	19.3 (14.0)
	–10	5.0 (< 0.1)	16.6 (1.71)
	–20	5.0 (< 0.1)	5.29 (1.19)
	–40	5.0 (< 0.1)	2.22 (0.96)
Mesotrophic fen (MES); WT: –0 cm	0	5.3 (0.2)	3.32 (0.83)
	–10	5.0 (< 0.1)	16.2 (14.9)
	–20	5.1 (0.1)	14.2 (4.72)
	–40	5.2 (< 0.1)	4.10 (1.95)

a. Negative values indicate depths below the water table level.
b. Mean (SD) from three replicate peat profiles.

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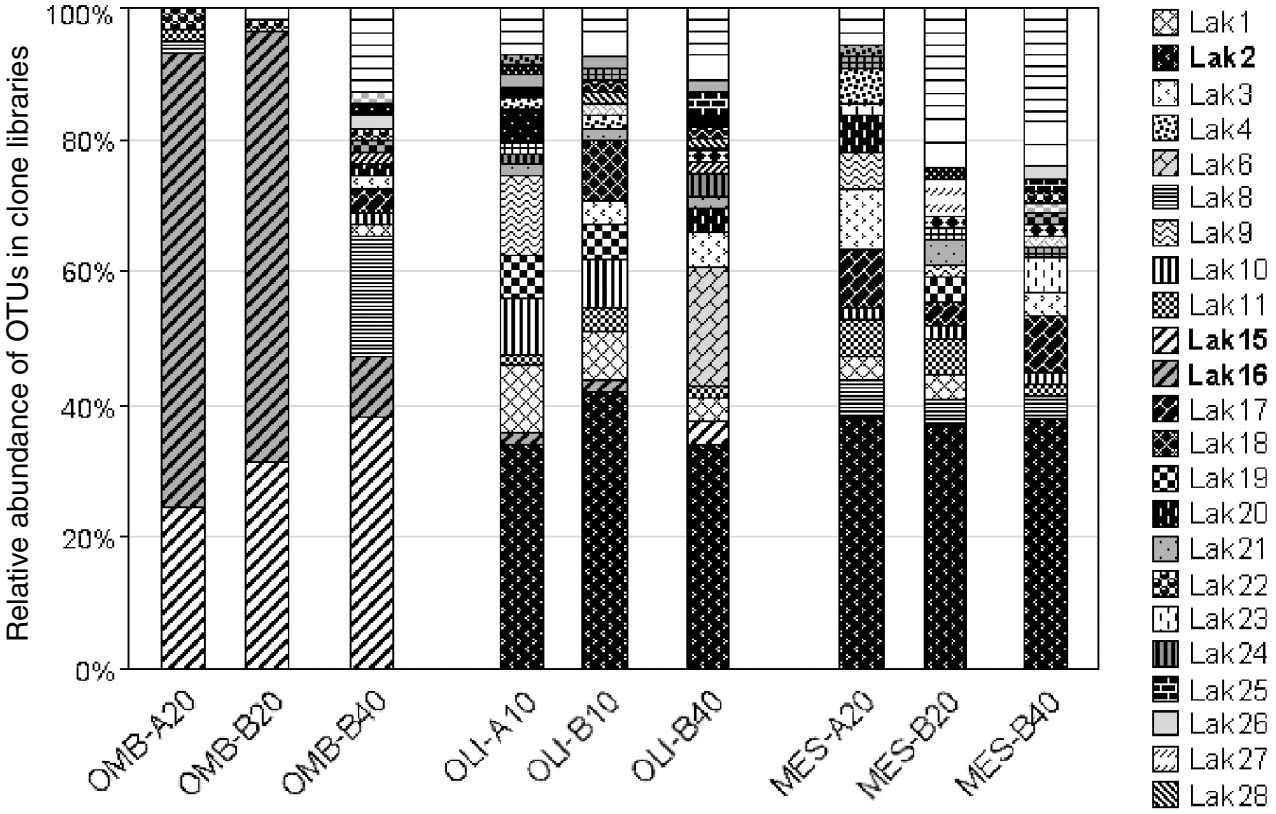

Fig. 1. Distribution of OTUs in *mcrA* clone libraries representing methanogen communities from three Lakkasuo study sites. The OTUs were defined by RFLP analysis of clones with two restriction enzymes (*Msp*I and *Taq*I). Mire type (OMB, ombrotrophic bog; OLI, oligotrophic fen; MES, mesotrophic fen), sampling depth (10/20 cm or 40 cm below water table level) and replicate peat profile (A, B) are indicated in the library names. White sections of columns represent OTUs unique to the particular library and detected in it only once or twice (see *Experimental procedures*). Other OTUs are designated in the legend (dominating OTUs in bold).

and 2). In contrast, change along the depth was less drastic in the oligotrophic fen, and in the mesotrophic fen no depth-related pattern was observed (OLI and MES communities in Figs 1 and 2). The difference between the bog and fen communities was reflected in their diversity. All indices (H', D and α) showed remarkably lower *mcrA* diversity in the upper layer of the bog site than in the two fen sites or in the deeper layer of the bog (Table 2). Although methanogen diversity in the deeper bog layer was similar to the diversity in the fens, community structure in this layer was clearly different from the structure in the fens (Fig. 2 and Table 2). There were no differences in diversity between the mesotrophic and oligotrophic fens or their upper and deeper layers.

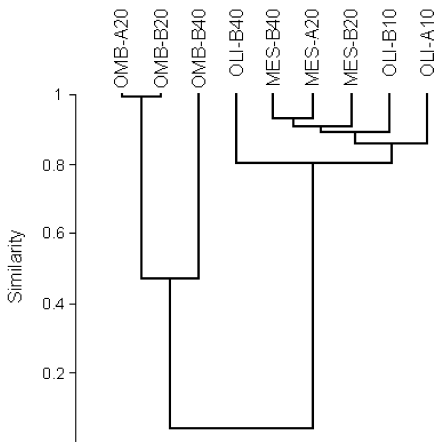

Fig. 2. Cluster analysis of OTU distributions from RFLP analysis of *mcrA* clone libraries. Distance matrix for the dendrogram was calculated using Morisita's similarity index. Libraries originate from three Lakkasuo mires (OMB, ombrotrophic bog; OLI, oligotrophic fen; MES, mesotrophic fen) from two depths (10/20 cm or 40 cm below water table level) and from two replicate peat profiles for each site (A, B).

Methanogen phylogeny

A majority of the 23 *mcrA* sequences representing the most abundant OTUs grouped with the Fen cluster (10 sequences) or the Rice cluster I (seven sequences, Fig. 3). Sequences of the dominating OTUs Lak15 and Lak16 from the bog grouped with the Fen cluster. They

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Table 2. Diversity indices calculated using OTU distributions from RFLP analysis of *mcrA* clone libraries.

	Clone library								
	Ombrotrophic bog			Oligotrophic fen			Mesotrophic fen		
Parameters	OMB-A20	OMB-B20	OMB-B40	OLI-A10	OLI-B10	OLI-B40	MES-A20	MES-B20	MES-B40
No. of clones analysed	61	61	55	59	55	55	55	54	58
No. of OTUs detected	5	4	21	20	18	21	16	24	27
Coverage (%)	97	97	69	78	82	79	87	74	66
Shannon diversity (*H'*)	0.85	0.77	2.26	2.40	2.21	2.41	2.23	2.58	2.60
Simpson's index (*D*)	0.54	0.53	0.19	0.16	0.20	0.16	0.18	0.16	0.16
Log series index (α)	1.29	0.96	12.4	10.7	9.32	12.2	7.58	16.6	19.7

Libraries originate from three Lakkasuo study sites (OMB, ombrotrophic bog; OLI, oligotrophic fen; MES, mesotrophic fen) from two depths (10/20 cm or 40 cm below water table level) and from two replicate peat profiles for each site (A, B).

were 99% similar to the *mcrA* sequence FenG retrieved earlier from Finnish mires (Galand *et al*., 2002; 2005a), and 98% similar to each other at amino acid level. Sequence Lak2, which dominated the fen libraries OLI and MES, was assigned to the order *Methanosarcinales* with closest sequence similarity to *Methanosaeta concilii* (86–88% amino acid identity). Two sequences fell outside any described methanogen lineages. One of them (Lak19) had highest similarity to *mcrA* sequences from a wetland in Florida (Castro *et al*., 2004). The other (Lak25) could not be affiliated with any available *mcrA* sequence, the best match being the *mcrA* gene of *Methanothermus fervidus* (67% amino acid identity). Sequence of Lak27 from the mesotrophic fen affiliated with the order *Methanobacteriales*. *mcrA* genes grouping with members of this order have not been detected previously in Finnish mires when the ME primers (Hales *et al*., 1996) were used (Galand *et al*., 2002, 2005a).

Bacterial phylogeny

To extend the microbial analysis in the upper water-submerged layer of the mires (with extremely low methanogen diversity in the bog), bacterial 16S rDNA was amplified, cloned and sequenced. Phylogenetic analysis distributed the clone sequences into 11 known bacterial phyla (Fig. 4). Sequences from the oligotrophic and mesotrophic fen sites represented nine and 10 phyla, respectively, whereas sequences from the bog represented four. The lineages containing most sequences were *Deltaproteobacteria* (19 sequences out of 68), followed by *Acidobacteria* (12 sequences) and *Verrucomicrobia* (12 sequences). Ten clones were closely related to cultivated bacterial species, with sequence identities ranging from 96% to 98%. Six of these clones showed high similarity to syntrophic deltaproteobacterial species *Syntrophobacter wolinii* (clones OMB-15, OMB-18, OMB-24) or propionate-degrading *Smithella propionica* (OMB-5, OMB-22, OLI-5). Clones OMB-4 and OLI-14 were related to *Geobacter metallireducens*, an anaerobic Fe(III) reducer. Clones OLI-7 and MES-2 resembled phylogenetically phototrophic *Alphaproteobacteria* species. The other bacterial clones grouped with environmental sequences or isolates, including several sequences obtained from peat (Brofft *et al*., 2002; Morris *et al*., 2002b; Sizova *et al*., 2003) and other acidic soils (Felske *et al*., 1998; Nogales *et al*., 2001). Clones MES-16 and MES-24 were 98–99% identical to clone FW34 from a forested wetland (Brofft *et al*., 2002), but they could not be reliably assigned to any bacterial lineage.

In addition to bacterial sequences, the Com primers detected nine 16S rDNA sequences related to *Crenarchaeaeota*. Six sequences (MES-1, MES-4, MES-6, MES-27, MES-28, OLI-24) from the fens were 97–99% similar to crenarchaeotal sequences VAL81 and VAL11 from a boreal forest lake (Jurgens *et al*., 2000). Sequences MES-14, OLI-23 and OMB-8 resembled Rice cluster IV sequences from a Siberian peat bog (Kotsyurbenko *et al*., 2004).

Discussion

As postulated, the diversity of methanogens decreased along the ecohydrological gradient from mesotrophic fen towards ombrotrophic bog. The bog, which represents the poorest end of the gradient, showed an exceptionally low diversity of methanogens in the upper peat layer. The two very similar OTUs Lak15 and Lak16, which totally dominated the bog libraries, belonged to the Fen cluster (Fig. 4), a novel group of *mcrA* sequences detected in Finnish mires (Galand *et al*., 2002; 2005a). The Fen cluster represents methanogens with pathways for methanogenesis remaining unconfirmed but which could be hydrogenotrophic, as the closest cultured relatives are hydrogenotrophs (order *Methanomicrobiales*). Lak15 and Lak16 may represent a single methanogen genus or species of the yet undescribed Fen cluster methanogens. An important characteristic of bogs is the dominance of ombrotrophic *Sphagnum* mosses in the vegetation. *Sphagnum* spp. outcompete other plants during mire develop-

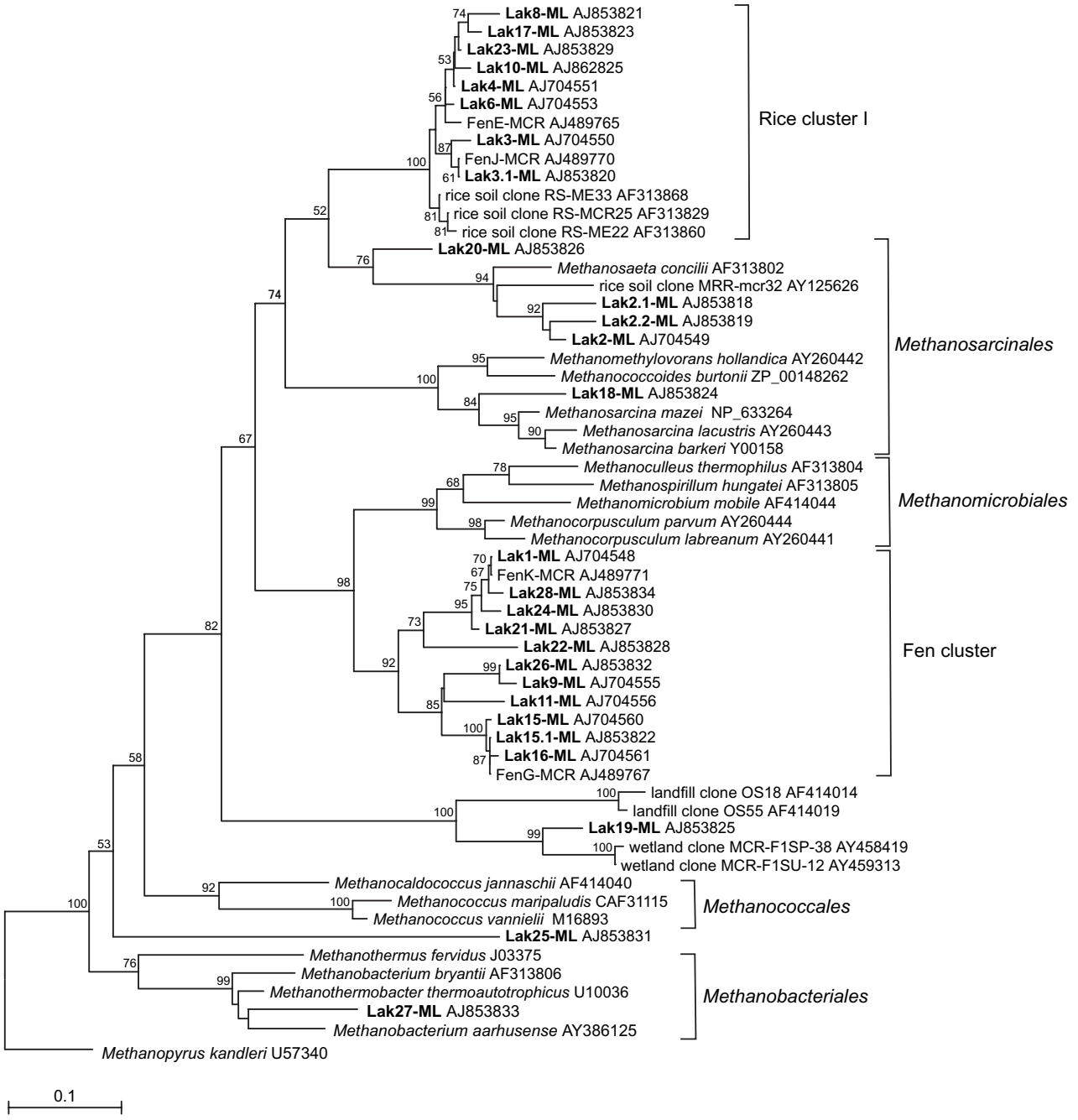

Fig. 3. Phylogenetic analysis of methanogen *mcrA* sequences from Lakkasuo study sites and reference sequences of described methanogen species and environmental clones. The neighbour-joining tree was constructed from derived amino acid sequences (137–145 aa) using *Methanopyrus kandleri* as outgroup. Names of the sequences from this study (in bold) refer to the OTU they represent. Number after OTU name indicates additional representatives of the same OTU. Bootstrap values >50% from 500 replicates are shown. The scale bar indicates 0.1 changes per amino acid position.

ment (Van Breemen, 1995) and further promote the already unfavourable growing conditions of nutrient-poor, acidic bogs (Clymo, 1984). The mosses trap nutrients and excrete acidic phenolic compounds, some of which have antibiotic properties (Banerjee and Sen, 1979; Verhoeven and Toth, 1995; Verhoeven and Liefveld, 1997). The extremely low methanogen diversity in the upper water-saturated layer of the bog could result from influence of *Sphagnum* mosses.

Community structure of methanogens changed along the ecohydrological gradient of the mire complex. Similarly to vegetation, the change was small between the

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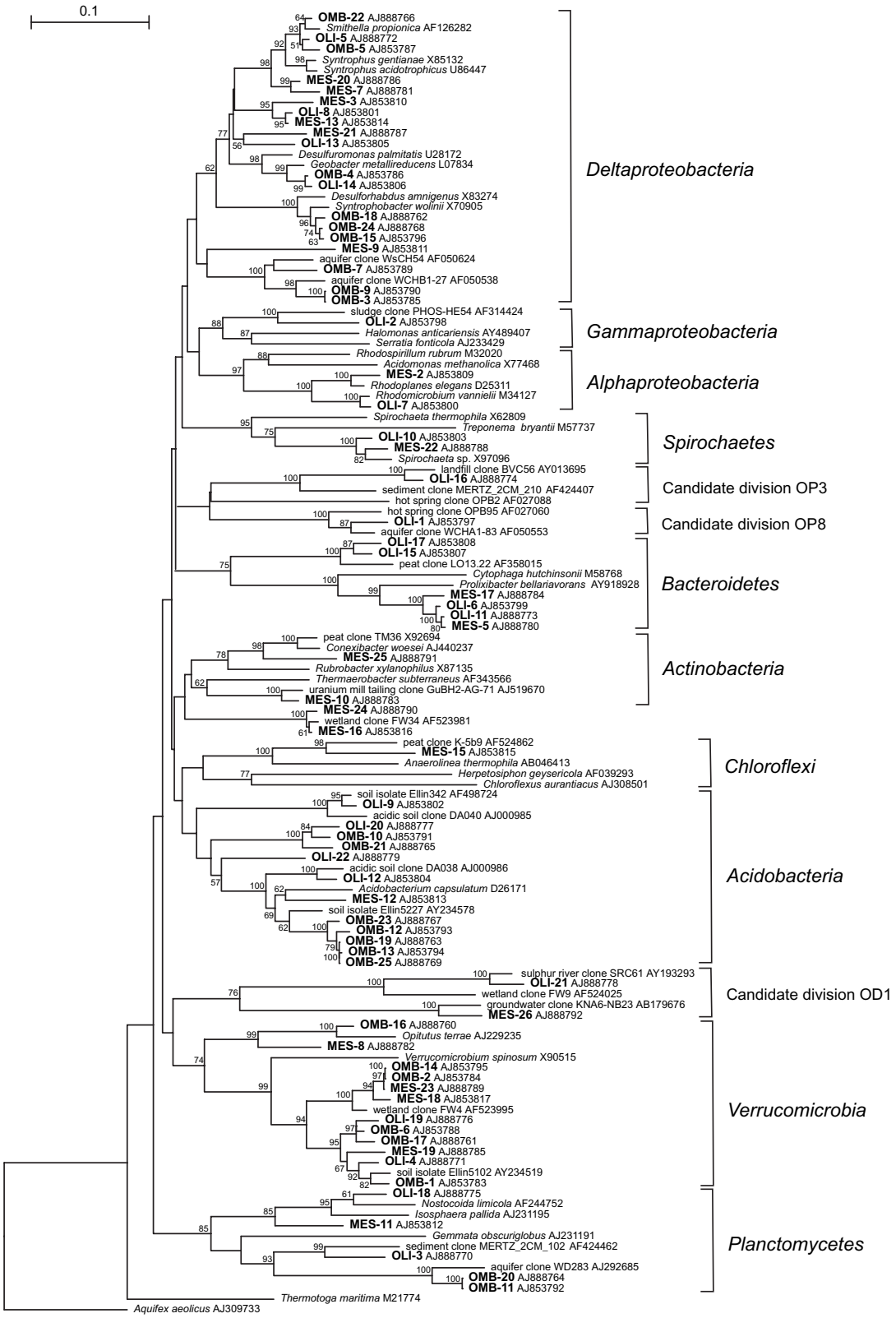

Fig. 4. Phylogenetic analysis of bacterial 16S rDNA sequences from Lakkasuo study sites (in bold; MES, mesotrophic fen; OLI, oligotrophic fen, OMB, ombrotrophic bog) and reference sequences of described bacterial species and environmental clones and isolates. The neighbour-joining tree was constructed from partial 16S rRNA gene sequences (368–382 bp in length) using *Aquifex aeolicus* as outgroup. Bootstrap values >50% from 500 replicates are shown. The scale bar indicates 0.1 changes per nucleotide position.

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mesotrophic and oligotrophic fens but large in transition from fen to bog. The bog's dominant *mcrA* sequences grouped with the Fen cluster, whereas the sequence dominating in the fens (Lak2) was related to the obligate acetotrophs of the family *Methanosaetaceae*. This suggests a possible difference in the proportion of acetotrophic methanogenesis between the fens and the bog. Such differences are highly plausible as site-related variations in the proportion of hydrogenotrophy have newly been described for peat samples taken from Lakkasuo during summer period (Galand *et al*., 2005b). Potential CH_4 production also varied along the gradient. The change in activity and methanogen community is probably connected to the differences in ecohydrology along the gradient: in the rich end of the gradient fens are receiving additional water and nutrients from the surrounding catchment area, but in the poor end bogs are exclusively fed by rain water. This leads to differences in abiotic and biotic conditions such as pH and vegetation (Laine and Vasander, 1996). The detected lower methanogenic activity in the bog (Table 1) is in agreement with the reports of lower CH_4 emissions measured (Nykänen *et al*., 1998) and lower degradation rates of organic matter for bogs than for fens (Aerts *et al*., 1999). This low degradation has been suggested to result from the unfavourable abiotic conditions (low pH, low nutrient levels) for microbial decomposition in bogs (Belyea, 1996). Another important factor for low activity may be the chemical composition of *Sphagnum*, which makes it resistant to decomposition (Verhoeven and Liefveld, 1997). In fens, higher abundance of vascular plants provides fresh carbon compounds for microbes, also to deeper peat layers through the root system (Chasar *et al*., 2000; Ström *et al*., 2003). This creates a more even depth distribution of labile carbon and could explain the even distribution of methanogenic communities with depth in the fens compared with the bog. Substrate quality may therefore explain the differences in methanogen communities between the fens and the bog and the putative differences in methanogenic pathways. Acetotrophic methanogenesis has been connected to labile carbon in surface peat, whereas hydrogenotrophic methanogenesis has been suggested to prevail in environments with less reactive, further decomposed carbon compounds (Hornibrook *et al*., 2000; Duddleston *et al*., 2002; Kotsyurbenko *et al*., 2004).

The drastic shift in methanogen diversity in transition from fen to bog raised the question if it would occur among bacteria as well, as they provide substrate for the CH_4 producers. Interestingly, we detected a difference in distribution of 16S rDNA sequences into bacterial lineages. The fens displayed more than twice as many phyla as the bog, where only *Deltaproteobacteria*, *Verrucomicrobia*, *Acidobacteria* and *Planctomycetes* were found. This is the first study to describe bacterial groups in connection to methanogens, as far as we know, specifically in the upper water-submerged layer of pristine mires. The majority of the sequenced bacterial 16S rRNA genes were related to *Deltaproteobacteria*, including propionate-degrading syntrophs. Syntrophic bacteria are known to provide H_2 to methanogens by interspecies H_2 transfer (Schink and Stams, 2002). The six clones closely related to known syntrophs (Fig. 4) could therefore be gene fragments from bacteria living in close relationship with methanogens, providing the substrate for hydrogenotrophic CH_4 production. All but one of the syntroph sequences were found in the bog, where substantial hydrogenotrophic methanogenesis has been detected (Galand *et al*., 2005b). A number of sequences belonging to the less known phyla *Acidobacteria* and *Verrucomicrobia* were also detected along the gradient, particularly in the bog. These are bacterial groups found in many different ecosystems with only a few cultured species (Barns *et al*., 1999; Chin *et al*., 1999; Buckley and Schmidt, 2001), and their ecological role still remains unknown. *Acidobacteria* have commonly been found in studies of peat environments (Rheims *et al*., 1996; Morris *et al*., 2002b; Horn *et al*., 2003; Sizova *et al*., 2003) and this group, along with different subdivisions of *Proteobacteria*, could be typical to peat habitats. A noteworthy finding was also the occurrence of *Planctomycetes*, which to our knowledge have not been detected by molecular analysis of peat before. As members of *Planctomycetes* are known to occupy aquatic environments (e.g. Hirsch and Muller, 1985; Gade *et al*., 2004), it is not surprising that they occur in water-submerged peat.

In summary, the comparison of methanogenic and bacterial marker genes at the different sites of the mire complex showed, for the first time, that drastic changes of both methanogenic activity and community occur along an ecohydrological nutrient gradient. Those changes are most likely driven by the ecohydrological conditions that, by shaping the vegetation composition, alter the availability of suitable substrates for the microbes. Our study also showed that the Fen cluster methanogens are well represented in boreal mires and may also be highly adapted to the special conditions found in ombrotrophic bogs. These results put forward the necessity to characterize the Fen cluster methanogens.

Experimental procedures

Experimental site and sample collection

Samples were collected in October 2002 from a raised bog complex, Lakkasuo, located in central Finland (61°35′–62°05′N, 23°50′–24°55′E). The area belongs to the southern boreal forest zone (Ahti *et al*., 1968). Study sites were selected to form an ecohydrological gradient from mesotro-

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phy to ombrotrophy (Laine *et al*., 2004). Study site MES is a mesotrophic fen, the vegetation of which is a mosaic of lawn and minerotrophic hollow level communities with high diversity. The field layer in both communities is characterized by sedges (*Carex rostrata*, *C. lasiocarpa*) and some herbaceous species, such as *Potentilla palustris* and *Menyanthes trifoliata*. In the drier lawn surfaces, the bottom layer is dominated by *Sphagnum* mosses (*S. fallax*, *S. flexuosum*, *S. magellanicum*), whereas in wetter hollow surfaces *Sphagnum subsecundum* is found together with *Warnstorfia exannulata* and *Utricularia intermendia*. Study site OLI is an oligotrophic fen, which consists of a fairly homogenous lawn level vegetation, dominated by *C. lasiocarpa* with some *Betula nana* in the field layer, and *Sphagnum papillosum*, *S. fallax* and *S. flexuosum* in the moss layer. Water table in both fen sites MES and OLI is near the surface and has small spatial and seasonal variation. Site OMB is an ombrotrophic bog. It is a mosaic of ecohydrological gradients shown as changing plant communities from wet hollows to intermediate lawns and finally to drier hummock communities. In addition to spatial variation, water level has large seasonal variations. *Eriophorum vaginatum*, together with *Andromeda polifolia* and *Rubus chamaemorus*, is the most abundant field layer species; *Sphagnum cuspidatum* dominates in the bottom layer of the hollows, *S. balticum* in the lawns and *S. fuscum* in the hummocks.

At each study site three replicate peat cores were collected from the lawn level with a box sampler ($8 \times 8 \times 90$ cm). From each peat core a 2 cm slice was taken as a sample at intervals of 10 cm using the water table level as a reference point. Samples were kept anoxic until further treatment. Slicing was performed immediately after sampling and subsamples for DNA analysis were frozen and stored at –20°C until DNA extraction.

Measurement of potential CH_4 production and pH

Potential endogenous CH_4 production was measured for peat samples from the three study sites from four depths of the submerged peat profiles ($n = 3$) with gas chromatography during laboratory incubation at anoxic conditions as reported by Galand and colleagues (2002). For pH measurement ($n = 3$), 10 ml of peat and 40 ml of distilled water were mixed in infusion bottles and pH was determined after an overnight incubation at room temperature (≈ 23°C). Two-way analysis of variance (ANOVA) followed by Tukey's test to compare the means was used to test the effect of site and depth on CH_4 production and pH.

DNA extraction and PCR amplification

Total DNA for analysis of methanogen and bacterial communities was extracted from 0.25 g of peat with UltraClean Soil DNA Kit (MoBio Laboratories).

The ML primer pair (Luton *et al*., 2002) was used to amplify a 470 bp region of methanogenic *mcrA* gene. An approximately 400 bp fragment of the V4-V5 region of bacterial 16S rRNA gene was amplified with primer pair Com1 and Com2 (Schwieger and Tebbe, 1998). The 50 μl PCR mixtures contained 20 pmol of appropriate primers, 200 μM dNTPs, 1 U Red Hot DNA polymerase (Advanced Biotechnologies) or 1.6 U Biotools DNA polymerase (B&M Labs), 1× PCR reaction buffer (supplied with the polymerase) and 1 μl of diluted total DNA as template (appropriate concentration determined empirically). Bovine serum albumin (0.6 μg μl^{-1}; Promega) was added to prevent PCR inhibition. Reaction conditions for methanogen PCR were initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 40 s, 55°C for 1 min and 72°C for 1 min 30 s, and final elongation at 72°C for 7 min. Bacterial PCR master mix was decontaminated before template addition with restriction enzyme *Sau*3AI. The master mix containing 4 U of *Sau*3AI (Promega) was incubated at 37°C for 30 min, followed by inactivation at 95°C for 3 min. Template was added and bacterial PCR was performed with following reaction conditions: initial denaturation at 95°C for 3 min, 35 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min 10 s, and final elongation at 72°C for 5 min. Reactions were performed in a GeneAmp PCR system 2400 or 2700 (Perkin Elmer). Amplification products were checked on agarose gels with ethidium bromide staining.

Cloning of methanogen and bacterial PCR products, RFLP and sequencing

Two methanogen *mcrA* clone libraries (from different replicate peat cores) and one bacterial 16S rDNA library per site were constructed using DNA obtained from upper peat layer (10 or 20 cm below water table; layer with highest potential CH_4 production in selected individual peat profiles) from the three Lakkasuo mire sites (MES, OLI and OMB). In addition, one *mcrA* library per site was constructed for the deeper peat layer (40 cm below water table; low CH_4 production). Polymerase chain reaction products of ML or Com primers were purified and cloned as described before by Galand and colleagues (2005a). Clone colonies were picked randomly for RFLP analysis (methanogen *mcrA* gene libraries) or for direct sequencing (bacterial 16S rRNA gene libraries). For RFLP, *mcrA* inserts in water-dispersed clone colonies were re-amplified with ML primers, and the PCR products were digested with restriction enzymes *Msp*I and *Taq*I in separate reactions. The restriction fragments were separated on 3% Synergel (Diversified Biotech) gel. Each *mcrA* clone was assigned into an *Msp*I RFLP group and a *Taq*I RFLP group based on the number and size of fragments detected. Restriction group data for both enzymes were used to place the clones into OTUs. Due to the high number of OTUs obtained in analysis with two restriction enzymes, OTUs detected only once or twice were not sequenced (white and unnamed coloured sections in Fig. 1). Colonies showing new distinct OTUs represented by at least three clones were selected for sequencing. Plasmid DNA was extracted from representative clones of these OTUs with Wizard Plus Minipreps DNA Purification System (Promega), and the *mcrA* inserts were sequenced using the vector primer T7. For analysis of bacterial 16S rRNA gene libraries, inserts of 25 clones from each library were sequenced from plasmid DNA or from clone PCR product.

Phylogenetic analysis

McrA sequences and 16S rDNA sequences were compared with sequences in databases with BLAST (National Center for

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Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were checked for chimeric PCR artefacts by using Chimera Check function of the Ribosomal Database Project (RDP-II, http://rdp.cme.msu.edu/), by inspection of sequence alignments and by construction of separate phylogenetic trees for 5′ and 3′ ends of the sequences. Five bacterial 16S rDNA sequences and two *mcrA* sequences were excluded as probable chimeras. Amino acid sequences were derived from *mcrA* nucleotide sequences. Sequences were aligned with CLUSTALW (European Biotechnology Institute, http://www.ebi.ac.uk/clustalw), and the alignments were checked manually.

Neighbour-joining trees with bootstrap values from 500 replicates were constructed with TREECON package (v. 1.3b; Van de Peer and De Wachter, 1994). Kimura models for DNA or amino acid sequences were used in distance calculations. The *mcrA* tree was verified by FITCH distance and parsimony analysis with PHYLIP package (v. 3.6b; J. Felsenstein, http://evolution.genetics.washington.edu/phylip.html). *McrA* and 16S rRNA gene sequence data have been submitted to EMBL database under Accession Nos AJ853778–AJ853834, AJ862825 and AJ888760–AJ888796.

Diversity indices and cluster analysis

Shannon diversity index *H′*, Simpson's index *D* and log series index α were calculated from distribution of OTUs in the *mcrA* clone libraries (Krebs, 1998; Galand *et al.*, 2002). Shannon index and log series index α were used to compare methanogen OTU richness. In general, both indices increase when the number of OTUs increases, but Shannon index takes also evenness of the community into account (Krebs, 1998). Simpson's index was used to quantify dominance of OTUs in libraries. It ranges from 0 (all OTUs equally abundant) to 1 (all clones belong to the same OTU). Similarities between OTU distributions of different samples were studied by cluster analysis, where pairwise Morisita's similarity indices (Krebs, 1998) for abundance data were calculated and displayed as a dendrogram. Clustering algorithm was the unweighted pair group method using arithmetic averages (UPGMA). Coverage of clone libraries was determined as in Galand and colleagues (2005a) to evaluate how well the number of detected OTUs reflects the total number of OTUs. PAST software (v. 1.26; O. Hammer and D.A.T. Harper, http://folk.uio.no/ohammer/past/) was used for cluster analysis and calculation of *H′*, *D* and α.

Acknowledgements

We thank Elina Heijari and Timo Sipilä for technical assistance. This work was supported by the Academy of Finland and the Research Foundation of University of Helsinki.

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