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Detection of methanogenic *Archaea* in peat: comparison of PCR primers targeting the *mcrA* gene

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Abstract

Methanogens (domain *Archaea*) have a unique role in the carbon cycle as producers of the greenhouse gas methane (CH_4). Methyl-coenzyme M reductase (MCR) is a vital enzyme in CH_4 production, and the *mcrA* gene coding for a subunit of MCR has been employed as a specific marker for the detection and differentiation of methanogen communities. A critical step in assessing environmental *mcrA* diversity is the selection of PCR primers. The objective of this study was to compare the diversity coverage of three published *mcrA* primer sets MCR, ME and ML (also known as MCR and Luton-*mcrA*) and their ability to discern methanogen communities in a drained peatland. The primers were applied to DNA extracts from unfertilised and ash-fertilised peat from two different depths. Amplified *mcrA* communities were cloned and sequenced, and the sequences were divided into operational taxonomic units (OTUs) by restriction fragment length polymorphism (RFLP) and sequence analysis. All primers recovered characteristic OTUs associated with the peat depths and treatments and confirmed a previous observation of low methanogen diversity. The minor differences in OTU ranges of the primers did not greatly affect the observed community composition. However, as the proportions of several OTUs varied strongly, the primers provided different quantitative representations of *mcrA* communities. We concluded that the ML and MCR primers had better amplification ranges than the ME set, but the use of MCR with peat samples was problematic due to poor amplification. Consequently, the ML primers were best suited for *mcrA* analysis of peatland methanogen communities.

Keywords: Methyl-coenzyme M reductase; RFLP; Methanogens; Peatlands; Diversity; Phylogeny

1. Introduction

The vast biodiversity of microbes in the environment has highlighted the challenge of relating microbial communities to ecosystem functions [37]. A straightforward approach to investigate the microbial populations involved in ecologically relevant processes is to extract total DNA directly from environmental samples and to amplify marker genes specific to the functional microbial group by PCR [5]. The PCR products can be directly cloned and sequenced or analysed by DNA fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), single strand conformation polymorphism (SSCP) or RFLP to reveal the microbial diversity of the functional communities [29,38,45].

Methanogenic *Archaea* are important actors in the biogeochemical cycling of carbon; they carry out the final stage of anaerobic degradation of organic matter with the unique ability to produce methane (CH_4). The significance of CH_4 as a strong greenhouse gas contributing to climate change has drawn attention to methanogenic soil ecosystems such as rice fields, sediments and peatlands. Methanogen populations in these environments have often been described using the archaeal 16S rDNA phylogenetic marker [9,21,27,31,42,43], but the high phylogenetic diversity of methanogens has hampered development of 16S rRNA gene-targeting PCR primers specific to methanogens [1]. Functional primers for exclusive detection of methanogens have been designed for the *mcrA* gene encoding the α subunit of methyl-coenzyme M reductase (MCR) [17,33,

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49]. In all known methanogens, MCR enters the final step in CH_4 synthesis, catalysing the reduction of a methyl group attached to coenzyme M with formation of CH_4 [12]. Members of the orders *Methanobacteriales* and *Methanococcales* additionally carry the corresponding *mrtA* gene for the isoenzyme MCRII [51]. An important advantage of *mcrA* as a marker gene is that its phylogeny is congruent with the 16S rRNA phylogeny of methanogens [30,33,49]. Recently, phylogenetically distinct *mcrA* genes have been discovered in marine anaerobic methane oxidizers (ANME-1 and ANME-2), in which MCR may be involved in reverse methanogenesis [18,19,28].

Although different *mcrA* primers have been widely employed to describe methanogen communities [8,15,26,30,33,36, 40], information allowing comparison of quality and amplification range of the primers in community analysis is scarce. Only a few *mcrA* studies have used two primer pairs in parallel [1,30, 39] or assessed the quantitative reliability of a primer set [32]. Luton et al. [33] showed that their primers amplify members from all five methanogenic orders, but the other primers [17,49] have not been as extensively tested on known species. Moreover, cultured taxa generally represent a tiny fraction of total microbial diversity [44], and clusters of environmental *mcrA* sequences have been recovered which are not closely affiliated with the available *mcrA* sequences of cultured methanogens [8, 15,30,40]. Testing the *mcrA* primers on diverse environmental samples is therefore of crucial importance.

Northern peatlands are important CH_4-emitting ecosystems [2,6,34] where methanogens reside in anoxic water-saturated peat. Studies on peatlands have retrieved 16S and *mcrA* sequences associated with uncharacterised methanogen groups, such as the Fen cluster [3,25,48]. The wide occurrence of uncultured methanogens makes peatlands interesting but challenging systems for PCR-based community analysis. Here we compared three sets of functional *mcrA* primers for analysing methanogen communities in peatlands. We used a method combining RFLP fingerprinting and sequencing of clone libraries, which has the advantage of combining high taxonomic resolution with fingerprinting comparison of samples. As methanogen communities in peatlands have been observed to change with depth [7,15,25], two peat depths were studied to target divergent communities. Our aim was to determine whether the use of different primer sets affects the observed methanogen community structure in a drained peatland, and more specifically, to assess how the primers may influence the relative abundances of operational taxonomic units (OTUs).

2. Materials and methods

2.1. Peat samples

Samples were collected from a drained peatland in northern Finland (64°30′N, 26°18′E). The sampling method and the experimental site consisting of ash-fertilised and unfertilised plots have been described previously [13]. One vertical peat core from each type of plot was used in this study. Samples for DNA analysis were taken from anoxic water-saturated peat 10 cm (depth 1) and 40 cm (depth 2) below water table level.

2.2. DNA extraction and PCR amplification

Total community DNA was extracted from 0.25 g of peat with UltraClean Soil DNA Kit (MoBio Laboratories). Fragments of the *mcrA* gene were amplified with three degenerate primer pairs with partially overlapping target regions (Fig. 1 and Table 1). The primer pair of Luton et al. [33] is referred to as ML instead of MCR to distinguish it from the earlier MCR primers of Springer et al. [49]. PCR was performed as reported previously [13] with the following reaction conditions: initial denaturation at 95 °C for 5 min, 35–40 cycles of 95 °C for 45 s, annealing at 46 °C (MCR), 50 °C (ME) or 55 °C (ML) for 45 s and 72 °C for 1 min 30 s, followed by a final extension at 72 °C for 7 min.

2.3. Cloning, RFLP analysis, sequencing and assignment of clones into OTUs

PCR products obtained with the three primer pairs were cloned [13], and ca. 63 (range 53 to 71) clones from each library were analysed by RFLP. Cloned inserts were reamplified with the respective primers and digested with the restriction enzyme *Msp*I (Promega). The restriction fragments were separated by agarose gel electrophoresis in 3% Synergel (Diversified Biotech). Clones that still exhibited only one full-length fragment after *Msp*I treatment were additionally digested with *Taq*I (Promega) to get distinguishable banding patterns for all clones. Analysis of the ME libraries had been presented earlier [13], but the data were treated differently in the present study.

Distribution of clones into operational taxonomic units (OTUs) was carried out in two stages: first by RFLP and then by sequence analysis. In preliminary grouping, clones of each

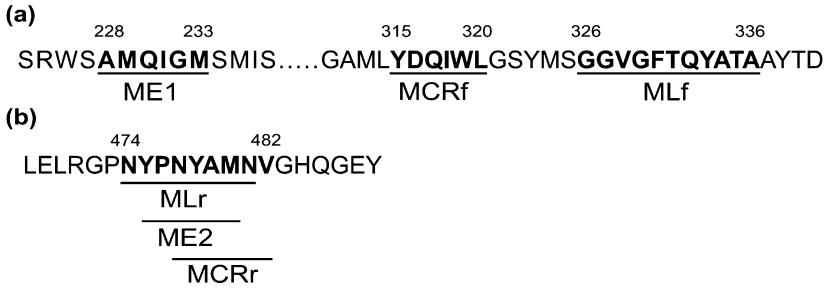

Fig. 1. Target sites of (a) forward and (b) reverse primers in McrA sequence. Residues are numbered according to McrA sequence of *Methanothermobacter thermoautotrophicus* (U10036).

Table 1
Properties of PCR primers targeting the *mcrA* gene

Primer	Sequence (5′–3′)	Length (bp)	Degeneracy[a]	Amplicon length (bp)	Reference
MCRf	TAYGAYCARATHTGGYT	17	48	~490	[49]
MCRr	ACRTTCATNGCRTARTT	17	32		
ME1	GCMATGCARATHGGWATGTC	20	24	~760	[17]
ME2	TCATKGCRTAGTTDGGRTAGT	21	24		
MLf	GGTGGTGTMGGATTCACACARTAYGCWACAGC	32	16	~470	[33]
MLr	TTCATTGCRTAGTTWGGRTAGTT	23	8		

[a] Number of different primer sequence variants as a result of degenerate bases.

primer set were grouped based on RFLP banding patterns. At least one clone from each of these primer-specific RFLP groups was sequenced. In the second stage, overlapping regions of all the sequences were compared, and RFLP groups with amino acid sequence identity of at least 98% were assigned to the same OTU. This grouping method allowed comparison of diversity between amplicons obtained with the different primers. Three OTUs with putative chimeric sequences were identified and excluded from further analysis based on analysis with the Bellerophon program [22] and inspection of pairwise sequence alignments.

2.4. Phylogenetic analysis

Deduced amino acid (aa) sequences of *mcrA* clones and reference sequences were aligned with ClustalW (http://www.ebi.ac.uk/clustalw/). Unambiguously aligned sections of the alignment (ca. 131 aa) were subjected to phylogenetic analysis. A maximum likelihood tree was inferred with PHYML [16] using JTT amino acid substitution model. Bootstrap values were generated from 100 samplings. Trees were also constructed using distance (FITCH) and maximum parsimony (PROTPARS) methods with Phylip software package (v. 3.64, J. Felsenstein, http://evolution.genetics.washington.edu/phylip.html).

2.5. Diversity indices and rarefaction analysis

Shannon diversity index $H' = -\sum_{i=1}^{S}(n_i/N)\ln(n_i/N)$ [46] and Simpson's index $D = \sum_{i=1}^{S}(n_i/N)^2$ [47] were estimated from the proportions of OTUs in each clone library with Past software package (v. 1.26, O. Hammer and D.A.T. Harper, http://folk.uio.no/ohammer/past). In both equations n_i is the number of clones assigned to OTU i in a library, N is the total number of clones analysed from the library and S is the total number of OTUs. Shannon diversity index increases with OTU richness and evenness of OTU distribution. Conversely, increasing values of Simpson's index indicate greater dominance of one or a few abundant OTUs. Rarefaction analyses to obtain richness curves were performed with Past software.

2.6. Gradient PCR and T-RFLP analysis

Amplification reactions with four annealing temperatures (49.2, 52.2, 55.4 and 56.9 °C) were performed with the ML primers in an Eppendorf Mastercycle Gradient thermal cycler using a reverse primer labeled with carboxyfluorescein (FAM). Total DNA extract from depth 1 (ash-fertilised plot) was used as template. The products were digested with *Msp*I, ethanol-precipitated and dissolved in formamide, and T-RFLP analysis was performed as described previously [35].

2.7. Nucleotide sequence accession numbers

The obtained *mcrA* nucleotide sequences have been submitted to the EMBL database under the accession numbers AM182234–AM182258.

3. Results

3.1. Depth and treatment-related distribution of mcrA communities

We evaluated three *mcrA*-targeting primer sets by comparing quantitative and phylogenetic composition of clone libraries and diversity index values. Samples from two depths of ash-fertilised and unfertilised peat were analysed to establish whether the primers could detect changes in methanogen communities with depth or treatment. Primer sets ML and ME gave a good yield of PCR products from environmental DNA extracts, but amplification efficiency of the MCR primers remained poor despite rigorous PCR optimisation, particularly for samples from depth 1. Moreover, 21% of the analysed clones in the MCR libraries of this depth contained non-*mcrA* sequences: either unidentifiable sequences with no open reading frames or sequences showing very low and only partial similarity to bacterial citrate synthase and NADH-plastoquinone oxidoreductase genes.

The *mcrA* clones obtained with the primers were assigned to a total of ten OTUs based on RFLP banding patterns and sequence similarity. Richness curves of all libraries except two approached a horizontal asymptote, indicating that the number of clones analysed was mostly sufficient (Fig. 2). Each primer set recovered eight OTUs, although not entirely the same ones, as four OTUs were not detected by all sets. All primers revealed a depth-related distribution of the six most abundant OTUs: OTUs B, G and H were associated with depth 1, and OTUs I, J and E with depth 2 (Fig. 3). When fertilised peat was compared with unfertilised peat, the major OTUs principally stayed the same, but all primer pairs showed a larger abundance of OTU E in fertilised peat.

3.2. Phylogenetic assignment of amplicons recovered by the different primers

Phylogenetic analysis of deduced amino acid sequences divided the McrA peptides into three well-identified clusters (Fig. 4). The sequences were affiliated with the Fen cluster (associated with the order *Methanomicrobiales*), the Rice cluster I (associated with *Methanosarcinales*) or the family *Methanosarcinaceae*. The six depth-characteristic OTUs belonged either to the Fen cluster or the Rice cluster I, and they were identified by all primer pairs. Only one OTU (F) was related to *Methanosarcinaceae*, and it was retrieved with primer pairs ML and MCR but not with ME. In addition, three minor OTUs assigned to the Fen cluster (A, C, D) were recovered with only one or two of the primer sets (Fig. 3).

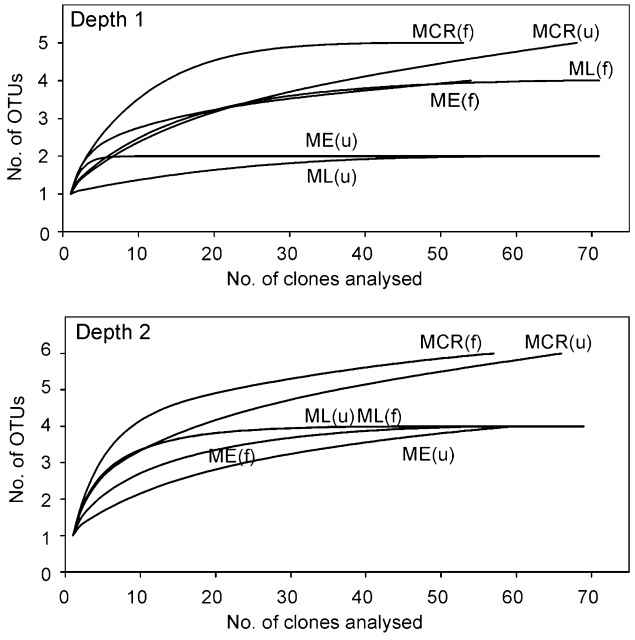

Fig. 2. OTU richness curves of *mcrA* clone libraries. The curves were obtained by rarefaction calculations and they represent libraries constructed with primer sets MCR, ME and ML for unfertilised (u) and ash-fertilised (f) peat from two depths.

3.3. Primer-related variations in community structure

Depending on the primer set, ratios of the dominant OTUs in the clone libraries varied markedly (Fig. 3). This discrepancy was most evident in libraries of depth 1, and it was more pronounced when the phylogenetic affiliations of the dominant OTUs were considered (Fig. 4). In MCR libraries of depth 1, the vast majority of clones belonged to the Fen cluster (OTU G), whereas in ML and ME libraries Rice cluster I OTUs (B, H) dominated. At depth 2 the primer-related variation in the proportions of the phylogenetic groups was not as substantial. Rice cluster I clones (OTU J) were, however, particularly abundant in the ME libraries, and the ML library of fertilised peat contained by far the largest share of the Fen cluster OTU E.

As diversity indices are frequently employed as a numerical tool for comparing microbial communities [20,23], we examined how the choice of primer pair affected the values of two widely used indices. Shannon diversity indices were in the same range for ME and MCR libraries from depth 1 (Fig. 5), and both primer sets indicated higher *mcrA* diversity than the ML set. At depth 2, in contrast, the ML and MCR sets showed larger values than ME. An increase in diversity with depth was seen with the ML set and possibly with MCR but not with the ME set. Simpson's index conveyed well the occurrence of a dominant OTU at depth 1 in the ML libraries and one MCR library, and at depth 2 in the ME libraries. Differences between ash-fertilised and unfertilised samples were generally small for both indices, but they were largest for ML and MCR libraries at depth 1.

Since annealing temperature can affect product ratios in PCR with degenerate primers [32,41], we performed initial tests by T-RFLP to find out whether changes in annealing temperature affect the products of the ML primers. Amplification reactions

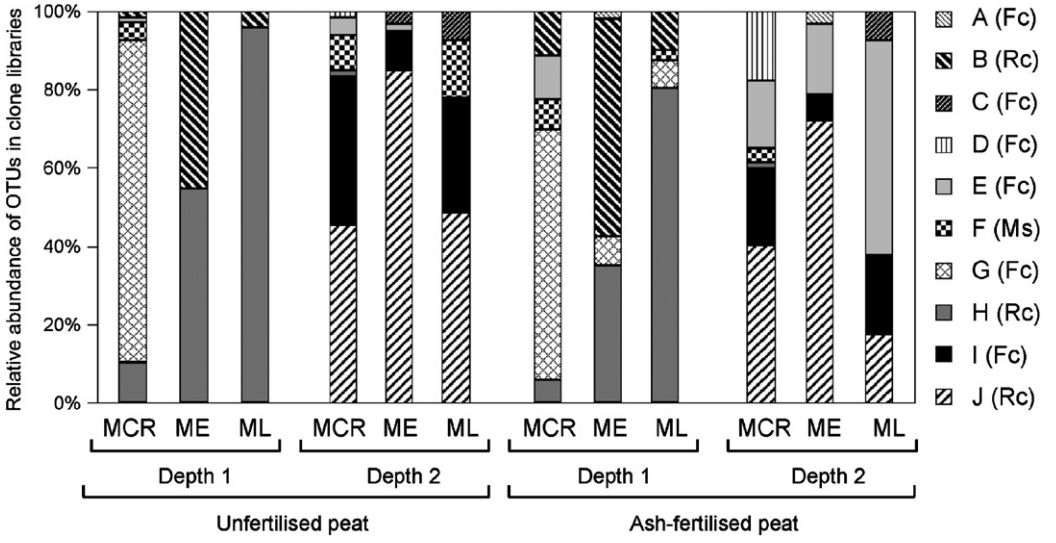

Fig. 3. Distribution of OTUs in clone libraries constructed with *mcrA* primer pairs MCR, ME and ML. Libraries were prepared using DNA from two peat treatments and depths. Phylogenetic affiliations of the OTUs are shown on the right (Fc, Fen cluster; Rc, Rice cluster I; Ms, *Methanosarcinaceae*).

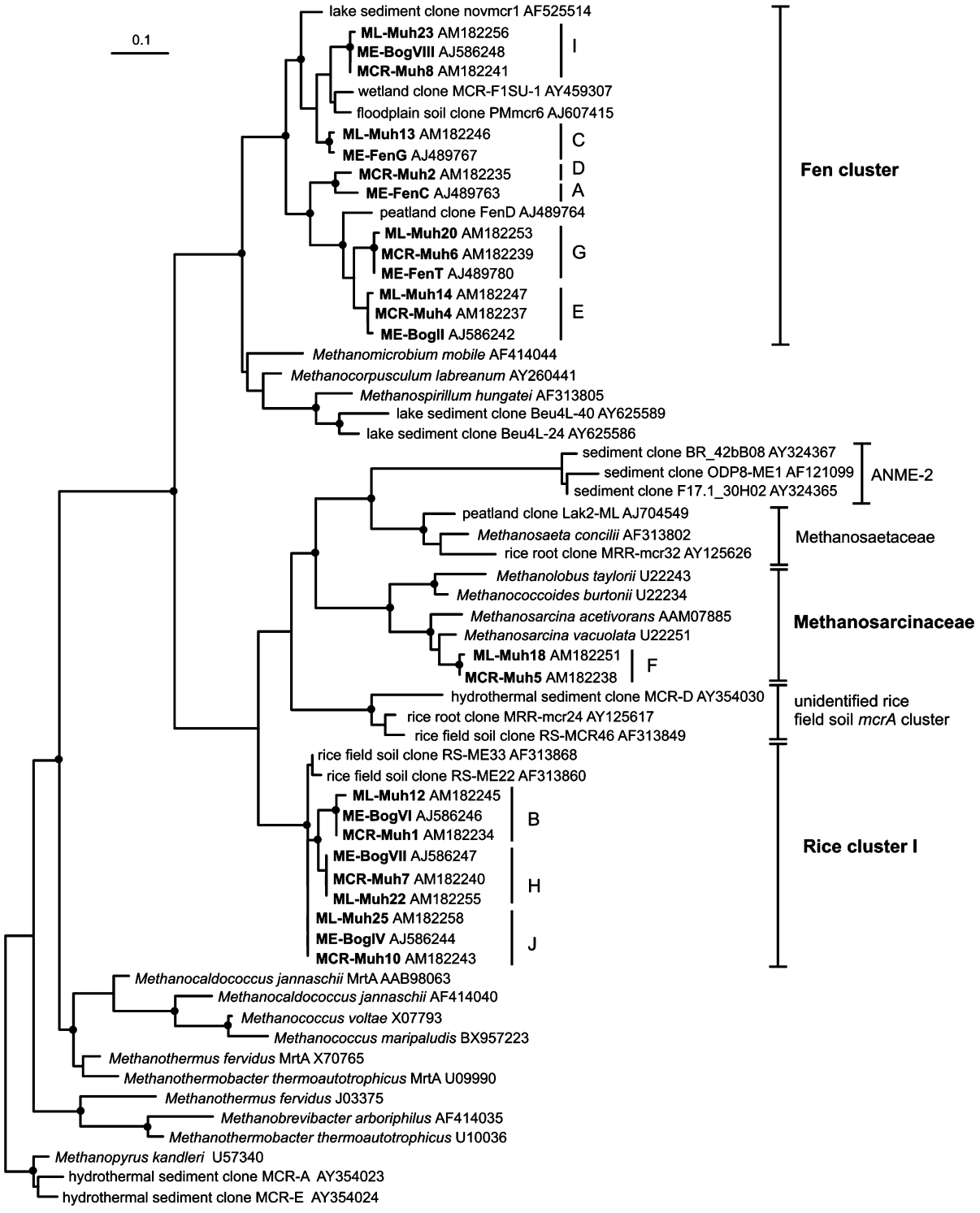

Fig. 4. Phylogenetic analysis of *mcrA* sequences amplified by primers MCR, ME and ML from peat (in bold) and of selected *mcrA* and *mrtA* reference sequences. Letters A–J refer to the OTUs that the sequences represent. The maximum likelihood tree was created from deduced amino acid sequences using *Methanopyrus kandleri* as outgroup. Nodes with bootstrap values of >75% are indicated (•). Scale bar indicates 0.1 changes per amino acid position.

prepared with four annealing temperatures ranging from 49 to 57 °C all displayed major peaks of 219 bp (phylogenetic affiliation unknown) and 250 bp (corresponding to in silico terminal fragments of OTUs B and H, see Fig. 3). The relative areas of these peaks showed no substantial variation (21–25% and 75–79% of total peak area, respectively).

4. Discussion

Detection of microbial diversity in the environment by PCR-based methods depends fundamentally on the primer set's ability to recover an assemblage of sequences that qualitatively (presence or absence of an OTU) and ideally also quantita-

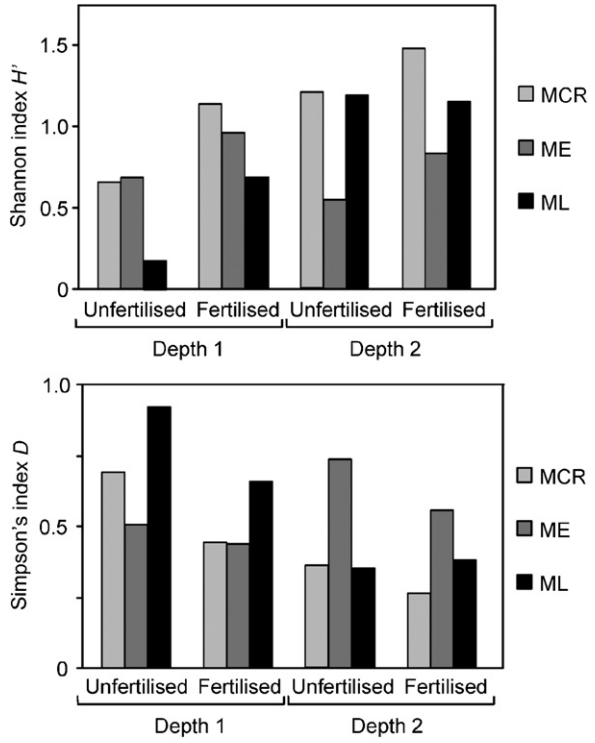

Fig. 5. Diversity of *mcrA* communities at two peat depths detected by primer pairs MCR, ME and ML. Shannon diversity index and Simpson's index were calculated from OTU distributions in clone libraries.

tively (relative abundance of an OTU) represents the natural community. The comparison of three different *mcrA* primer sets revealed that the primers differed substantially in quantitative amplification. The qualitative methanogen community composition, on the other hand, was not strongly dependent on the primer pair: All primers recovered the same set of dominant OTUs belonging to the Fen cluster and the Rice cluster I, two novel methanogen groups previously detected in peatlands [4,7,15,25]. All primers also showed a distinct change in methanogen communities with peat depth and smaller differences with ash amendment, supporting the results of an earlier study conducted with the ME primers [13]. In that previous work, a small group of closely related Fen cluster sequences corresponding to OTUs E and G here were found almost exclusively in ash-amended plots. The occurrence of these Fen cluster OTUs became more evident when the results of three primer sets were considered, giving additional support to the contribution of OTU E. Other differences between unfertilised and fertilised plots were not supported by all primers. Analysis with the ML and MCR sets also confirmed the low *mcrA* diversity previously detected with the ME primers by both RFLP analysis of clone libraries and DGGE. Neither ML nor MCR consistently detected higher OTU richness or diversity, with the possible exception of two MCR libraries that may have contained additional OTUs (Fig. 2). The low diversity was not unexpected because the studied peatland is nutrient-poor, has been drained by ditching, and showed low CH_4 production potential [13]. Low *mcrA* diversity has also been detected in other northern peatlands [25,36].

The amplification ranges of the primers were surprisingly similar, and the few minor OTUs that were only detected by some of the primers did not greatly affect the general community composition. In some cases the slight differences in detected OTUs may reside in the amounts of clones analysed and not in the inability of the primers to detect the OTU. For instance, *Methanosarcinaceae* sequences were not recovered with the ME primers, but in the previous study of the same site a small number of *Methanosarcinaceae* clones were retrieved using these primers [13]. The ME set has also retrieved *Methanosarcinaceae* in several other studies [24,30,40]. On the other hand, when a peat sample from an oligotrophic peatland was examined using both the ME primers and a methanogen-targeting 16S gene primer pair, the 16S primers detected *Methanosarcinaceae* but the ME primers did not [14,15]. The ME set might therefore possibly discriminate against some *Methanosarcinaceae* members in addition to its previously reported inability to detect the other *Methanosarcinales* family *Methanosaetaceae* [1,30]. The ME set also failed to detect an unidentified rice field soil *mcrA* cluster and *Methanobacteriaceae mrtA* [30,39]. As the ML and MCR primers have been shown to amplify all these groups and a broad selection of other taxa [8,10,11,26,30,33,39], they have a wider amplification range than the ME primers. Yet ME produces considerably longer amplicons than the ML and MCR sets and thus provides more phylogenetic information. The ME set would therefore still be a good choice for *mcrA* amplification when detection of the groups mentioned above is not relevant.

The dissimilar OTU ratios and diversity estimates (Figs. 3 and 5) that the three primer sets produced implied, as suggested previously [8,30,32,33], that at least some of the sets provided a quantitatively inaccurate community structure. A well-recognised limitation of PCR-based methods is that amplification does not always preserve the ratios of different templates due to biases such as PCR selection [41,50,52,53]. Selective amplification particularly affects PCR with degenerate primers. The primer variants exhibit variable binding affinities, leading for instance to preferential amplification of templates with a GC-rich primer binding site [41,53]. Conversely, mismatches between primers and a template can result in low amplification efficiency and underrepresentation of the template sequence. Shortage of *mcrA* sequence data unfortunately hinders in silico analysis of primer binding sites, and possible primer-specific primer-template mismatches can mostly not be determined. The sequence comparisons that were possible revealed, surprisingly, that the MLf primer had five to six mismatches against the Rice cluster I sequences retrieved by the ME and MCR primers, but Rice cluster I OTUs were not underrepresented in the ML libraries.

Biased amplification by the mechanisms discussed above could have affected the two depths differently due to their distinct OTU compositions, and might explain the greater primer-dependent variation in community structure at depth 1 (Fig. 3). Another form of PCR bias, template reannealing, may occur during PCR when a high concentration of a product has accumulated and similar products and templates reanneal to each other, inhibiting primer binding and further amplification of a

product. It has been proposed to depend on the primer pair and could occur here because it would particularly affect template mixtures with low sequence diversity [50]. Although we found no substantial differences in diversity between the two depths (Fig. 5), depth 1 had lower OTU richness (five OTUs in total against eight at depth 2). Another possible explanation is the high degeneracy of the MCR primers, which produced an aberrant community structure for depth 1. A low annealing temperature was used in PCR with these primers to counter the low product yield for depth 1, and amplicon ratios detected with the MCR primers have been demonstrated to change depending on the annealing temperature of PCR [32]. The temperature influences binding affinities of different variants of degenerate primers, and with the highest degree of degeneracy of the three sets (Table 1) the MCR set may be particularly sensitive to changes in annealing temperature. To assess whether the least degenerate ML primers are similarly susceptible to amplification conditions, we performed initial tests by varying the annealing temperature in PCR. Although no noticeable change in product ratios was observed, the T-RFLP fingerprinting method may not have sufficient resolution to detect finer differences, and further analysis using a different method or sample is required to confirm this preliminary observation.

In conclusion, all the *mcrA* primers were able to differentiate peatland methanogen communities with depth and ash treatment and identified the same taxa with minor differences. The use of three primer sets revealed, however, that each primer pair provided a quantitatively different community structure, emphasising the unreliability of quantitative conclusions based on *mcrA*-PCR for other than comparative purposes. The poor performance of the MCR set, frequently used in studies of rice field methanogens [10,30], demonstrates how the utility of a primer pair strongly depends on the studied environment. The shortcomings in amplification range of the ME primers concerning *Methanosarcinales* left the ML pair of Luton et al. [33] as the best performer in analysis of peatland *mcrA* communities.

Acknowledgements

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