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PLANT MICROBE INTERACTIONS

Diversity and Community Structure of *Archaea* Inhabiting the Rhizoplane of Two Contrasting Plants from an Acidic Bog

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Abstract Plant root exudates increase nutrient availability and influence microbial communities including archaeal members. We examined the archaeal community inhabiting the rhizoplane of two contrasting vascular plants, *Dulichium arundinaceum* and *Sarracenia purpurea*, from an acidic bog in upstate NY. Multiple archaeal 16S rRNA gene libraries showed that methanogenic *Archaea* were dominant in the rhizoplane of both plants. In addition, the community structure (evenness) of the rhizoplane was found markedly different from the bulk peat. The archaeal community in peat from the same site has been found dominated by the E2 group, meanwhile the rhizoplane communities on both plants were co-dominated by *Methanosarcinaceae* (MS), rice cluster (RC)-I, and E2. Complementary T-RFLP analysis confirmed the difference between bulk peat and rhizoplane, and further characterized the dominance pattern of MS, RC-I, and E2. In the rhizoplane, MS was dominant on both plants although as a less variable fraction in *S. purpurea*. RC-I was significantly more abundant than E2 on *S. purpurea*, while the opposite was observed on *D. arundinaceum*, suggesting a plant-specific enrichment. Also, the statistical analyses of T-RFLP data showed that although both plants overlap in their community structure, factors such as plant type, patch location, and time could explain nearly a third of the variability in the dataset. Other factors such as water table, plant replicate, and root depth had a low contribution to the observed variance. The results of this study illustrate the general effects of roots and the specific effects of plant types on their nearby archaeal communities which in bog-inhabiting plants were mainly composed by methanogenic groups.

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Introduction

The surface of plant roots, i.e., the rhizoplane, is traditionally considered an unlikely habitat for active populations of methanogenic *Archaea* because of the diffusion of oxygen (O_2), through root tissues or soil in shallow depths, into the surrounding soil and because methane (CH_4) production is considered to be a strictly anaerobic process. However, several reports indicated that root surfaces and surrounding soils in rice paddies contain active methanogenic communities with higher potential CH_4 production than nearby bulk soil, and that these communities were mainly composed of uncultured archaeal groups [23, 24]. These findings support the importance of root surfaces in CH_4 production from commonly flooded, anaerobic ecosystems with abundant plant coverage such as rice paddies as well as peatlands.

Peatlands store nearly a third of all terrestrial carbon (C) and are known as the largest natural source of CH_4 emissions to the atmosphere [3]. Because of the magnitude of stored C, several studies have examined the methanogens inhabiting bulk peat soil from bogs, the most common type of peatland. These studies have found several novel euryarchaeal groups [7, 14, 22], some of which have been isolated recently [5, 8]. However, the relationship between methanogenic *Archaea* and vascular plants in these sites has not yet been evaluated.

Vascular plants play an important role in CH_4 production in bogs as a source of substrates through decomposition of dead plant material by aerobic and anaerobic bacteria as well as the release of substrate-rich root exudates [3]. The active substrate contribution by roots can be of particular importance for CH_4 production in bogs since bogs are reported as likely substrate-limited systems. This view is supported by studies that manipulated photosynthesis rates (C assimilation) of vascular plants from peatlands and found that the allocation of recently fixed C to the roots of certain species affected substrate quality and CH_4 formation [30, 37]. These reports also showed that there is a stronger substrate-based coupling of root surface and methanogens in oligotrophic (bog) than in minerotrophic (fen) sites [30], and that different plant types led to different rates of acetate formation [37]. These studies did not assess the composition of the methanogenic communities inhabiting the root surfaces of the different plants or the same plants under different environmental regimes. A community assessment using molecular markers, such as the 16S rRNA gene, can provide insights for identifying associations of methanogens with plants and plant types.

Thus, given the importance of root surfaces for the CH_4 dynamics in bogs, this study was aimed at characterizing the diversity and composition of the archaeal communities inhabiting the root surface of two dominant but physiologically contrasting vascular plants from an acidic bog (McLean Bog) in upstate New York (NY): *Dulichium arundinaceum* (three-way sedge) and *Sarracenia purpurea* (pitcher plant).

Material and Methods

Study Site and Sampling

The study site is locally known as McLean Bog and is located in Tompkins County, upstate NY, USA (42° 05′N, 75° 00′W). This site is an ombrotrophic bog with near 6 m deep peat and acidic pH (3.6–4.0). *Sphagnum* mosses and vascular plants including *D. arundinaceum, S. purpurea*, and evergreen shrubs from the *Ericaceae* family dominated the vegetation of the site. *Eriophorum vaginatum* ("cotton sedge") was also present although with a sparse distribution. We selected *D. arundinaceum* and *S. purpurea* for this study based on their abundance, different morphology and physiology, and accessibility.

D. arundinaceum and *S. purpurea* rhizoplane samples for clone libraries were collected in October 2003. Additional samplings for T-RFLP analysis were done in August and October 2004, and July 2005. For all rhizoplane samplings, we used the same set of four points that satisfied the following conditions in McLean bog: (a) two points were located in separated sections where the water table (WT) was at peat surface level and the other two were in separated sections with WT near 20–25 cm below the surface, (b) each point held two nearby plant patches with either *D. arundinaceum* or *S. purpurea* but not both (each patch was near 0.5 m in diameter). Each point had a unique location (a, b, c, and d), and the distance between points with the same or different WT level were 15 and 10 m, respectively (Supplemental Figure 1). Two plants of *D. arundinaceum* and *S. purpurea* were taken at each of the four sampling points; thus, a total of 16 plants were collected every sampling time. For T-RFLP analysis, the root of each plant was further subdivided in two subsamples according to their root depth resulting in 32 samples. Samples were retrieved by cutting a block of peat (circa. 10 cm long, 10 cm wide, and 25 cm high) containing the complete root system of an adult plant with some surrounding peat. The peat blocks were transported to the laboratory in polypropylene bags held at 4°C. Upon arrival, roots were immediately separated from the peat material with sterile forceps followed by gentle rinsing with sterile water for 30 s. Rinsing was done twice.

Bulk peat samples were collected within 24 h before or after rhizoplane samplings. Duplicated samples were collected in a transect parallel to the sampled plant patches. Samples were obtained from depths similar to the position of root systems in both plants (15–25 cm below peat surface).

Sample Standardization, Microbial Cell Separation, and DNA Extraction

A linear correlation between root length (centimeter) and root surface (square centimeter) was established for each plant (Fig. 1) using a high-resolution scanner and image analysis with the MacRHIZO software as described previously [16]. After standardization, 25 cm^2 of root surface area was used for subsequent analyses since that area was found to yield enough DNA for consistent polymerase chain reaction (PCR) amplifications for all primers and all samples in the study.

To avoid PCR interference by co-extracted plant DNA (not shown), we adapted a protocol for releasing microbial cells from surfaces and sediments [27] followed by DNA

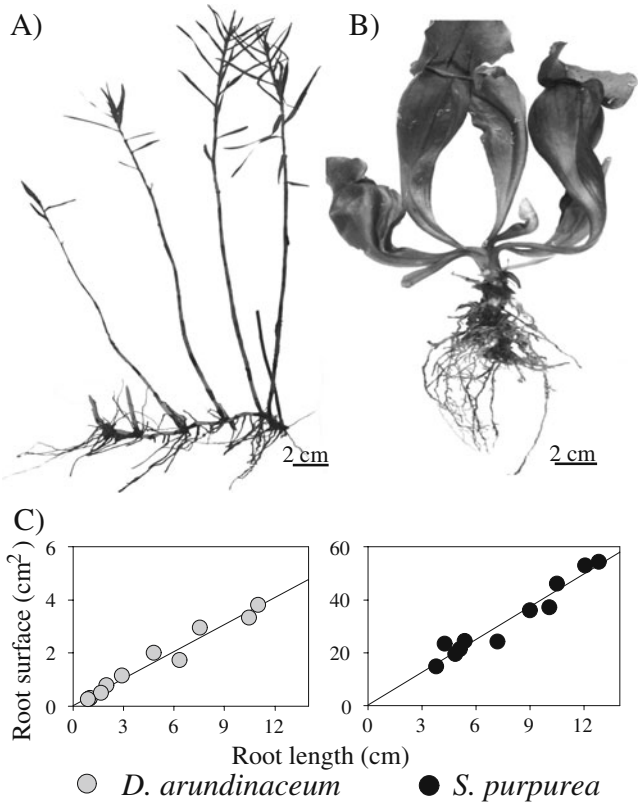

Figure 1 **a** *Dulichium arundinaceum* (three-way sedge) full plant including its rhizome–stolon root type. **b** *Sarracenia purpurea* (pitcher plant) full plant including vertical root type. **c** Plot of samples (*dots*) and linear correlation (*line*) between root length (L) and root surface area (S) calculated for *D. arundinaceum* ($S=(0.331\times L)+0.0075$, $r=0.99$) and *S. purpurea* ($S=(4.111\times L)-0.3959$, $r=0.95$), respectively. Note one order of magnitude difference in *y*-axis

extraction as described: the microbial cells were released from root surfaces into a liquid phase by placing the root fragments in a sterile tube containing 6 mL of sodium pyrophosphate solution (0.01%) and ten glass beads (5 mm, Fisher Scientific). The tubes were sonicated for1 min (Branson 510 Bath Sonicator at 135 W) and vortexed for 1 min, and these were repeated five times. The roots were disposed with sterile forceps, and the solution was pelleted by centrifugation (20 min at 14,000×*g*). The supernatant was disposed and resulting pellets were used for DNA extraction with the Soil Clean DNA® extraction kit (Q-BIOgene). Microscopy revealed that pellets contained mostly microbial cells and a few fragments of plant debris; 0.5 g of bulk peat were directly used for DNA extraction as previously described [7]. Extracted DNA was quantified by image analysis of DNA stained with 0.01% ethidium bromide and compared against calf thymus DNA standards using the Fluor-S™ MultiImager and accompanying Quantity One® software (Bio-Rad).

16S rRNA Gene Amplification, Cloning, and Phylogenetic Analysis

Three archaeal 16S rRNA gene primer sets (Table 1) were used in separate PCR reactions with the following conditions: 5 min at 94°C, followed by 26 cycles of 1 min at 94°C, 1.5 min at corresponding annealing temperature (Table 1) and 72°C for 1.5 min, plus a final step of 6 min at 72°C. Reactants used in PCR reactions were as previously described [7]. PCR products were verified by 1% agarose gel electrophoresis.

Rhizoplane clone libraries were prepared by pooling the PCR products of triplicate reactions, for a plant of each type (*D. arundinaceum* or *S. purpurea*) and for each of the three different primer sets, using the TA Cloning kit® (Invitrogen). This produced six libraries that were subsequently screened for the correct insert size using the m13 primers as indicated by the kit manufacturer. An average of 40 clones per libraries were selected for restriction analysis with a mix of 10 U of HaeIII and HhaI enzymes (New England Biolabs) following the specifications of the manufacturer. Representative clones of unique restriction patterns (72 total) were selected for forward and reverse sequencing using a 3730 xl sequencer at the Cornell Life Sciences Core Facility (Ithaca, NY). Sequences were aligned using the ARB software [26] and the latest release of the Greengenes ARB database (http://greengenes.lbl.gov/). An alignment with long sequences (≥1,000 bases) obtained from the clones, plus nearly complete 16S rRNA gene reference sequences and close relatives in the database was exported from ARB, and a base

Table 1 16S rRNA gene primers used in this study

Name	Estimated coverage	AT (°C)	Sequence (5′ → 3′)	Reference
A751F UA1204R	*Archaea*	53	GCYTAAAGSRICCGTAGC TTMGGGGCATRCIKACCT	[2]
1AF 1100R	Mainly *Euryarchaeota*	55	TCYGKTTGATCCYGSCRGAG TGGGTCTCGCTCGTTG	[15]
89Fb Arc915R	Mainly *Crenarchaeota* and some *Euryarchaeota*	48	ACGGCTCAGTAACRC GTGCTCCCCCGCCAATTCCT	[6]

AT annealing temperature

phylogenetic tree was constructed by means of Bayesian inference using the software MrBayes3 [31]. The base tree was imported back into ARB and shorter sequences (~500 bases long, mostly from the A751-U1204R library) were added without changes in tree topology using the ARB parsimony tool. Overall tree topology was confirmed using the maximum likelihood and neighbor-joining methods, as implemented in ARB, for the base tree as well as the sequences of each library accompanied by reference and close relatives sequences overlapping the amplified fragments. Sequence identity among all samples was calculated on their overlapping positions. To reduce potential grouping artifacts from comparing sequences with little overlap to others (e.g., A751-U1204R with 1Af-1100r and 8Fb-Arc915r libraries), we also required all grouped sequences to share a similar identity to a close reference sequence within a percentage of variability determined by the identity cut off used for the grouping (3% variability in 97% identity cut off).

Sequences have been deposited in the NCBI GenBank database under the following accession numbers: FJ822542-FJ822613.

T-RFLP Analyses

For T-RFLP analysis, root samples were subdivided into two subsamples: high (0–10 cm) and low (10–25 cm), according to their depth with reference to the beginning of the root system. This yielded a total of 32 samples for T-RFLP for each sampling time. After collecting the equivalent of 25 cm^2 from each root subsample, the root fragments were processed to release microbial cells and extract their DNA as described above.

We evaluated two primer sets for T-RFLP analysis (1Af-1100r and 89Fb-Arc915r in Table 1) by labeling the 5′ end of the forward and reverse primers with 6-carboxy-1,4-dichloro-2′,7′-dichloro-fluorescein and carboxifluorescein (6-FAM), respectively. PCR products were digested with different restriction enzymes or a combination of them as predicted by the in silico digestion of sequences. The 1AF-1100R primer set using a 6-FAM-labeled reverse primer and HhaI/Sau96I digestion was selected for further analysis. The T-RFLP protocol has been described in detail previously [7].

Statistical Analyses

Rarefaction analysis of the clone libraries for each separate primer or all libraries combined was done using ARB-generated distance matrices analyzed with the program DOTUR as described previously [35].

We used the T-RFLP data to evaluate the community composition as well as the contribution of the different variables recorded under our sampling protocol as follows. Prior to the statistical analysis, each T-RFLP profile was labeled according to their source (bulk peat or rhizoplane), plant type (*D. arundinaceum* or *S. purpurea*), plant replicate (I or II), patch position (a to d), water table (submerged or not), root depth (high or low), and sampling time (August 2004, October 2004, or July 2005). T-RFLP data quality check and formatting were done using the T-RFLP EXpedited (T-REX) online tool (http://trex.biohpc.org/index.aspx) [9] addressing several aspects of the dataset as further detailed. True T-RFs (peaks) were identified from background noise and pseudo peaks removed. T-RFs were aligned within a 0.5 base size variation range for comparison among samples. All traces were standardized to a total of 100 relative units (cumulative peak area). Traces were formatted into tabulated matrices containing TRF area data and labels for plant type, as well as other spatial and temporal variables. Subsequently, matrices containing TRF area data were evaluated for the contribution of each variable in the community conformation using the Additive Main Effects and Multiplicative Interaction (AMMI) model [10], also available in the T-REX tool. Data matrices were exported for evaluation with Non Metric Multidimensional Scaling (NMDS) plots. NMDS was performed using the meta.MDS function of the vegan package (http://vegan.r-forge.r-project.org/) as implemented in the R software with the following settings: Jaccard distance matrix and root square auto transformation.

Results

Sampling the Rhizoplane Community

The root morphology of *S. purpurea* as compared with *D. arundinaceum* is markedly different (Fig. 1) and required a sampling technique that would allow us to compare the same total area of the rhizoplane. For this, we established the linear correlation between root length and root surface area for each plant (r=0.99 and 0.95, Fig. 1). These correlations were used to estimate a similar rhizoplane area (25 cm^2) for each plant type. We observed a nearly one order of magnitude difference in the total root length needed for sampling the equivalent root surface between plants (*D. arundinaceum* with thinner roots than *S. purpurea*). Nevertheless, the selected sample size (root area) yielded roughly similar amounts of DNA from both plants with extracted DNA concentrations ranging between 10–80 ng uL^{-1} among all samples.

Archaeal Community Composition in Rhizoplane

The analysis of multiple 16S rRNA gene libraries revealed a diverse archaeal community associated with the rhizo-

plane of *D. arundinaceum* and *S. purpurea*. The majority of phylotypes were related to euryarchaeal groups that have been reported previously from peat soils and other methanogenic environments (Fig. 2). Phylotypes were associated with well-known methanogenic groups, including *Methanosarcinaceae* (MS), *Methanosaetaceae* (MT), and *Methanobacteriaceae* (MB), as well as recently cultured groups, such as group E2′ (E2) and group E1′ (E1) in the order *Methanomicrobiales*, and rice cluster-I (RC-I) in the novel order Methanocellales [33]. We also identified several phylotypes associated with as yet uncultured euryarchaeal and crenarchaeal groups, including RC-III, group 1.3b and 1.1c (Fig. 2).

The distribution of all clones showed that three euryarchaeal groups dominated the rhizoplane of both plants: MS, RC-I, and E2 (Fig. 3). These three groups represented the biggest community fraction independent of the primer used in the construction of the libraries (Fig. 4). Taking the six different libraries together, these three groups contributed 70–78% of all clones (Fig. 3).

When considering a finer level of resolution for the grouping of phylotypes estimated at 97% sequence identity, some plant-specific differences were evident for MS and RC-III (Fig. 3). MS was represented by a larger fraction of *S. purpurea* clones due to the higher presence of a MS.2 subcluster (14% vs 3% in *D. arundinaceum*), while the MS.1 subcluster was present on both plants at similar proportions. The RC-III.1 showed an opposite distribution representing 23% of the clones from *D. arundinaceum* and only 1% in those from *S. purpurea*. MB and group G1.1c were only detected in *S. purpurea* samples suggesting a potential plant-specific pattern. The crenarchaeal groups (G1.3b and G1.1c) represented only a small fraction of the rhizoplane community in both plants.

Analysis of Diversity Coverage by Different Primers

In this study, none of the three primer sets (Table 1) recovered sequences from all detected archaeal groups; instead, there was a different coverage for each primer set (Table 1). The 1AF-1100R primers recovered only euryarchaeal sequences from all euryarchaeal groups observed in this study except RC-III and the MS.1 subcluster. The 89Fb-915R primer pair had the broadest coverage and retrieved euryarchaeal as well as crenarchaeal sequences, although it did not recover members of the MT or RC-III.1. Primers A751-1204 recovered euryarchaeal and crenarchaeal groups as well, but missed several groups including MT, MB, MS.2, RC-I.1, RC-III.2, and group G1.1c.

The rarefaction analyses of the combined libraries (clones from all three primers analyzed together) indicated that the overall survey reached a near full coverage plateau at 97% sequence similarity (Fig. 5). When analyzing libraries by different primer sets, the 89Fb-915R pair had the most similar plot to that obtained from combining all libraries at 97% similarity, followed by 1A-1100R and then A751-1204. Interestingly, the A751-1204 rarefaction plot was the closest to reaching complete saturation (asymptotic plateau). Thus, A751-1204 had the lowest coverage of the groups recovered from the rhizoplane samples. Similar trends were observed at 99% identity with a higher number of groups and less asymptotically shaped rarefaction curves, indicating that at this level of phylogenetic resolution any individual primer was not close to full saturation.

T-RFLP Community Analysis

The labeling of the 1100R primer combined with a double digestion with the HhaI/Sau96I restriction enzymes resolved most of the detected euryarchaeal groups in which each T-RF could be associated with a single group. In contrast, the 89Fb-Arc915R set had a low resolution at the phylogenetic level of order or below; T-RFs were not associated with a single group, but rather with three or more (data not shown). Then, the 1Af-1100R set was selected for further analysis considering these results in addition to previous results where the association of T-RF with euryarchaeal groups was confirmed by digestion of multiple clones and pure cultures [7]

In agreement with the results from clone libraries, the analysis of standardized T-RFLP profiles showed that MS, RC-I, and E2 made up the greatest proportion of the archaeal community in the rhizoplane of both plants (Fig. 6a). Bulk peat community had a different structure than the rhizoplane of both plants being highly dominated by E2. Additional patterns were observed when analyzing the profiles separated by plant. MS was the most dominant member in the rhizoplane of both plants (39–42%), and was less variable fraction in *S. purpurea* than in *D. arundinaceum* roots. Also, the distributions of RC-I and E2 were clearly different between the two plant species. The average RC-I fraction was twice as abundant on *S. purpurea* (27%) than on *D. arundinaceum* (13%), while the opposite was observed for E2, which was more than twice as abundant on *D. arundinaceum* (28%) than on *S. purpurea* (11%). Similar distributions were observed for all three sampling times (data not shown).

The complementary NMDS and AMMI analyses were used to evaluate possible sources or associations of the variability in community structure with plant type and spatial or temporal components. NMDS ordination analysis of each sampling time showed that, although samples did not fully separate into isolated groups, they still formed overlapping clusters according to plant (*D. arundinaceum* and *S. purpurea*) and patch position (a to d) as shown in subpanel b of Fig. 6. The NMDS analysis also showed that the distribution of the T-RFs in the NMDS space formed

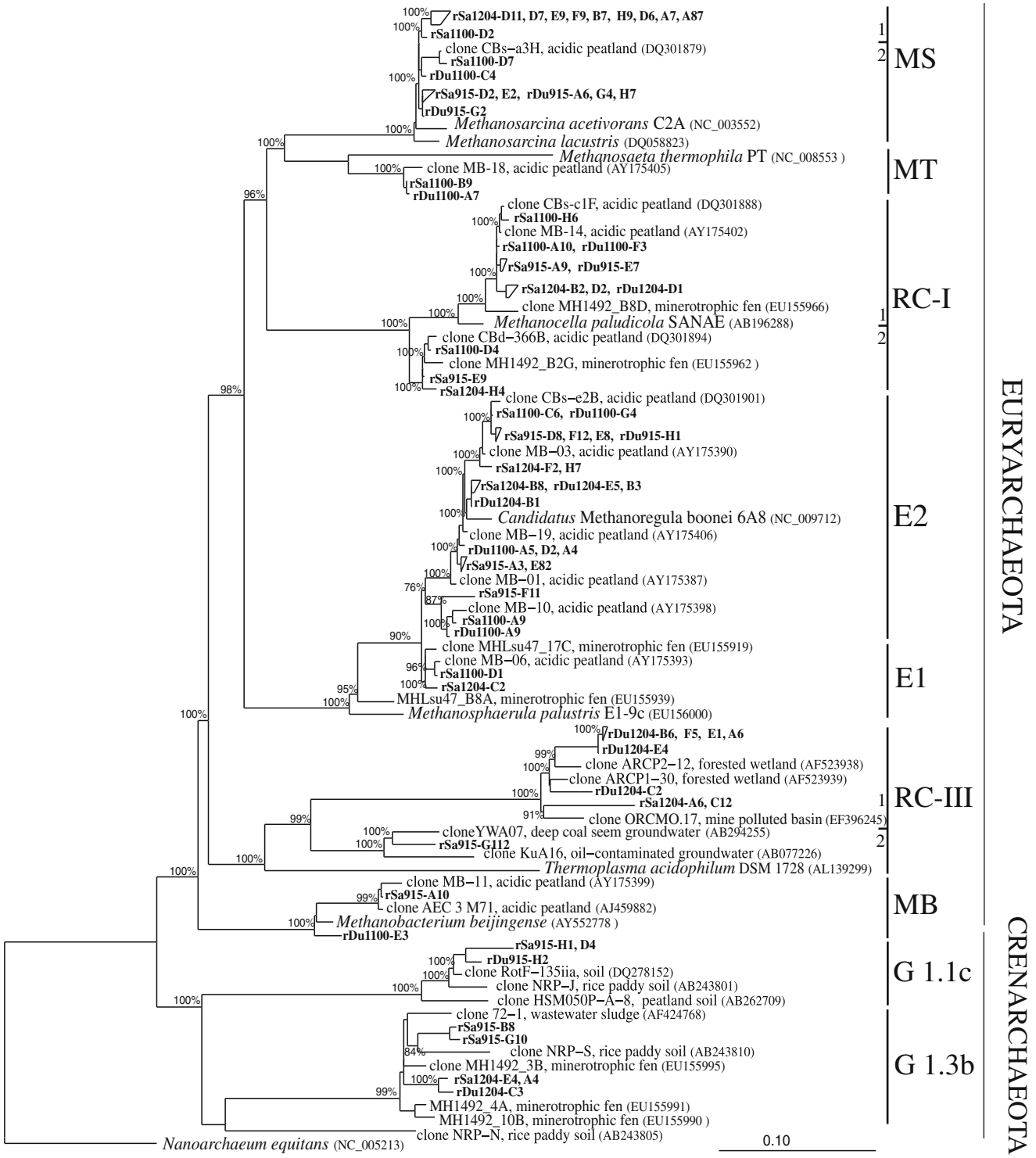

Figure 2 Phylogenetic tree of representative 16S rRNA gene sequences. *MS Methanosarcinaceae*, *MT Methanosaetaceae*, *RC-I* rice cluster I, *E2* group E2, *E1* group E1 (E1 and E2 are novel members in the order *Methanomicrobiales*), *RC-III* rice Cluster III, *MB Methanobacteriacea*, *G 1.3b* Group G1.3b, and *G1.1c* Group G1.1c. *1* and *2* represent subclusters at 97% sequence similarity within a group. Clone names (*bold letters*) include initials for the rhizoplane (r) and plant: *D. arundinaceum* (Du) and *S. purpurea* (Sa), as well as the primer used for generating the library: 1AF-1100R (1,100), 89Fb-915Arc (915), and A751F-UA1204R (1,204). Posterior probability as calculated by Bayesian inference is indicated next to their corresponding tree node

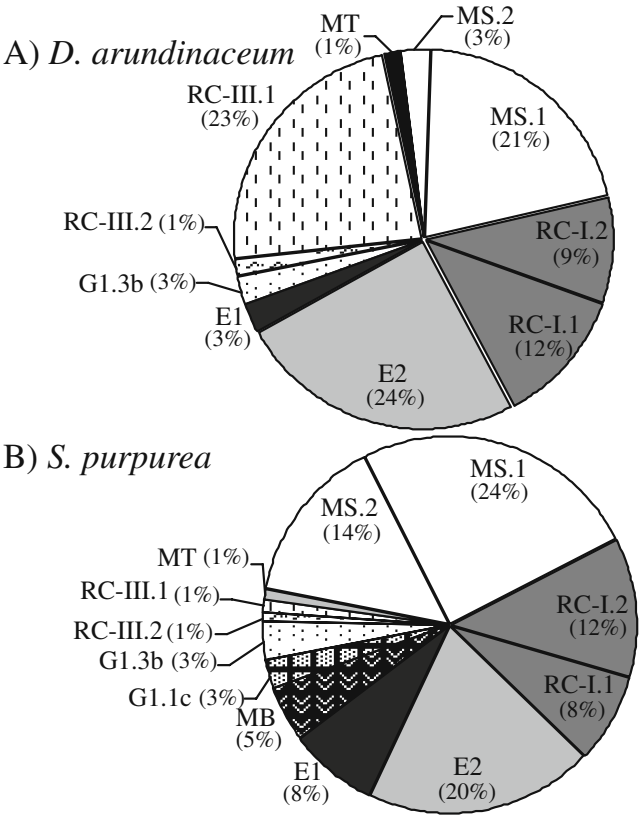

Figure 3 Clone distribution of combined 16S rRNA gene libraries from *D. arundinaceum* and *S. purpurea* rhizoplane samples. Data summarizes the accumulated clone distribution of a total of six clone libraries (225 total clones) as described in the methods section. The legend of the detected archaeal groups is the same as that given in Fig. 2

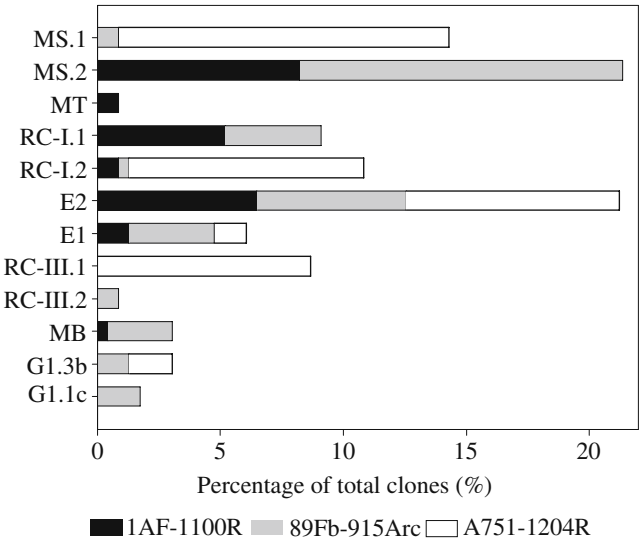

Figure 4 Archaeal groups and the percentage of clones (225 total clones) recovered with each primer set used in this study. Sequences from *D. arundinaceum* and *S. purpurea* samples were combined and distributed by each primer set. The legend of the detected archaeal groups is the same as that given in Fig. 2

the following groups: E1 closest to E2, RC-II closest to an RC-I, MS closest to MT and the second RC-I, and MB/RC-III as single member group. This group distribution is similar to the phylogenetic relationships among the euryarchaeal groups represented by the T-RFs. When the dataset included multiple sampling times, the NMDS analysis showed similar patterns (plant type and patch clustered samples) as well as sampling time as an additional source of clustering with more overlapping or less delimited groups (Supplemental Figure 2). Other variables such as water table height, root depth and plant replicate did not exhibit a clustered distribution (not shown).

Similar clustering was also observed in the AMMI plots (not shown). In addition, the analysis of the partitioning of the variation in the dataset, as done by the AMMI model, allowed quantifying the contribution of the T-RFs (main effects) and the response to each variable or combination of variables (interaction effects). Among the interaction effects, plant type explained the highest percent of variation (12%), closely followed by patch position (11%) and time (4.8%), as shown in Table 2. The remaining variables had an individual contribution of less than 2% which was similar to or smaller than the noise fraction (Table 2). Plant type, patch position, and time were responsible for nearly 30% of the total dataset variability, and this fraction increased to 34% when all the variables recorded in the study were included. The distribution of interaction effects within the AMMI analysis results are in agreement with the clustering observed in the NMDS analysis.

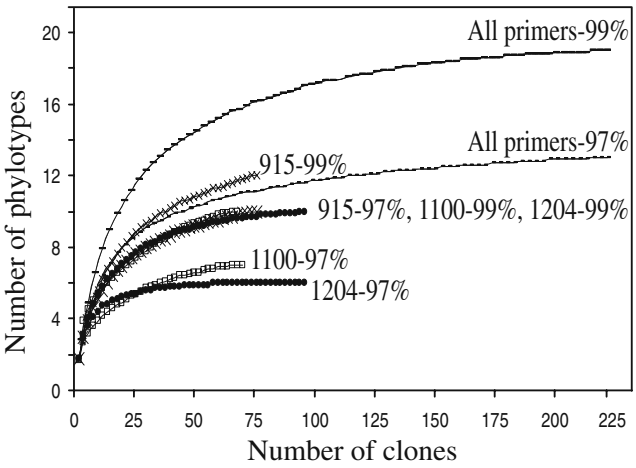

Figure 5 Rarefaction analysis at 97% and 99% similarity of all 16S rRNA gene sequences separated by primer set. *Lines* in the plot are labeled with the reverse primer used for generating the library; 1AF-1100R (1,100), 89Fb-915Arc (915), and A751F-UA1204R (1,204), followed by the percent of similarity (97% or 99%) used in the rarefaction analysis

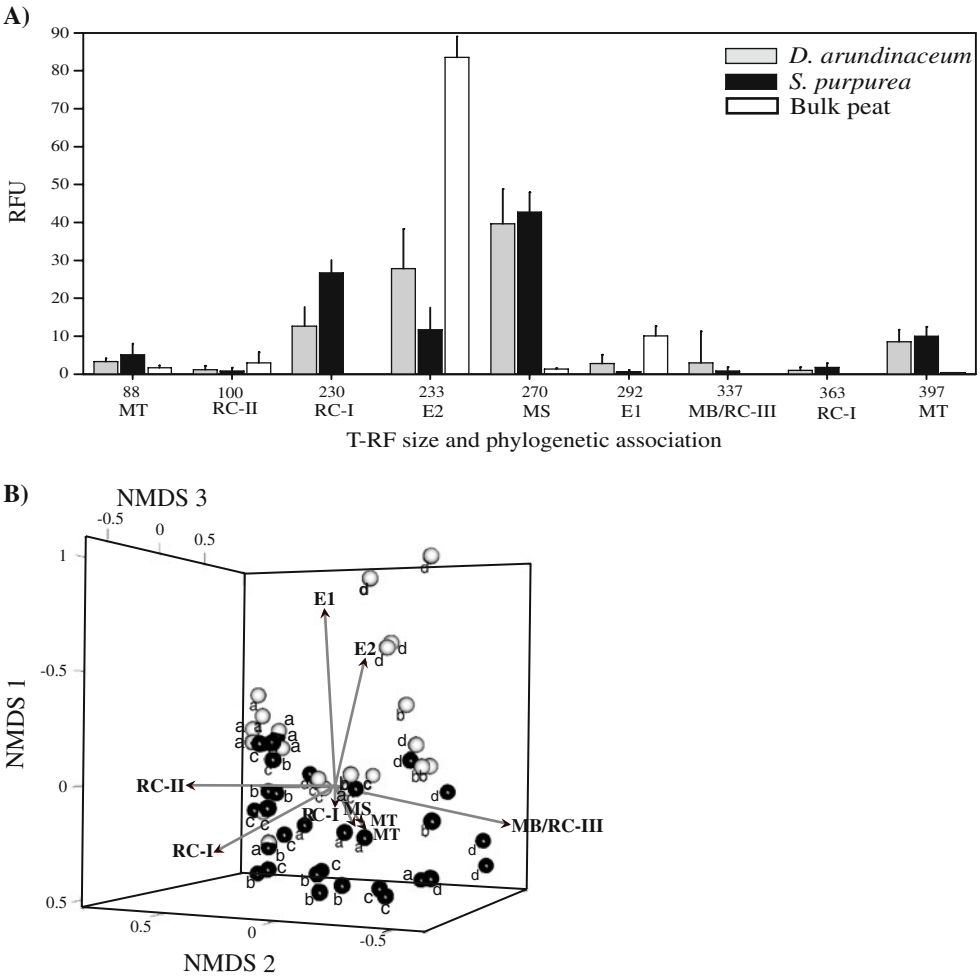

Figure 6 **a** Analysis of the average values and standard deviations of T-RF from samples collected in August 2004. T-RFs were standardized by peak area. *RFU* relative fluorescent units. **b** NMDS plot of rhizoplane samples only. Samples were labeled by bulk peat (*white bars* in **a**); plant: *D. arundinaceum* (*gray bars* and *spheres* in **a** and **b**, respectively) and *S. purpurea* (*black bars* and *spheres* in **a** and **b**, respectively); and by location of the patch sampled (*a*, *b*, *c*, and *d* in **b**). Rhizoplane analyses included 52 of 64 of samples: 12 samples were excluded after evaluating the quality of T-RFLP traces with the T-REX tool. After similar T-REX evaluation, seven of eight bulk peat samples were used in section A. The legend of archaeal groups associated with the different T-RFs is the same as that given in Fig. 2

Discussion

Root exudates stimulate microbial heterotrophic activity [28] and thus increase microbial O_2 consumption in the rhizoplane. Along with root respiration, these heterotrophic activities lead to a mosaic of microaerophilic and near-anoxic conditions in the rhizoplane and rhizosphere [17, 40]. Additionally, high heterotrophy also contributes to increased accumulation of metabolic byproducts such as acetate and H_2/CO_2 [28], two key methanogenic substrates. Our results

Table 2 AMMI analysis results from the August 2004 sampling (n=52) as well as the combined data from the tree sampling times (n=125)

Only T-RFLP traces that passed quality tests including a minimum of four out of nine T-RF (implemented in T-rex tool) were used in the analysis. All values in the table are expressed as percentages of variability (%). Main effects correspond to the variability associable to the populations (T-RFs) while Interaction effects correspond to the variability associable to environmental variables (Pattern) or random sample variability (Noise)

	August 2004 samples			All samples		
	Main effects	Interaction effects		Main effects	Interaction effects	
	T-RFs	Pattern	Noise	T-RFs	Pattern	Noise
Single variables						
Plant (Pl)	86.5	12.7	0.9	87.9	11.8	0.3
Patch (Pt)	86.2	11.1	2.7	90	8.7	1.3
Time (T)	–	–	–	94.5	4.8	0.7
Water table	96.8	1.9	1.3	96.8	2.7	0.5
Plant rep	97.6	1.1	1.29	97.8	1.6	0.7
Root depth	99.7	0	0.33	99.1	0.5	0.4
Combined variables						
All	60.62	34.1	5.22	60.9	34.5	4.6
Pl, Pt, T excluded	–	–	–	92.4	5.1	2.5

indicate that methanogens associate with root surfaces. The archaeal community in the rhizoplane of two plants sampled from the McLean bog was mainly composed of methane-producing *Archaea* (Fig. 2). Previous 16S rRNA gene surveys of bulk peat from the same and another nearby bog revealed a similar archaeal richness with groups such as MS, MT, RC-I, E2, E1, and MB being present across sites [7]. However, the rhizoplane community is markedly different from bulk peat when considering the community structure (evenness) as represented by the distribution of clones (Fig. 3) and T-RFLP data (Fig. 6a). The *Archaea* in the rhizoplane of both plants was dominated by MS, RC-I, and E2, and formed a more even community than the E2-dominated one from the bulk peat.

Only observations of rhizoplane and rhizosphere samples from rice paddies have shown some similarity to our findings in the rhizoplane of bog-inhabiting plants. Rice roots and associated soil also harbor MS and RC-I as their main community members [23]. Indeed, MS and RC-I have been shown as active methanogens in rice roots [24], using acetate and H_2 accumulated in root surfaces from the fermentation of excreted photosynthates as demonstrated in ^{14}C-isotope labeling experiments [11]. *Methanosarcina* is a known acetate-utilizing methanogen also capable of using methylated compounds and in some cases, H_2/CO_2 [4]. Thus, MS abundance in the rhizoplane is supported by its ability to use various byproducts derived from root exudates or decaying roots, its capacity to tolerate low oxygen levels by different morphological and physiological mechanisms [41], and the high availability of acetate around roots. High acetate availability has been shown from root exudates of plants inhabiting oligotrophic peatlands such as *E. vaginatum* (cotton sedge) [32], where acetate was found at one to two order of magnitude higher than any other detected organic acids [32]. High acetate concentrations eliminate the lower-acetate threshold advantage of the only other known acetate-utilizing methanogen, MT [18]. MT was only a small fraction of the methanogenic *Archaea* found on the roots of *D. arundinaceum* or *S. purpurea* in our study, and has also been found as a smaller fraction on rice rhizoplane than surrounding soils [23]. In peatlands, MT has only been found to dominate over MS in the bulk peat of minerotrophic fens [8, 14], which usually have low acetate concentrations in soil pore water.

RC-I was the next most abundant member of the rhizoplane community and has also been found in significant proportions on rice roots and paddy bulk soil samples (20 to 50% T-RF fraction), as well as in densely sedge-inhabited minerotrophic fen peat (up to 14% in clone libraries) [8, 23]. RC-I has been detected in bulk peat from bogs only as a small fraction [7], suggesting its better performance in labile-substrate-rich habitats, such as root surfaces. The release of complex substrates and a broad suite of active bacterial degraders [25] makes the rhizoplane an excellent place for syntrophic interactions. Interestingly, the isolation of a RC-I member, strain SANAE, from a rice paddy was only possible when using a syntrophic-coculture technique that maintained low levels H_2 in the medium [34]. Higher H_2 affinity and likely adaptation to low-H_2 environments of RC-I members is supported by the observed inhibition of rice root-associated RC-I when incubated under high H_2 concentrations [24]. Genomic data suggest that RC-I is also well adapted to low oxygen conditions, such as those around roots, by containing multiple oxygen-insensitive and oxygen-detoxifying enzymes [13]. Together, these observations suggest that the RC-I group is a root-associated methanogen of importance when considering root-controlled methane production. The RC-I group has been classified recently as the order *Methanocellales* [33].

The E2 group also represented a significant fraction of the *Archaea* in the rhizoplane of both plants. This novel group from the order *Methanomicrobiales* has been found to be dominant (up to 90% of the total *Archaea*) in bulk peat from shallow layers in MacLean bog [7]. Also, E2 has been seen as a significant or dominant fraction in the archaeal community in the peat of other oligotrophic peatlands [14, 36], suggesting its adaptation to low-nutrient environments. In fact, the first strain of this group, "*Candidatus* Methanoregula boonei", was isolated using a low-nutrient low-ionic-strength medium [5]. The H_2/CO_2 utilizing "*M. boonei*", requires low amounts of acetate for growth [5] and cultures appear to be highly sensitive to oxygen. Then, the following hypotheses can be considered to explain the significant presence of E2 in the rhizoplane despite its apparent oligotrophic preference and O_2 sensitivity. Gene surveys have shown that the E2 group is diverse and composed of several subclusters (H. Cadillo-Quiroz; unpublished), some of which could possess better traits for persisting in the rhizoplane than the "*M. boonei*" isolate. Alternatively, the high numbers of E2 in the bulk peat could passively supply large numbers of E2 to the rhizoplanes of both plants.

Several other archaeal groups, including presumably non-methanogenic *Archaea*, formed smaller fractions of the rhizoplane community of either or both plants. These groups have been reported to be abundant community members in places with likely higher nutrient availability than peat from bogs. For instance, MB has been observed as a highly significant member of river sediment communities [19], RC-III was enriched from rice paddies under rich heterotrophic conditions [20], and E1 was one of the dominant members in a minerotrophic fen [8]. The crenarchaeal groups detected in this study, group G1.3b and G1.1c, have been observed as a major fraction of a grassland rhizosphere [29] and acidic forest soil communities [21]. Root exudates and subsequent decomposition could provide

these groups with suitable substrates for heterotrophic or heterotrophic-related growth; however, their potential association with root surfaces has not been elucidated.

T-RFLP analysis supported the findings from the clone library analyses. MS, RC-I, and E2 were the dominant groups on the rhizoplane contrary to the almost exclusively E2-dominated bulk peat (Fig. 6a). Additionally, the inclusion of multiple T-RFLP processed samples allowed the identification of other patterns relating these methanogens to environmental conditions (Fig. 6; Table 2). In the T-RFLP profiles, RC-I was a larger fraction on the roots of *S. purpurea* than on *D. arundinaceum*. A higher average and less variability could suggest that MS is more important in *S. purpurea* but the high variability of MS in *D. arundinaceum* made the differences between plant types not significant in our data set. In fact, *D. arundinaceum* supported a more variable community containing MS and E2 as highly variable fractions. *S. purpurea* ("pitcher plant") is a carnivorous plant whose leaves (pitchers) are specialized for capturing insect prey followed by fast nutrient uptake, as well as more efficient photosynthesis [12]. In addition, *S. purpurea* is a long-lived perennial plant (>50 years) whose roots are slowly but constantly renewed. *S. purpurea*'s efficiency in producing photosynthates and in nutrient assimilation could influence the quality of their root exudates. Meanwhile, *D. arundinaceum* ("three-way sedge"), as many other sedges in peatlands, is a seasonal plant [1] whose secondary roots senesce and get reabsorbed or degraded at the end of each growing season. Then it is reasonable to hypothesize that both plants could provide differential conditions, which in turn could affect the structure of the surrounding archaeal community.

The apparent influence of plant type on the archaeal community was supported by the significant amount of profile variability (12%) associated with this component in the AMMI analysis (Table 2). The use and suitability of the AMMI analysis for T-RFLP data has been discussed in detail elsewhere [10] but it is worth mentioning its capacity to focus the analysis in the response of the T-RFs to imposed treatments or environmental variables. Patch location and time contributed 11% and 4.8% of the observed variance and were the next most important variables, while variables such as water table and root depth did not account for significant differences. This hierarchy in the variables we recorded is congruent with other findings where CH_4 production has been seen dependent on plant type, mosaic spatial distribution of substrates and conditions for plants and microbes, as well as seasonal changes affecting the vegetation [38, 39]

Interestingly, other variables found important in bulk peat, including water table and depth, had only minor contributions to the overall variability according to the AMMI analysis. This could be explained by the common location of *D. arundinaceum* roots under the water table via large stems in raised sections, and that *S. purpurea* formed tight colonies in usually water-filled depressions. However, when interpreting the importance each variable using the T-RFLP data from this study, it is important to keep in mind the rough level of T-RF resolution, in our case close to the family taxonomic level. Although several T-RFLP settings were tested seeking a more phylogenetically resolved association for each T-RF, the high conservation of 16S rRNA sequences constrained the final results. Low contributing variables at the family level could drive differences at finer phylogenetic levels such as genus or species.

Finally, in terms of 16S rRNA gene library coverage, the multiple primer approach allowed for a more detailed survey of the archaeal groups. Rarefaction analysis at 97% sequence identity indicated the survey was close to reaching sampling saturation (Fig. 5). None of the primer sets used in this study (Table 1) recovered sequences from all groups detected (Fig. 4). Surprisingly, the A751-UA1240R which was designed to target all *Archaea* [2], did not recover sequences of as many groups as the primers designed for mainly *Euryarchaea* [15] or *Crenarchaea* [6]. Based on these results we suggest a multi primer approach separately targeting euryarchaeal and crenarchaeal groups as preferred over a single 'universal' primer set.

In summary, in our knowledge this is the first report for peatlands showing that the rhizoplane archaeal community has a different structure than in peat soils- each location having different dominant members. Also, the roots of the plants examined in this study appear to exert different effects on their surrounding archaeal community colonizing the root surfaces. This is of importance for considering the effects of vegetation change in peatlands as well as the expansion of vegetation cover. Thus, the archaeal community composition and structure in the rhizoplane should be considered when evaluating methanogenesis and plant-controlled CH_4 emissions from peatlands.

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