

A wide variety of putative extremophiles and large beta-diversity at the Mars Desert Research Station (Utah)

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Abstract: Humankind's innate curiosity makes us wonder whether life is or was present on other planetary bodies such as Mars. The EuroGeoMars 2009 campaign was organized at the Mars Desert Research Station (MDRS) to perform multidisciplinary astrobiology research. MDRS in southeast Utah is situated in a cold arid desert with mineralogy and erosion processes comparable to those on Mars. Insight into the microbial community composition of this terrestrial Mars analogue provides essential information for the search for life on Mars: including sampling and life detection methodology optimization and what kind of organisms to expect. Soil samples were collected from different locations. Culture-independent molecular analyses directed at ribosomal RNA genes revealed the presence of all three domains of life (Archaea, Bacteria and Eukarya), but these were not detected in all samples. Spiking experiments revealed that this appears to relate to low DNA recovery, due to adsorption or degradation. Bacteria were most frequently detected and showed high alpha- and beta-diversity. Members of the *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Gemmatimonadetes* phyla were found in the majority of samples. Archaea alpha- and beta-diversity was very low. For Eukarya, a diverse range of organisms was identified, such as fungi, green algae and several phyla of Protozoa. Phylogenetic analysis revealed an extraordinary variety of putative extremophiles, mainly Bacteria but also Archaea and Eukarya. These comprised radioresistant, endolithic, chasmolithic, xerophilic, hypolithic, thermophilic, thermoacidophilic, psychrophilic, halophilic, haloalkaliphilic and alkaliphilic micro-organisms. Overall, our data revealed large difference in occurrence and diversity over short distances, indicating the need for high-sampling frequency at similar sites. DNA extraction methods need to be optimized to improve extraction efficiencies.

Received 17 November 2010, accepted 28 December 2010, first published online 4 February 2011

Key words: Mars analogue, extremophiles, beta-diversity, desert, DNA recovery.

Introduction

The study of terrestrial extreme environments has raised expectations to find life outside our planet. Today, we know that not only is life present in the most unimaginable extreme environments on Earth, but it can also be diverse (e.g. Amaral Zettler *et al.* 2002). Extremophiles are present in all three domains of life (Archaea, Bacteria and Eukarya) (Stan-Lotter 2007).

Mars has been selected as a prime target for the search for life, with robotic exploratory missions such as sample return being the major goal and ultimately paving the way to human exploration (Worms *et al.* 2009). If life currently exists on Mars, it had to adapt to extreme conditions: very low temperatures (average daily temperature -60°C), dryness, high UV

radiation, strongly oxidizing conditions and a thin carbon-rich atmosphere ($\sim 95\% \text{CO}_2$) (Grady 2007).

On Earth, particular deserts reveal extreme Mars-like surface characteristics. Examples include the cold Antarctic desert McMurdo Dry Valley which is considered to have the coldest, driest and oligotrophic soils (Cary *et al.* 2010) and the arid Atacama desert in Chile which has very low levels of organics and soil bacteria (Gómez-Silva *et al.* 2008).

The Mars Desert Research Station (MDRS) in southeast Utah is a Mars analogue field research facility used to prepare human missions to Mars and to study its geology, biology and environmental conditions. Like Mars (Greeley *et al.* 2004), its soils are very dry and subjected to wind erosion (Godfrey 1997). The surface at MDRS is shaped by fluvial erosion resembling Mars' landscape and reveals gullies similar to those

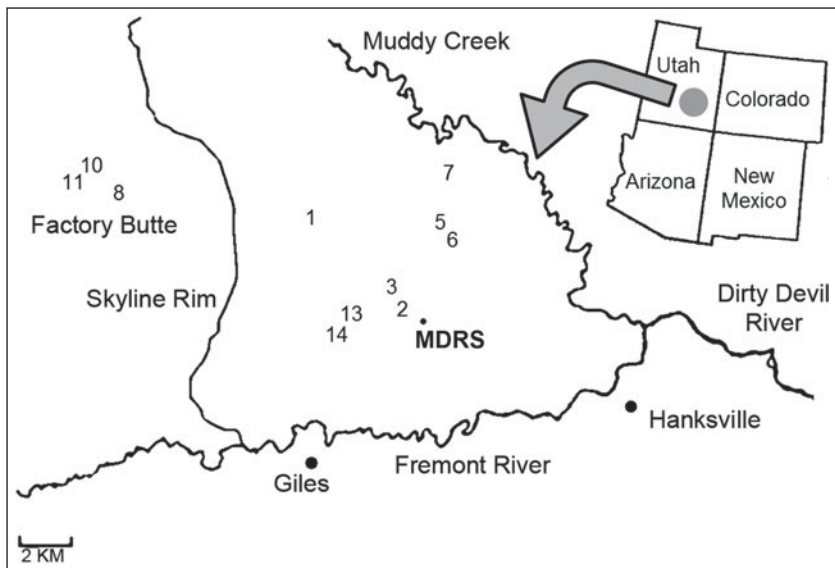


Fig. 1. MDRS desert sampling locations in the southeast area of Utah with USA four corners context map.

found on Mars (Malin & Edgett 2000). MDRS is located in a cold arid desert with an average annual temperature of 12 °C (with –36 and +46 °C as the lowest and highest temperatures recorded; plus daily variations of 20 °C) and 140 mm annual average precipitation, with often months without any precipitation (Godfrey *et al.* 2008).

Of particular interest is that MDRS is a shale desert (Godfrey 1997) and not a sand desert such as those previously studied for their microbiology (e.g. Prestel *et al.* 2008; Gommeaux *et al.* 2010). Mineralogy comparable to Mars is encountered at MDRS. Martian mineralogy consists of silicates (olivine, pyroxene and plagioclase), phyllosilicates (clay minerals such as montmorillonite and nontronite), evaporites (sulfates such as gypsum and jarosite), iron oxides (haematite and magnetite), iron oxyhydroxides (goethite and ferrihydrite) and traces of carbonates (Poulet *et al.* 2005; Chevrier & Mathé 2007; Grady 2007). At MDRS, sedimentary deposits of sands, evaporites and clays are found; gypsum and a variety of clay minerals have been detected (Borst *et al.* 2010; Kotler *et al.* 2011). In addition, MDRS is located in an area characterized by red-coloured hills, soils and sandstones due to the presence of iron oxides (e.g. hematite) (Chan *et al.* 2004; Ormö *et al.* 2004), the same mineral compound that gives Mars' surface the red colour (Catling & Moore 2003).

We conducted a cultivation-independent, rRNA gene-based characterization of all three domains of life at MDRS. We aimed at describing their alpha- and beta-diversity in this Mars analogue. In particular, we wanted to establish in how far the communities in this shale desert differ from those in previously studied sand deserts and how this may relate to the Mars-like physicochemical parameters. This information is deemed important in the search for life on Mars: it will contribute to optimizing sampling strategies and life detection methods, and suggests what type of organisms to expect on Mars.

Material and Methods

Site description and sampling

Soils were sampled nearby the MDRS during the EuroGeoMars campaign (24 January – 1 March 2009) from different locations (Fig. 1; Table 1) (Foing *et al.* 2009; Ehrenfreund *et al.* 2010). MDRS is situated northwest of Hanksville (Utah) at a high altitude, approximately 1314 m. Samples were collected from various geological formations (Table 1). The Morrison Formation belongs to the late Jurassic period (about 208–144 millions of years ago) when a large part of Utah was desertified, causing dune sands, salt and gypsum deposits (Chronic 1990). During this period, thick layers of volcanic ashes were also deposited (Borst *et al.* 2010). Mancos Shale and Dakota Sandstone are from the Cretaceous period (about 144–166 millions years ago) when Utah was occupied by the last invading sea carrying near-shore sandstone plus coal deposits and deep-water shale (Chronic 1990). Physicochemical parameters of the various samples are summarized in Table 1 and were determined by Orzechowska *et al.* (2011), mineralogy analysis was performed by Kotler *et al.* (2011) and amino acids content by Martins *et al.* (2011). Soil samples were sent to VU University Amsterdam for the detailed culture-independent molecular analyses described in this paper. On-site PCR analysis at MDRS is reported in Thiel *et al.* (2011).

Microbial community profiling

DNA was extracted using two DNA isolation kits, in order to account for possible differences between extraction procedures, according to the manufacturer's protocol: FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH; 0.5 g soil sample) and PowerSoil DNA Kit (MO BIO Laboratories, Solana Beach, CA, USA; 0.25 g soil sample). The amount of double-stranded DNA (dsDNA) was determined using the

Table 1. Characteristics of soils sampled at the MDRS. Sample locations refer to the position shown in Fig. 1. Adapted from Orzechowska et al. (2011). Sample P-11 was not analysed.

Sample location	Coordinates	Altitude (m)	Depth	Formation	pH	Texture of soil % of each layer			Organic matter (%)
						Sand	Silt	Clay	
P-1	N38.43621° W110.81943°	1350	Surface	Mancos Shale/ Tunuck	7.77–8.37	10	90		2
P-2	N38.40746° W110.79280°	1382	Surface	Morrison	8.11–8.93		100		1
P-3	N38.40737° W110.7921°	1375	Surface	Morrison	8.33–9.15	25	75		1
P-5	N38.42638° W110.78342°	1400	Cliff	Morrison	8.88–9.28	25	25	50	2
P-6	N38.42638° W110.78342°	1400	Cliff	Morrison	7.56–8.37	25		75	2
P-7	N38.45424° W110.79092°	1357	Surface	Morrison	8.97–10.01	45	45	10	2
P-8	N38.43755° W110.88725°	1482	Surface	Mancos Shale	7.55–8.22		100		4
P-10	N38.43896° W110.89001°	1500	Surface	Mancos Shale	7.60–8.42	50	50		5
P-11	N38.43896° W110.89001°	1500	30 cm	Mancos Shale	–	–	–	–	–
P-13	N38.40630° W110.79547°	1405	Surface	Dakota	7.41–8.75	80	10		2
P-14	N38.40630° W110.79547°	1405	15 cm	Dakota	7.56–8.21	40	40	20	3

Quant-iT PicoGreen dsDNA kit (Invitrogen, Breda, the Netherlands) following the manufacturer's instructions.

16S rRNA gene fragments of Bacteria and Archaea, and 18S rRNA gene fragments of Eukarya were amplified by PCR. A total volume of 25 µl was used in each PCR reaction, containing 0.4 µM forward and reverse primers; 0.4 mg/ml bovine serum albumin (BSA; New England BioLabs, Leusden, The Netherlands); 12.5 µl Fidelitaq PCR Master Mix (2×) (USB Corporation, Cleveland, OH); 8.5 µl DNase and RNase-free water (MP Biomedicals, Solon, OH) and 1 µl template. For Bacteria, primers F357 and R518 (Muyzer *et al.* 1993) were used. The PCR programme consisted of an initial denaturation at 94 °C for 5 minutes; 35 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s; plus a final elongation step at 72 °C for 8 minutes. Due to the faint PCR products initially obtained, a second PCR (with GC clamp on the F357 primer) was performed on the PCR products without GC clamp. For Archaea, a nested PCR was performed: PRA46f and Univ0907r (first set) and PARCH340f-GC and PARCH519r (second set) (Øvreås *et al.* 1997; Vetriani *et al.* 1999). For both steps, an initial denaturation was performed at 94 °C for 4 minutes; 35 cycles of 94 °C for 30 seconds, 54 °C for 1 minute and of 72 °C for 1 minute; plus a final elongation step at 72 °C for 5 minutes. For Eukarya, primers Euk1A and Euk516r-GC were used (Diez *et al.* 2001). We did an initial denaturation at 94 °C for 130 seconds; 35 cycles of 94 °C for 30 seconds, 56 °C for 45 seconds and 72 °C for 130 seconds; plus a final elongation step at 72 °C for 7 minutes.

PCR products were profiled by denaturing gradient gel electrophoresis (DGGE; Bio-Rad DCode Universal Mutation Detection System). Markers consisting of a mixture of 12 different bacterial 16S rRNA gene fragments were used alongside the samples to aid in normalization. Gels were 8% polyacrylamide (37.5:1 acrylamide/Bis) with a denaturing gradient of 30/55% (for Bacteria), 30/70% (for Archaea) or 20/35% for (Eukarya). Electrophoresis was performed in 1×TAE (Tris-acetate-EDTA) buffer during 3.5 hours at 60 °C and 200 V. Gels were ethidium bromide stained, illuminated under a Vilber Lourmat (TCP-20-M) UV

transilluminator and photographed. All DGGE pictures were converted, analysed and normalized with Gel Compar II (Applied Maths, Belgium). Similarity values were calculated using Pearson correlation and Dice similarity coefficients, and visualized by unweighted paired group method with arithmetic means (UPGMA) cluster analysis. An indication for alpha-diversity was obtained by counting the number of bands in a DGGE profile, while beta-diversity was measured by the Dice coefficient, with 0 indicating large beta-diversity and 1 low beta-diversity.

Phylogenetic analysis of community members

The 16S rRNA gene fragments for Bacteria and Archaea and 18S rRNA gene fragments for Eukarya were amplified. The PCR reactions were prepared the same way as described in the previous section. The nearly full-length bacterial 16S rRNA gene was amplified with primers F8 and 1512R (Felske *et al.* 1997) and the PCR programme had an initial denaturation at 94 °C for 5 minutes; 35 cycles of 94 °C for 30 seconds, 54 °C for 30 seconds and of 72 °C for 90 seconds; plus a final elongation step at 72 °C for 7 minutes. For Archaea, the primers were ARCH46F (Øvreås *et al.* 1997) and ARCH1017R (Barns *et al.* 1994) and the programme included an initial denaturation at 94 °C for 3 minutes; 35 cycles of 94 °C for 30 seconds, 55 °C for 1 minute and of 72 °C for 1 minute; plus a final elongation step at 72 °C for 10 minutes. For Eukarya, primers and PCR conditions were identical to those used for community profiling, except for the primers that were without GC clamp. PCR products were purified with GeneJET PCR Purification Kit (Fermentas, St Leon-Rot, Germany) and cloned with the pGEM-T Easy Vector System (Promega, Madison, WI, USA). A total of 744 clones were screened, 144 Archaea clones (24 per sample), 480 Bacteria clones (96 for P-1 and P-2 and 48 for the other samples) and 120 Eukarya clones (24 for each sample). Clones with the correct size insert were selected after PCR with T7 and SP6 vector primers. Subsequently, these clones were screened by amplified rDNA restriction analysis (ARDRA analysis) with RsaI (New England BioLabs, Leusden, The Netherlands) (5 units per

Table 2. Detection of the three domains of life in various samples (for location, see Fig. 1) from the MDRS, in relation to the used extraction kit (PowerSoil and FastDNA). Amount of dsDNA in DNA extracts is indicated as micrograms per gram of soil. Recovery of a DNA spike is indicated as a percentage

Sample name	Sample location	Bacteria		Archaea		Eukarya		DNA amount (µg/g)		Spike recovery %
		PowerSoil	FastDNA	PowerSoil	FastDNA	PowerSoil	FastDNA	PowerSoil	FastDNA	
P-1	Mancos Shale/ Tunuck	+	+	+	+	+	+	<0.08	<0.04	0.8
P-2	Morrison	+	–	–	–	–	–	<0.08	<0.04	4.6
P-3	Morrison	+	+	+	+	–	–	<0.08	<0.04	5.6
P-5	Morrison	+	–	–	–	–	–	<0.08	<0.04	0.4
P-6	Morrison	–	–	–	–	–	–	<0.08	<0.04	0.0
P-7	Morrison	–	–	–	–	–	–	<0.08	<0.04	2.9
P-8	Mancos Shale	–	+	–	+	–	+	<0.08	0.40	123.0
P-10	Mancos Shale	+	+	+	+	+	+	<0.08	<0.04	35.4
P-11	Mancos Shale	–	+	–	+	–	+	<0.08	0.17	23.2
P-13	Dakota	+	+	+	+	+	+	3.86	6.58	528.7
P-14	Dakota	–	–	–	–	–	–	<0.08	<0.04	0.0
	Total of positives	6	6	4	6	3	5			

'+' indicates amplification and '–' indicates no amplification.

reaction, 3 hours incubation at 37 °C). At least one representative for each unique ARDRA profile per clone library was subjected to DGGE analysis and sequencing. DGGE was performed to further help to select for different cloned sequences, using the INGENY phor U-2 system (Ingeny International BV, Goes, The Netherlands) and the same denaturant gradients as described above. Sequencing was performed on clones presenting a unique combination of ARDRA and DGGE profiles by Macrogen Europe (Amsterdam, The Netherlands). Contig assembly was done using DNA Baser software. The assembled sequences were compared with sequences deposited in GenBank using BLAST (Altschul *et al.* 1990). Sequence alignment was performed by using ClustalW. Distance analysis of unambiguously aligned sequences using the correction of Jukes & Cantor (1969) and bootstrap re-sampling (100 times) were performed. The distance matrix was used to construct a phylogenetic tree using the neighbour-joining method (Saitou & Nei 1987). All steps in this phylogenetic analysis were carried out in MEGA version 4 (Tamura *et al.* 2007).

Testing for PCR inhibitors

Samples for which no PCR amplification was obtained were checked for the presence of PCR inhibitors. One µl of *Shewanella putrefaciens* DNA (20 ng/µl in water) was added to 1 µl template and subjected to bacterial 16S rRNA PCR reactions. The lack of amplification would indicate the presence of PCR inhibitors.

Quantification of DNA recovery from soil samples

An 81 bp PCR fragment of the yeast *Saccharomyces cerevisiae* hexokinase 1 (YHXX1) gene was used as spike and added to soil samples (0.5 ng/µl, final concentration) prior to DNA extraction with the PowerSoil DNA Kit (MO BIO Laboratories, Solana Beach, CA, USA) and subsequent quantitative

PCR. This gene was chosen as it was not likely to be present in the soil samples. This assumption was confirmed by the lack of amplification from unspiked samples. Extractions (spiked and unspiked) and qPCR reactions were performed in duplicate. The amount of YHXX1 recovered was determined by qPCR (Postmus *et al.* 2008) and compared with a 'water control' (extraction of water plus spike only) in order to account for loss of the YHXX1 fragment in the extraction procedure. The primers used in generating the spike fragment and in qPCR were Y-HXX1-f (5'-CCCAGCTTCCCAAACAAGA-3') and Y-HXX1-r (5'-TGGCCTTCGACATTTGGAATA-3'). A total volume of 22 µl was used in each qPCR reaction, containing: 2.5 µl forward and reverse primers mix; 12.5 µl 2 × DyNAmo HS SYBR Green qPCR master mix (Finnzymes, Espoo, Finland); 0.6 µl 50 × ROX (Finnzymes, Espoo, Finland); 6.4 µl DNase and RNase-free water (MP Biomedicals, Solon, OH) and 3 µl template. Tenfold serial dilutions of the YHXX1 gene in the range from 10⁻⁵ to 1 ng/µl were used for the standard curve, along with negative controls (DNase- and RNase-free water). The qPCR programme was: 50 °C for 2 minutes, 95 °C for 15 minutes, 40 cycles (95 °C for 15 seconds, 60 °C for 30 seconds, 72 °C for 27 seconds) plus 72 °C for 10 minutes, 95 °C for 15 seconds, 60 °C for 1 minute, 95 °C for 15 seconds and 60 °C for 15 seconds.

Accession numbers

Nucleotides sequences HQ910241-HQ910388 were deposited in GenBank.

Results

PCR analysis on extracted DNA revealed that all three domains of life were present at the MDRS site, but could not be found in all samples; also the detection was dependent on the extraction kit applied (Table 2; see Fig. 1 for sample locations and Table 1 for sample characteristics). Bacteria were

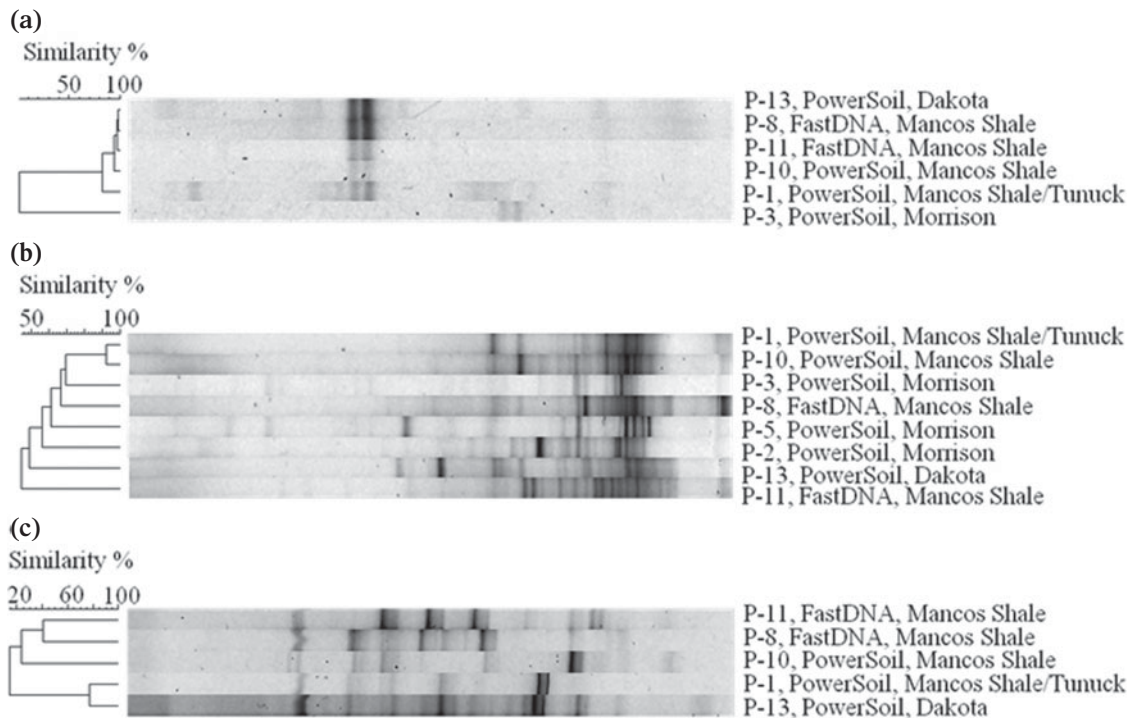


Fig. 2. UPGMA cluster analysis of DGGE profiles of rRNA gene fragments of (a) Archaea 16S rRNA (30–70% denaturant gradient), (b) Bacteria 16S rRNA (30–55% denaturant gradient) and (c) Eukarya 18S rRNA (20–35% denaturant gradient) in the MDRS desert soil samples after Pearson correlation analysis. Percentage (%) similarity represents the Pearson correlation between DGGE bands. For each lane, the sample designation, DNA extraction kit used and location are indicated.

most often detected (73% of samples) and Eukarya least (45%). All three domains of life were present in samples P-1, P-10 and P-13, irrespective of the DNA extraction method applied. Amplification from sample P-3 was also not affected by the extraction method used, however, Eukarya were not detected. Only after DNA extraction with the PowerSoil kit Bacteria were detected in samples P-2 and P-5. In contrast, only DNA extracted with the FastDNA kit allowed the detection of all domains of life in samples P-8 and P-11. PCR on samples P-6, P-7 and P-14 was never successful (Table 2).

The lack of amplification was not due to the presence of PCR inhibitors, PCR products were obtained when *Shewanella* DNA was added to the PCR reaction. Thus, amplifiable DNA in the DNA extracts was too low to be detected. To test whether the low-DNA contents were due to low biomass content or due to low extraction efficiencies, soil samples were spiked with a known quantity of DNA, extracted and the spike quantified. Samples P-1 till P-7 had very low recovery (0–5.6%) of spike. Samples P-6 and P-14, which were negative in PCR amplification for all three domains of life, had no significant recovery of spike at all (0%, Table 2). In contrast, samples P-8 till P-13 revealed high recovery (>23.2%) and showed good amplification for the three domains of life (Table 2). The spiking experiment suggests that the main problem is low recovery of DNA for several samples due to DNA being adsorbed and/or degraded in the original soil.

DGGE-based community fingerprinting provided information on the microbial diversity. Bacteria DGGE profiles revealed the highest alpha-diversity as indicated by a higher

number of bands per sample (23 ± 6 (Standard deviation – SD), $n=8$) than the Archaea (3 ± 1 , $n=6$, bands) and Eukarya (13 ± 4 , $n=5$, bands) (Fig. 2). The similarity of community profiles generated by independent DNA extractions (FastDNA and PowerSoil kit) on the same sample was high: $91 \pm 8\%$ ($n=4$) for Archaea and $70 \pm 23\%$ ($n=4$) for Bacteria. The similarity of DGGE bacterial profiles from independent PCRs on the same DNA extract was $89 \pm 5\%$ ($n=6$), indicating high PCR reproducibility.

Low diversity in Archaea

Archaea were encountered in six samples, including all Mancos Shale samples (P-1, P-8, P-10 and P-11) plus P-3 (Morrison) and P-13 (Dakota). Both DGGE profiling (Fig. 2(a)) and sequencing (Fig. 3) revealed that Archaeal alpha- and beta-diversity was low, e.g. DGGE profiles contained on average only three bands, while the average Dice similarity coefficient, used as indicator for beta-diversity, was $55 \pm 44\%$.

Most samples, except for P-3, clustered together (Fig. 2(a), >83%) and revealed the same double-banding pattern (due to the use of degenerate primers). These bands corresponded to identical sequences closest related (>99% similarity) to an uncultured chasmolithic *Crenarchaeota* archaeon present in tuff at a high-altitude arid desert environment in Tibet (Fig. 3). This sequence was dominant (>95% of clones) in each sample, with the exception of P-3 where it was only present at 8.7%. The closest cultured species is thermophilic *Candidatus Nitrososphaera gargensis* (93–95%). Thus, this sequence was closely related to extremophiles (Table 3).

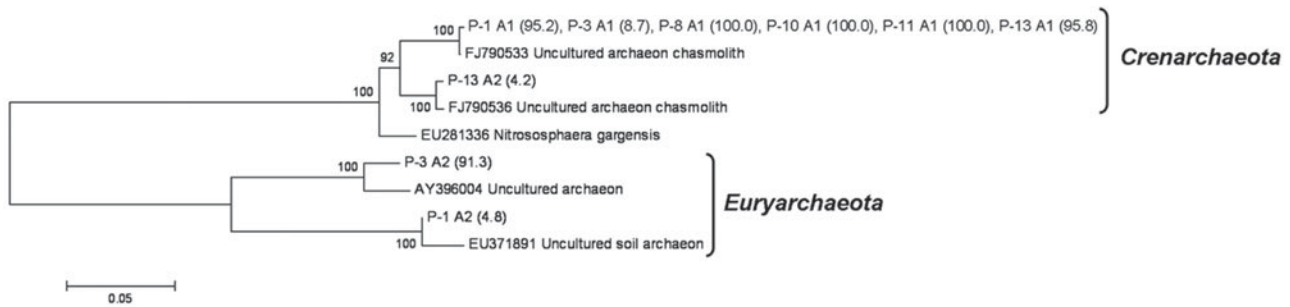


Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences of Archaea in MDRS desert soil samples. The analysis comprised neighbour-joining analysis with Jukes–Cantor and bootstrapping. Each clone is represented by the respective sample name, initial of domain (A – Archaea) and an additional number to distinguish between different clones in the same sample. The percentual contribution of a clone to the clone library of a sample is indicated in parentheses, the scale bar indicates substitutions per site.

P-3 showed a double banding at a lower migration position than the other samples (Fig. 2(a)), which corresponded to clone P-3 A2 (91.3% dominant in P-3) being closest related (96%) to uncultured marine *Euryarchaeota* (Fig. 3). Next to these two sequences, only two other Archaea sequences were obtained, at low frequency and only encountered in a single sample (Fig. 3).

High diversity in Bacteria

In general, bacterial DGGE profiles were complex (Fig. 2(b)). The community profiles of P-1 and P-10 samples were most similar (92%), both being Mancos Shale samples (but from different geological members, Tunuck and Blue Gate, respectively). However, distinct profiles, no clear clustering and an average Dice coefficient of $47 \pm 10\%$ revealed a large beta-diversity. Large diversity and differences between sampling locations were also indicated by detailed phylogenetic analysis (Figs. 4 and 5).

The 239 clones with correct size insert revealed 121 unique sequences. Fig. 5 shows the relative contributions of different Bacteria phyla to the communities at the various MDRS sampling locations. It is noteworthy that samples very near to each other such as P-5 and P-6 (only few cm apart) and P-13 (surface) and P-14 (15 cm deep) revealed very different results: for samples P-6 and P-14 amplification was not successful (Table 2).

Members of the *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Gemmatimonadetes* phyla were encountered most frequently (>6 samples) and with significant contributions (Figs. 4 and 5; 8–31% on average). Less frequently found, but still in at least four samples, were *Chloroflexi* and *Deinococcus-Thermus* (each contributing on average 10% of clones). Other phyla were detected in two or less samples. Striking was the dominant occurrence (32%) of *Cyanobacteria* in sample P-13 only (Fig. 5).

Sequences closest related to several types of extremophiles or to sequences encountered in other extreme environments were observed for all samples revealing the presence of Bacteria (Table 3: 37 sequences, corresponding to 71 clones, 30% of clones). These comprised radioresistant, endolithic,

chasmolithic, xerophilic, hypolithic, thermophilic, thermoacidophilic, psychrophilic, halophilic, haloalkaliphilic and alkaliphilic organisms. However, often similarities to known extremophiles were less than 97% and/or closest related sequences belonged to uncultured species encountered in extreme environments, and thus care needs to be taken in assigning them unequivocally to extremophiles, and hence we refer to these sequences as putative extremophiles.

Twelve unique sequences from a total of six samples were closest related to radioresistant (and desiccation tolerant) species and were found in the order *Actinomycetales* (1 sequence; 96% similarity), genus *Rubrobacter* (six sequences; 92–97% similarity) and order *Deinococcales* (five sequences; 89–98% similarity) (Fig. 4 and Table 3). Several dominantly occurring clones were found to be associated with radioresistant *Rubrobacter*, i.e. P-8 B2 (13.2% in P-8) and P5-B3 (12.5% in P-5) and within *Deinococcales* P-3 B8 (12.5% in P-3) and P-5 B2 (12.5% in P-5). Also dominant was P-3 B7 (12.5% in P-3) close in tree to *Deinococcales* but from the TM7 phylum.

Many sequences related to putative extremophilic *Actinobacteria* were encountered: P-3 B1 (12.5% in P-3), P-3 B4 (12.5% in P-3) and P-8 B4 (2.6% in P-8) were closest (>96% similarity) related to an uncultured actinobacterium in a cryptoendolithic community in the cold desert of McMurdo Dry Valleys, Antarctica. P-8 B11 (2.6% in P-8) is 95% similar to an uncultured actinobacterium observed in methane hydrate-bearing deep subsurface marine sediments in Nankai Trough (Japan). All these clones clustered with the order *Acidimicrobiales*, as also clone P-13 B18 (2.4% in P-13), which was 92% similar to *Acidimicrobiaceae* bacterium IC-180, an iron-reducing thermoacidophilic actinobacterium. The family *Acidimicrobiaceae* comprises mesophilic or moderately thermophilic, acidophilic, iron oxidizing and reducing Bacteria (Itoh et al. 2010). Clones P-2 B2 (20% in P-2), P-5 B8 and P5 B4 (in total 12.6% in P-5) and P-10 B15 (3% in P-10) were closest related (97–99% similarity) to an uncultured actinobacterium from the hyperarid core of Atacama Desert, thus indicative of putative xerophilic micro-organisms. P5 B4 (6.3% in P-5) was also related (94%) to *Sporichthya* sp. from an ice core (China) and therefore potentially psychrophilic.

Table 3. Types of putative extremophiles found in MDRS desert soil samples

Location	Phylotype	Closest related sequences	Similarity %	Domain	Putative extremophilic characteristics	Clone % (per domain)	
Mancos Shale/ Tunuck	P-1 A1	Uncultured <i>Crenarchaeota</i> archaeon (FJ790533)	99%	Archaea	Chasmolithic	95.2	
	P-1 B-16	Uncultured <i>Chloroflexus</i> sp. (AB257647)	88%	Bacteria	Endolithic/ cryptoendolithic	7.7	
Morrison	P-2 B2	Uncultured actinobacterium (EF016815), hyperarid core of Atacama Desert	98%	Bacteria	Xerophilic	20.0	
	P-2 B3	Uncultured <i>Salegentibacter</i> sp. (EU328040)	96%	Bacteria	Halophilic	28.0	
	P-2 B5	Uncultured actinobacterium (FJ152905), highly alkaline saline soil	97%	Bacteria	Haloalkaliphilic	8.0	
	P-2 B6	<i>Salibacillus</i> sp. YIM-kkny3 (AY121430)	94%	Bacteria	Halophilic	4.0	
	P-2 B9	Uncultured <i>Deinococcus</i> sp. (DQ366013)	97%	Bacteria	Radioresistant	4.0	
Morrison	P-3 A1	Uncultured <i>Crenarchaeota</i> archaeon (FJ790533)	99%	Archaea	Chasmolithic	8.7	
	P-3 B1	Uncultured actinobacterium (AY250866), McMurdo Dry Valleys, Antarctica	99%	Bacteria	Endolithic/ cryptoendolithic	12.5	
	P-3 B4	Uncultured actinobacterium (AY250866), McMurdo Dry Valleys, Antarctica	96%	Bacteria	Endolithic/ cryptoendolithic	12.5	
	P-3 B5	Uncultured actinobacterium (AY053479), Gulf of Mexico	97%	Bacteria	Gas hydrate/deep sea	12.5	
	P-3 B8	<i>Truepera radiovictrix</i> (DQ022077)	91%	Bacteria	Radioresistant	12.5	
Morrison	P-5 B2	Uncultured <i>Deinococcus</i> sp. (DQ514091)	97%	Bacteria	Radioresistant	12.5	
	P-5 B5	Uncultured <i>Deinococcus</i> sp. (DQ366013)	98%	Bacteria	Radioresistant	6.3	
	P-5 B3	<i>Rubrobacter radiotolerans</i> (NR 029191)	92%	Bacteria	Radioresistant	12.5	
	P-5 B4	Uncultured <i>Sporichthya</i> sp. (DQ418541), ice core, China	94%	Bacteria	Psychrophilic/ Cryophilic	6.3	
	P-5 B8	Uncultured actinobacterium (EF016815), hyperarid core of Atacama Desert	98%	Bacteria	Xerophilic	6.3	
Mancos Shale	P-8 A1	Uncultured <i>Crenarchaeota</i> archaeon (FJ790533)	99%	Archaea	Chasmolithic	100	
	P-8 B2	Uncultured <i>Rubrobacter</i> sp. (AY571811)	96%	Bacteria	Radioresistant	13.2	
	P-8 B4	Uncultured actinobacterium (AY250866), McMurdo Dry Valleys, Antarctica	96%	Bacteria	Endolithic/ cryptoendolithic	2.6	
	P-8 B7	<i>Kineococcus</i> sp. 1P02MC (EU977818)	96%	Bacteria	Radioresistant	2.6	
	P-8 B8	Uncultured <i>Rubrobacter</i> sp. (EU341239)	94%	Bacteria	Radioresistant	5.3	
	P-8 B11	Uncultured actinobacterium (AY093455), Nankai Trough, Japan	95%	Bacteria	Gas hydrate/deep sea	2.6	
	P-8 B15	Uncultured <i>Thermomicrobium</i> sp. (AM259923)	92%	Bacteria	Thermophilic	2.6	
	P-8 B16	<i>Rhodospirillaceae</i> bacterium 262-8 (AB510913)	93%	Bacteria	Endolithic/ cryptoendolithic	2.6	
Mancos Shale	P-8 B20	<i>Rubrobacter radiotolerans</i> (NR 029191)	94%	Bacteria	Radioresistant	2.6	
	P-10 A1	Uncultured <i>Crenarchaeota</i> archaeon (FJ790533)	98%	Archaea	Chasmolithic	95.4	
	P-10 B5	<i>Salibacillus</i> sp. YIM-kkny3 (AY121430)	92%	Bacteria	Halophilic	15.2	
Mancos Shale	P-10 B15	Uncultured actinobacterium (EF016815), hyperarid core of Atacama Desert	99%	Bacteria	Xerophilic	3.0	
	P-11 A1	Uncultured <i>Crenarchaeota</i> archaeon (FJ790533)	98%	Archaea	Chasmolithic	95.4	
	P-11 B1	<i>Truepera radiovictrix</i> (DQ022077).	89%	Bacteria	Radioresistant	5.1	
	P-11 B4	<i>Halomonas phoceae</i> (AY922995)	97%	Bacteria	Halophilic	7.7	
	P-11 B5	Chibacore 1500 (AB038407)	98%	Bacteria	Alkaliphilic	7.7	
	P-11 B8	Uncultured actinobacterium (FJ152905), highly alkaline saline soil	94%	Bacteria	Haloalkaliphilic	2.6	
	P-11 B11	Uncultured <i>Thermomicrobium</i> sp. (AM259923)	92%	Bacteria	Thermophilic	5.1	
	P-11 B12	Uncultured <i>Salegentibacter</i> sp. (EU328040)	95%	Bacteria	Halophilic	2.6	
	P-11 B20	<i>Alkalispirillum</i> sp. ACO5 (FJ976681)	92%	Bacteria	Haloalkaliphilic	2.6	
	Dakota	P-13 A1	Uncultured <i>Crenarchaeota</i> archaeon (FJ790533)	99%	Archaea	Chasmolithic	95.8
		P-13 A2	Uncultured <i>Crenarchaeota</i> archaeon (FJ790536)	99%	Archaea	Chasmolithic	4.2
P-13 B3		<i>Chroococidiopsis</i> sp. CC1 (DQ914863)	95%	Bacteria	Hypolithic	12.2	
P-13 B12		<i>Chroococidiopsis</i> sp. CC1 (DQ914863)	94%	Bacteria	Hypolithic	2.4	
P-13 B16		Uncultured <i>Rubrobacter</i> sp. (AY571811)	97%	Bacteria	Radioresistant	2.4	
P-13 B18		Acidimicrobiaceae bacterium IC-180 (AB517669)	92%	Bacteria	Thermoacidophilic	2.4	
P-13 B22		Uncultured <i>Rubrobacter</i> sp. (AY571811)	94%	Bacteria	Radioresistant	2.4	
P-13 E3		<i>Dunaliella salina</i> (DQ447648)	99%	Eukarya	Halophilic	57.1	

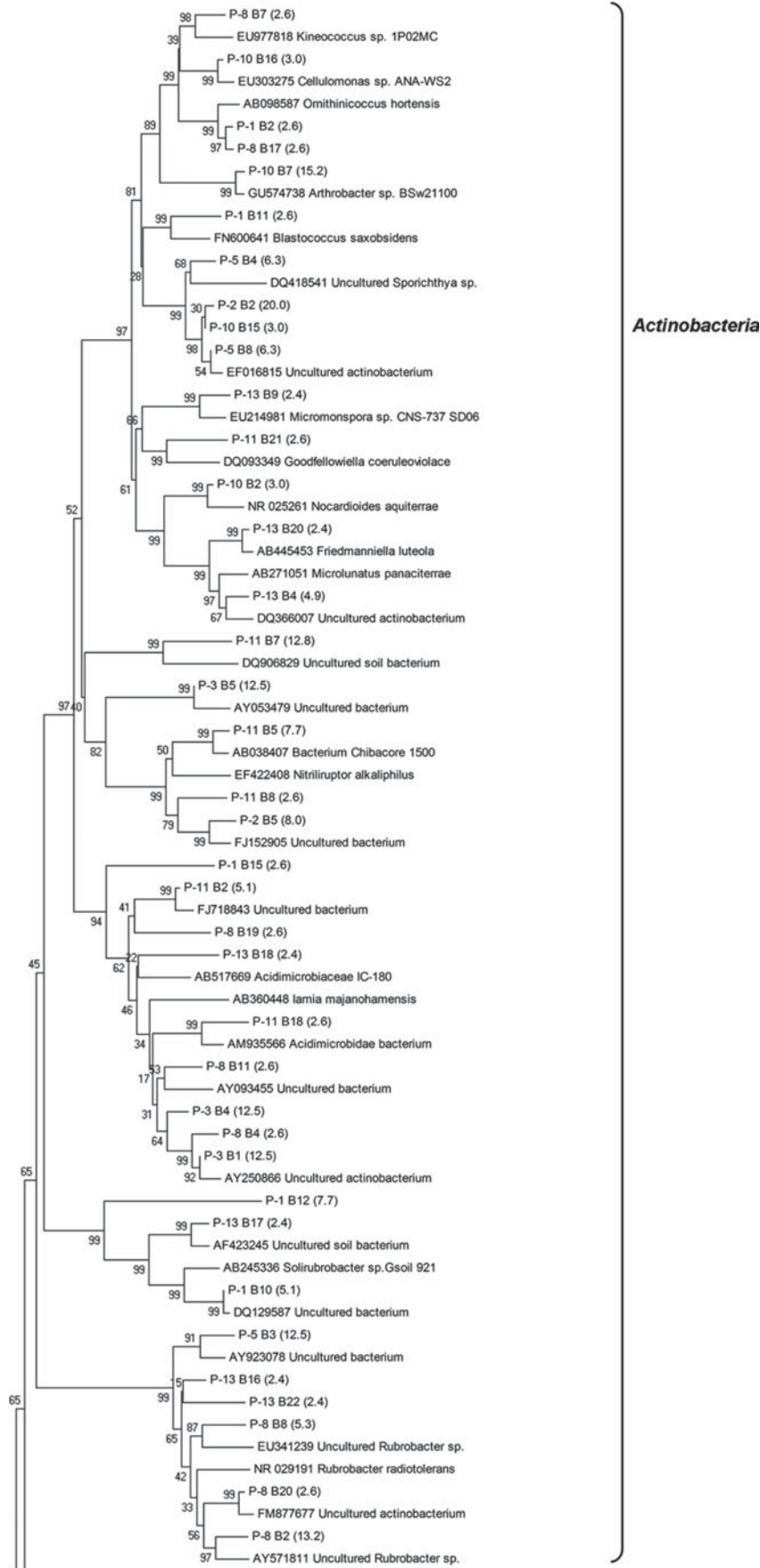


Fig. 4. (Cont.)

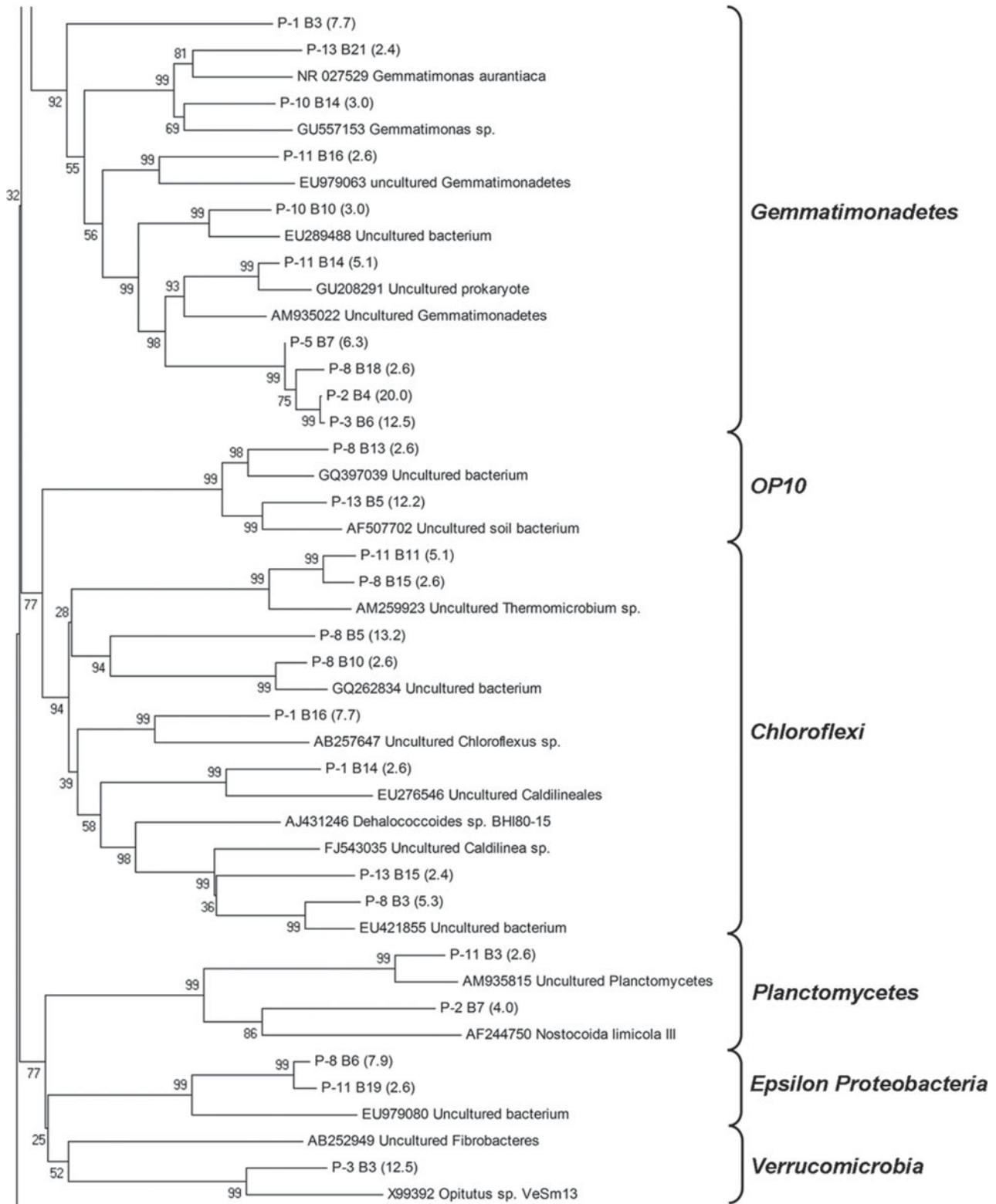


Fig. 4. (Cont.)

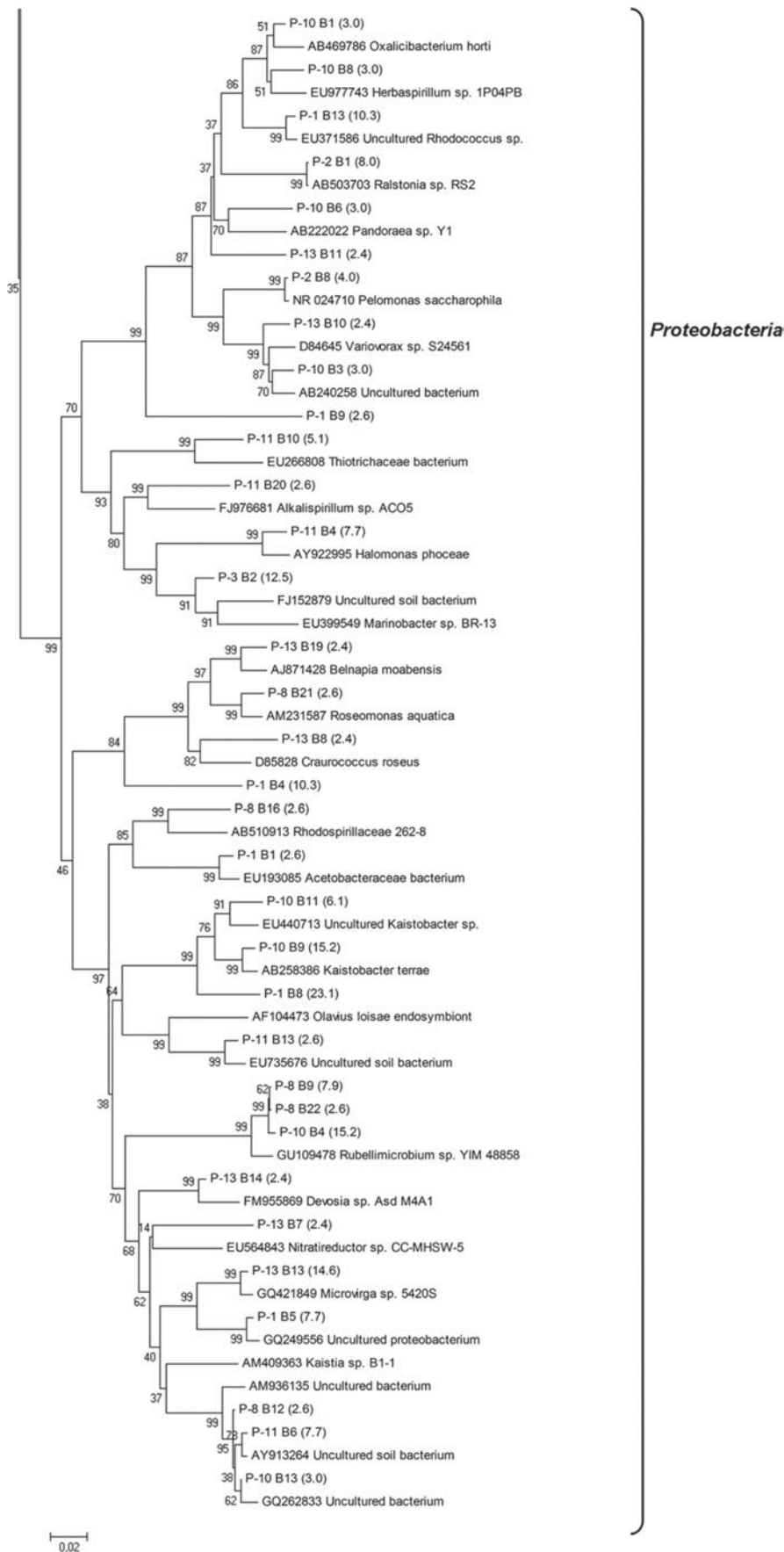


Fig. 4. (Cont.)

Belonging to a cluster of haloalkaliphilic actinobacteria were P-2 B5 (8% in P-2) and P-11 B8 (2.6% in P-11), they were closest related (94–97% similarity) to an uncultured actinobacterium present in a highly alkaline saline soil. P-2 B5 was also 94% similar to *Nitriliruptor alkaliphilus*. P-11 B5 (7.7% in P-11) falls into the same group and showed 98% similarity to an alkaliphilic bacterium isolated from a depth of 1500 m. P-3 B5 (12.5% in P-3) was 97% similar to an actinobacterium associated with Gulf of Mexico gas hydrates.

P-1 B-16 (7.7% in P-1) was 88% similar to a *Chloroflexus* sp. sequence found inside a dolomite rock in central Alps (Switzerland). Several other Chloroflexi clones (P-8 B15, P-11 B11) were closest related (both 92% similarity) to thermophilic *Thermomicrobium* sp.

P-8 B16 (2.6% in P-8) was 93% similar to cryptoendolithic *Rhodospirillaceae* bacterium 262–8 found in Antarctic white rock.

Cyanobacteria were only encountered in sample P-13. P-13 B3 (12.2%) and P-13 B12 (2.4%) were 95% and 94% similar to endolithic, desiccation- and radiation-resistant *Chroococciopsis* sp. CC1. Clone P-13 B2 (14.6% of clones) was 99% similar to *Trichocoleus sociatus* and P-13 B6 (2.4%) 99% similar to *Microcoleus vaginatus* SAG, species encountered in biological crusts in the deserts of northwestern Negev/ Sinai region (Büdel & Veste 2008).

Clones P-2 B6 (4% in P-2) and P-10 B5 (15.2% in P-10) revealed, respectively, 94% and 92% similarity to the halophilic *Salibacillus* sp. YIM-kkny3. Other potential halophiles are P-11 B20 (2.6% in P-11; 92% similar to haloalkaliphilic *Alkalispirillum* sp. ACO5) and P-11 B4 (7.7% in P-11; 97% similar to halophilic *Halomonas phoceae*). P-2 B3 (28% in P-2) and P-11 B12 (2.6% in P-11) were 96% and 95% similar to halophilic *Salegentibacter* sp. in the phylum *Bacteroidetes*.

In addition to putative extremophiles, several other sequences were present in significant numbers (>10% in a sample), especially sequences closest related to soil bacteria: P-11 B7 and P-13 B5 were closest related to different uncultured soil bacteria; P-3 B3 to *Opitutus* sp. VeSm13 from anoxic rice paddy soil; P-5 B6 to *Flexibacteraceae* bacterium 24M from dry soil; P-1 B8, P-10 B11 and P-10 B9 to *Kaistobacter* species; and P-10 B4, P-8 B9 and P-8 B22 to *Rubellimicrobium* sp. YIM 48858. A number of dominant sequences were closest related to uncultured bacteria from contaminated sediments and low-level-radioactive-waste site: P-3 B7 (*TM7* phylum), P-8 B5 and P-8 B10 (both *Chloroflexi*). Dominant sequences closest related to bacteria detected in marine environments were also observed: P-10 B7 was closest related (98% similarity) to *Arthrobacter* sp. BSw21100 isolated from seawater in the Northern Bering Sea (although *Arthrobacter* species are generally soil bacteria); P-3 B2 closest

related (94%) to *Marinobacter* sp.; P-1 B4 90% similar to *Roseomonas aquatica*. Other dominant sequences were P-1 B13 closest related (98%) to uncultured *Rhodococcus* sp. found in oyster shell (*Rhodococcus* may occur in soil, water and eukaryotic cells) and P-13 B13 related to airborne *Microvirga* sp. 5420S.

Moderate diversity in Eukarya

Eukarya were detected in five samples, the same samples where Archaea were found, with the exception of sample P-3 (Table 2). Eukaryotes were not detected in the oldest geological formation sampled (Morrison Formation) and P-14 (Oyster Reef, Dakota). Both DGGE profiling (Fig. 2(c)) and sequencing (Fig. 6) revealed that the Eukarya diversity (13 ± 4 bands) present in these desert soil samples is in between those of Archaea (3 ± 1 bands) and Bacteria (23 ± 6 bands) (Fig. 2). Eukarya DGGE profiles did not reveal clear clustering; the profiles of P-1 (Mancos Shale/Tunuck) and P-13 (Dakota) were the most similar (77% similarity). Large beta-diversity was revealed by an average Dice coefficient of 41 ± 9%.

The community profiles (Fig. 2(c)) correspond to a diverse range of Eukarya, such as fungi, a mollusc, green algae, a moss and several phyla of Protozoa (Fig. 6). Protozoa were found in all samples that had positive amplification for Eukarya and contributed strongly to Eukarya communities. The protozoa *Cercozoa* (with average abundance of 14 ± 16% SD, *n* = 4 samples), *Tubulinea* (at 45 ± 41% SD, *n* = 4) and *Ciliophora* (at 26 ± 17% SD, *n* = 3) were most frequently encountered. The slime mold *Mycetozoa* (at 12 ± 3% SD, *n* = 2) was encountered only in two samples. A species closest related to the halophile green algae *Dunaliella salina* (*Chlorophyta*) was dominant (57%) in P-13 and was the only Eukarya sequence potentially indicative of an extremophile (Table 3). Sequences related to parasitic *Apicomplexa* were dominant in P-1 (80%). Other Eukaryotes, such as parasitic *Ichthyophonida* protozoa, fungi belonging to *Blastocladiomycota* and *Zygomycota*, a *Mollusca* and a moss (*Streptophyta*) were only minorely (<10%) present in one sample.

Discussion

Microbial community composition of MDRS in comparison with other deserts

Bacteria were the most frequently observed and diverse domain of life at MDRS. Its bacterial community composition in general concurred with the bacterial community composition of other deserts studied in detail using culture-independent molecular techniques: the cold desert McMurdo Dry Valley in Antarctica that is regarded as having the driest, oligotrophic and coldest soils (Cary et al. 2010), the arid and hot Atacama

Fig. 4. Phylogenetic tree based on 16S rRNA gene sequences of Bacteria in MDRS desert soil samples. The analysis comprised neighbour-joining analysis with Jukes–Cantor and bootstrapping. Each clone is represented by the respective sample name, initial of domain (B – Bacteria) and an additional number to distinguish between different clones in the same sample. The percentual contribution of a clone to the clone library of a sample is indicated in parentheses, the scale bar indicates substitutions per site.

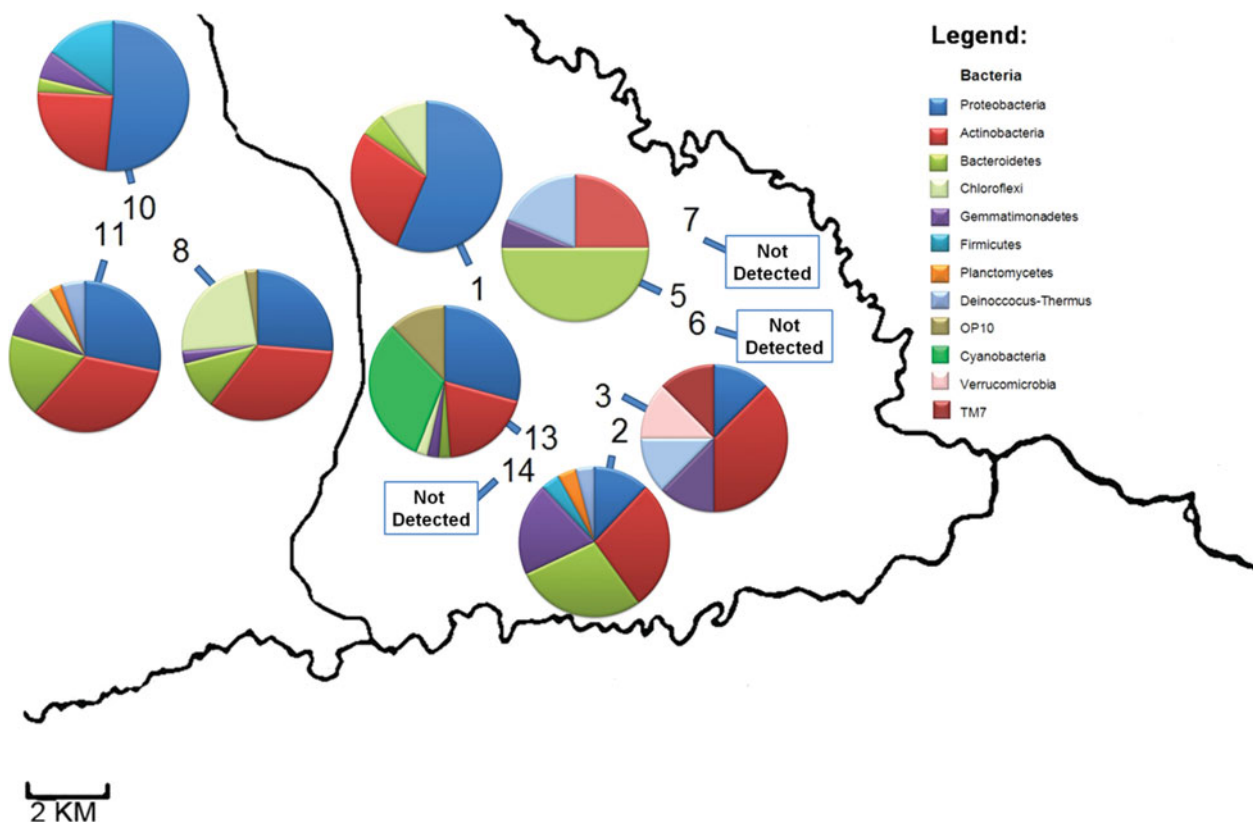


Fig. 5. Relative contributions of Bacteria phyla to clone libraries derived from samples taken at various locations at MDRS.

Desert (Drees *et al.* 2006; Connon *et al.* 2007; Lester *et al.* 2007), the hot desert of Tataouine (South Tunisia) (Chanal *et al.* 2006), the desert of arid southeast Morocco region (Gommeaux *et al.* 2010), Namib Desert (Namibia) believed to be the oldest desert on Earth (Prestel *et al.* 2008) and the Sonoran desert in Arizona (Nagy *et al.* 2005). Members of the *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Gemmatimonadetes* phyla were dominant in the majority of MDRS samples and common in most other deserts with a few exceptions. For example, *Proteobacteria* were absent from McMurdo Dry Valley in Antarctica although this phylum is prevailing in other Antarctic locations (Yergeau *et al.* 2007). Radiation- and desiccation-resistant *Deinococci* were also widely found in this study and in other deserts. Especially *Deinococcus radiodurans* has been studied as a model for potential Martian life, since it is potentially capable of surviving Martian conditions (de la Vega *et al.* 2007). The sharpest contrast with other previously studied deserts was in the *Acidobacteria*, *Planctomycetes* and *Firmicutes*. These phyla were only observed in low numbers in a few MDRS samples, while they were more abundantly present in most other deserts.

In arid deserts, microbiota often inhabit rocks and form biological soil crusts (Torsvik & Øvreås 2008). Biological crusts are rich in cyanobacteria and many types of Eukarya such as fungi, mosses, green algae and lichens (Lewis & Lewis 2005; Bamforth 2008; Büdel & Veste 2008). Cyanobacteria sequences were found at MDRS, indicative for the presence of

biological crusts. The endolithic, desiccation and radiation-resistant cyanobacterium *Chroococcidiopsis* (Cockell *et al.* 2005) is common in hot and cold deserts (Friedmann & Ocampo-Friedmann 1985; Büdel & Wessels 1991; Wood *et al.* 2008), but was only detected in P-13 at MDRS. Also *Chroococcidiopsis* has been previously studied as a model organism for survivability studies under Martian conditions, it survives inside of 1 mm of rock in the Martian UV radiation environment (Cockell *et al.* 2005).

The eukaryotic diversity in our samples revealed fungi, a mollusc, an extremophile eukaryotic green algae *Dumaliella salina*, a moss and several phyla of Protozoa and also comprised species previously observed in biological crusts: the bacterivorous flagellate *Heteromita globosa* and the amoeba *Hartmanella cantabrigiensis* were observed in crusts on the Colorado Plateau, Utah (Bamforth 2008). *Heteromita* is widely distributed in soils; in Antarctic environments it is able to survive freeze–thaw cycles (by encystment and excystment) (Hughes & Smith 1989).

In contrast to Bacteria and also Eukarya, Archaea diversity was low, but revealed strong dominance in most samples by putative chasmoliths (Table 3). Archaea were also hard to detect in other cold (Brambilla *et al.* 2001; Bottos *et al.* 2008; Pointing *et al.* 2009; Cary *et al.* 2010) and hot deserts (Nagy *et al.* 2005; Chanal *et al.* 2006), and if present, Archaea diversity was low, while high diversity in Bacteria was observed. Aller & Kemp (2008) suggested that differences in diversity might imply that Archaea could live in microniches,

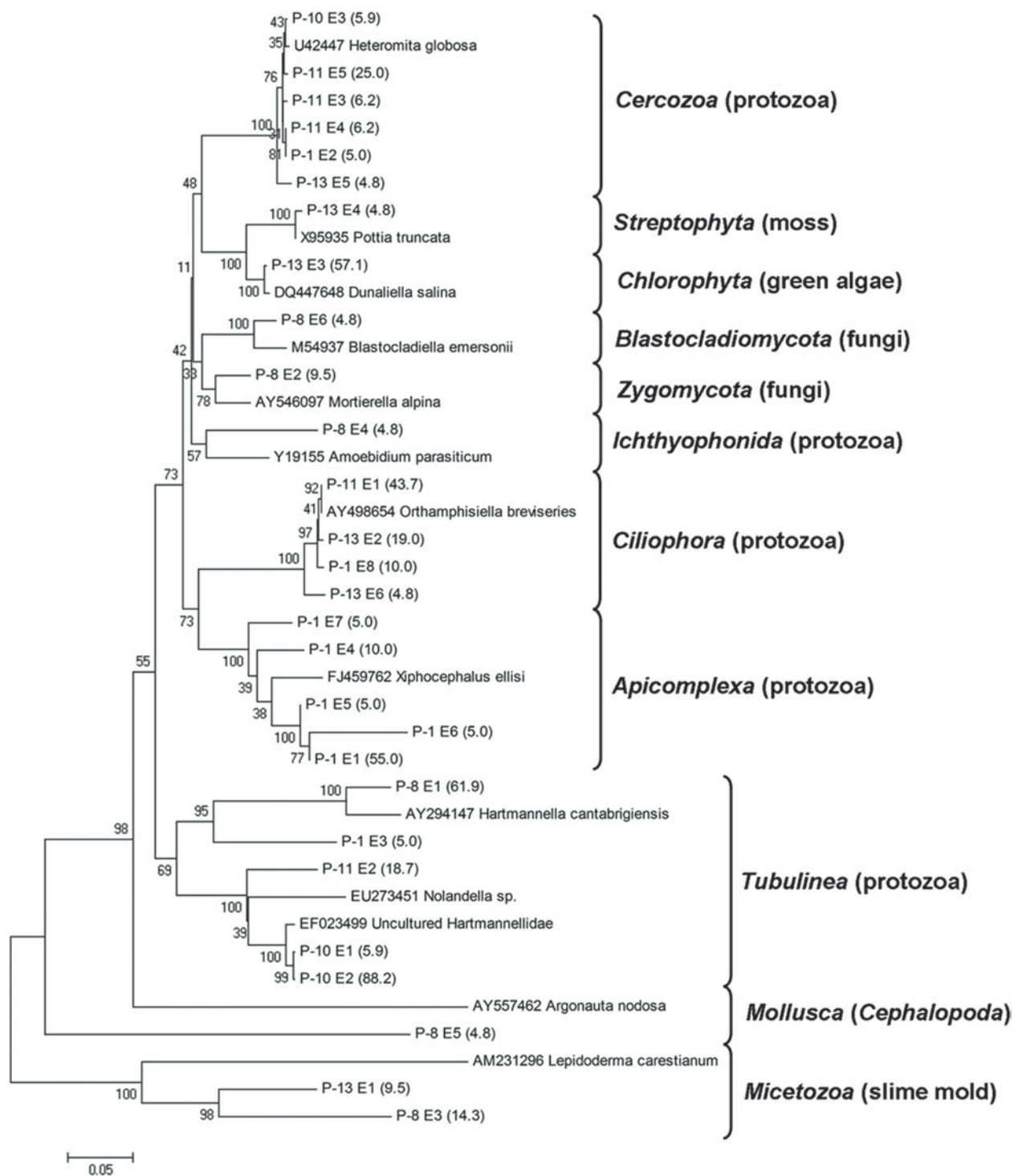


Fig. 6. Phylogenetic tree based on 18S rRNA gene sequences of Eukarya in MDRS desert soil samples. The analysis comprised neighbour-joining analysis with Jukes–Cantor and bootstrapping. Each clone is represented by the respective sample name, initial of domain (E – Eukarya) and an additional number to distinguish between different clones in the same sample. The percentual contribution of a clone to the clone library of a sample is indicated in parentheses, the scale bar indicates substitutions per site.

while Bacteria are more broadly distributed or live in different microniches in the same environment. Other possible explanations are that metabolic energy costs for Archaea might be too big for providing diversity or that substrate availability may limit their diversity (Aller & Kemp 2008).

Relation between the occurrence of putative extremophiles and environmental conditions

Putative extremophiles were detected in all samples that had successful PCR amplification. Caution needs to be taken in

attributing phenotypes, such as extremophile characteristics, solely on the basis of phylogeny and the ideal would be to complement studies like the one presented here with culturing or other functional approaches.

Soils were slightly alkaline explaining why putative alkaliphilic organisms were detected in several samples. Alkalinization of soils (pH increase from 8 to 10.5) has previously been attributed to *Chroococcidiopsis* strains during photosynthesis (Wessels & Büdel 1995; Büdel 1999). A similar effect was also observed for *Microcoleus vaginatus* in southeast Utah (Garcia-Pichel & Belnap 1996). These species were detected in P-13, but may have widely contributed to the high pH around MDRS.

MDRS is a cold desert subject to large daily variation in temperature (20 °C), with −36 °C and 46 °C at its extremes. The variation in temperatures might explain the presence of both thermophiles and, to a lesser degree, psychrophiles/cryophiles.

Water availability and precipitation are low in this desert environment, 140 mm annual average precipitation with sometimes many months without precipitation (Godfrey *et al.* 2008). Therefore, it is not a surprise that putative xerophiles and desiccation-tolerant organisms (a characteristic often associated with radioresistance) were found.

MDRS is subjected to high UV radiation and the region is also known for its uranium deposits. Sedimentary rocks from the Triassic and Jurassic periods in this region enclose uranium originated from volcanic rocks; mines in this region were explored in the 1950–1960s (Chronic 1990). Salt Wash member of Morrison formation (just below Brushy basin member) of the Colorado Plateau has been a main source of uranium (Keller 1958). Putative endolithic, chasmolithic and hypolithic micro-organisms were also frequent which is not surprising since their presence is common in deserts. Rocks are known to provide a shelter for organisms and also protect from harmful UV radiation and dehydration (e.g. Vitek *et al.* 2010). Concerning other soil properties such as salinity, different putative halophiles or haloalkaliphiles could be found in several samples (Bacteria and Eukarya) P-2, P-10, P-11 and P-13 (Table 3).

Spatial heterogeneity in microbial communities, in relation to environmental factors

High beta-diversity was observed at MDRS. The variation in physicochemical characteristics of MDRS might help to understand the large spatial heterogeneity in community composition. Factors that are known to influence soil community composition and that are variable in these samples are: pH (Fierer & Jackson 2006), geochemical properties and salinity (Cary *et al.* 2010). Mineralogy also influences microbial community composition and diversity (Boyd *et al.* 2007; Carson *et al.* 2009). Different clay and mineralogy composition were observed in MDRS samples (Kotler *et al.* 2011).

Differences were noted between the geological formations: Mancos Shale samples and Dakota (P-13) had in general a higher content of organics and revealed higher DNA recovery,

higher number of successful PCR amplification and higher bacterial diversity than the Morrison formation samples.

Even over small distances large differences were observed. Dakota Sandstone samples P-13 (surface) and P-14 (15 cm deep) were collected near MDRS station from a top of a hill consisting of fossil oyster shells. Their results were strikingly different: PCR was successful for all domains (and kits) for P-13, while P-14 PCRs were never successful.

Relations between the microbial community composition and the geochemistry, mineralogy and physicochemical parameters are further discussed in Ehrenfreund *et al.* (2011).

Low DNA recovery in relation to site characteristics

Some soils and specially clays are known to adsorb DNA (e.g. Saeki & Sakai 2009). This may occur by ligand exchange: DNA phosphate groups may bind via ligand exchange to OH groups of iron or aluminium oxide minerals or of phyllosilicates (Saeki & Sakai 2009).

In addition, arid soils as well as Mars can contain high quantities of the strong oxidant perchlorate (Hecht *et al.* 2009). It has been postulated that the Mars Viking results were obscured by the chemical oxidation of organic matter by perchlorate at high temperatures (Navarro-González *et al.* 2010). We cannot exclude that during the heat generated in the mechanical shearing of cells in our isolation procedures, DNA was lost. However, in our results there appears to be a striking relation between the presence of clay (Table 1) and low recovery (Table 2). Therefore, it is important to use a spiking technique, as employed in this study, which allows the quantification of how much DNA is being lost during extraction and optimize procedures to minimize loss. Our results imply that we might be underestimating the actual diversity in our samples due to losses of minor species in the extraction procedures. Samples with a high DNA recovery had a higher number of successful PCR amplification and also higher bacterial diversity was observed.

Implications for the search for life on Mars

All domains of life were detected at MDRS, and this single environment proved to be quite diverse especially concerning Bacteria, revealing many different types of putative extremophiles. Our data revealed large difference in occurrence and diversity over short distances, indicating the need for high-sampling frequency at similar sites. Also minerals (e.g. clay phyllosilicates) may complicate the extraction and detection of biomarkers (like DNA). Therefore, we underline the importance of using an external spike to account for possible biomarker losses in extraction, ‘not detected’ is not a synonym for ‘is it not present’. The potential loss during extraction also implies the need to use sensitive biomarker detection: amplification-based methods are then a strong option for the detection of current life.

Acknowledgements

The EuroGeoMars 2009 campaign was organized and supported by the International Lunar Exploration Working

Group (ILEWG), NASA Ames Research Centre and ESA/ ESTEC. We gratefully acknowledge the contribution of the EuroGeoMars 2009 campaign crew and the mission support team, in particular Carol Stoker, Jhony Zavaleta and Cora Thiel were critical to the campaign and acquisition of samples. The research was funded by a grant from the Netherlands Organisation for Scientific Research (NWO/SRON User Support Programme Space Research, project 'Molecular detection of life on Mars' ALW-GO- PL/07-11).

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