Microbial Biodiversity of Thermophilic Communities in Hot Mineral Soils of Tramway Ridge, Mt. Erebus, Antarctica

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Abstract

Only a few studies have looked at microbial biogeography in soils and whether microorganisms are endemic to an area is still debatable. Tramway Ridge, a geothermal area on Mount Erebus, Antarctica, provides a unique opportunity due to its isolation and extreme conditions to explore the possibilities of microbial endemism and to identify novel Bacteria and Archaea. This site was chosen for a culture-independent study with a preliminary culturing survey for bacterial communities along three temperature gradients (65°C – 2.5ºC). In addition, a physico-chemical analysis was undertaken to identify which environmental factors were driving the different diversity along the transects. An automated rRNA intergenic spacer analysis (ARISA) was used to assess the diversity across the transects using Bacteria and Cyanobacteria-specific primers and results showed that temperature and pH were the main drivers for these communities. Due to its unique physico-chemical and ARISA profile, a hot temperature site (T-3A, 65ºC) was chosen for further investigation by bacterial and archaeal 16S rDNA clone libraries. Unique rDNA types among the 78 bacterial and 83 archaeal clones were identified by restriction fragment length polymophisms and 18 bacterial and 5 archaeal operational taxonomic units (>97% identity) were observed. All of the bacterial sequences were deeply branching and loosely affiliated with other recognised bacterial divisions, with 40% of the sequences not affiliated to any genus. The archaeal clones were found to be deep-branching and sequences clustered together within the Crenarcaheota. In addition, two strains of Bacilli were isolated. The novel microorganisms show that the Tramway Ridge communities are unique from organisms found in other environments and show that “Everything is (not) everywhere”.

Preface

As part of writing this thesis, I wanted to write a journal article so that I could share my work with the wider scientific community. This thesis has been written in three parts with the first chapter as a literature review and a background on the research. The second chapter has been written as a journal article, which will be submitted to Environmental Micrbiology shortly after this thesis has been submitted. The third chapter is a general conclusion and discussion, which discribes the work that has been completed and future work that will provide us with a more comprehensive overview of the microbial communities at Tramway Ridge. The appendix includes a journal article that was submitted to Environmental Microbiolgy with Tom Niederberger as the first author. I have included it into the appendix as I was a co-author for the article, in which I worked with one sample, LV High 1-3 as part of a learning process for when I obtained my own samples to process from Mt. Erebus. In the study, I extracted DNA from the soil samples, ran denaturing gradient gel electrophoresis (DGGE) and performed PCR to produce products for cloning. I then cloned the PCR product, sequenced and edited them.

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Chapter 1 : Literature Review

1.1 Extreme environments and microbes

An extremophile is an organism that thrives in extreme environments. Extremes include physical extremes (for example, temperature, radiation or pressure) and geochemical extremes (for example, desiccation, salinity, pH, oxygen species or redox potential). Extremophiles include single-celled organisms from all three domains (Bacteria, Archaea and Eukaryota), as well as multicellular organisms and vertebrates. Some examples of extreme environments are hotsprings and geysers, deep-sea hydrothermal vents, hypersaline environments, evaporates, deserts, ice, permafrost and snow, as well as in the atmosphere (Rothschild and Mancinelli, 2001).

The discovery of life in extreme environments is one of the most exciting areas of current research. It has made the search for extraterrestrial life and the possibility of transferring life from other celestial bodies such as Mars and Europa more plausible (Rothschild and Mancinelli, 2001; Ellery *et al.,* 2003; Marion, 2003). Recently, microbes have been found in the anoxic basins off the coast of Sicily and there is growing evidence that Mars and Europa may provide hypersaline environments rich in magnesium chloride. Traces of methane have been detected on Mars, which some scientists believe could be a by-product of extremophilic methanogens (Phillips and Parnell, 2006; Hallsworth *et al.,* 2007).

Extremophiles are also important for the biotechnology industry in areas such as agriculture, chemical synthesis, laundry agents and pharmaceuticals (Bruins *et al.,* 2001; Horikoshi, 1999; Zeikus *et al.,* 1998). For example, DNA polymerases have been isolated from the hyperthermophiles, *Thermus aquaticus*, *Thermotoga maritime*, *Thermococcus litoralis*, *Phyrococcus woesii* and *Pyrococcus furiosus*, for use in the polymerase chain reaction (PCR). The biotechnology industry is expanding with the isolation of new organisms, the identification of novel

compounds and pathways, and through molecular and biochemical characterisation of cellular components.

Our understanding of biodiversity and evolutionary biology has benefited by research into the most extreme of extremophiles, with many new taxa being discovered (Woese 1987; DeLong and Pace, 2004). For example, in 2004, a halophilic archaeon was isolated from unsterilised salt crystals taken from a 250-million-year-old Salado formation in southeastern New Mexico. The microbe was unique in that it had three dissimilar 16S rRNA genes. Due to it having a unique phenotype, lipid pattern and phylogeny, the new genus and species given was *Halosimplex carlsbadense* (Vreeland *et al.,* 2004).

The study of extremophiles has also given insight into the metabolism required to survive in extreme conditions and has led us to understand protein stability and function at low and high temperatures (Hollien and Marqusee, 1999; Russell, 2004).

1.2 Hot soils and microbial communities

Soil is a poorly characterized microbial environment. It has been established that the genetic diversity of soil has an estimated 6400 to 38000 prokaryotic species per gram. On the other hand, less than 0.3% of the microscopically observed microorganisms present are culturable by standard techniques (Janssen *et al.,* 2002; Pettit, 2004; Walsh *et al.,* 2005). Only a handful of studies have looked at microorganisms in hot temperature soils and little is known about the diversity and ecology of these communities.

1.2.1 Hot soils as environments for microbes

The majority of hot soils are found on geothermally heated grounds such as those that occur near hot springs (Kimble *et al.,* 1994; Norris *et al.,* 2002; Kumar *et al.,* 2004). They can also be found in desert soils (Nagy *et al.,* 2005) and decaying matter, such as compost and peat bogs (Strom, 1985; Blanc *et al.,* 1997; Dees and Ghiorse, 2001).

One study looking at microbial communities in geothermally heated grounds was performed by Norris *et al.,* 2002, who studied the microbial species diversity across a landscape in Yellowstone National Park where there had been a soil temperature increase associated with geothermal activity. They assessed bacterial and archaeal community composition along a thermal gradient using 16SrDNA and rRNA fingerprinting techniques and found that the majority of clones belonged to the *Acidobacteria* (51%) and *Planctomycetes* (18%) divisions. They concluded that the selective pressure of temperature significantly reduced the diversity of the soil microbial community. In another experiment looking at geothermally heated grounds, soil samples were collected from two hot springs, Sohldhar and Ringidad, both located in the Garhwal region of Uttaranchai Himalaya, India. The soil samples were analysed for physical, chemical and microbial properties and microscopy was used to identify bacteria, yeast and filamentous organisms. Of the 58 aerobic isolates, 53 were gram positive bacilli, with gram positive anaerobic oval rods observed in soils with temperatures up to 60°C. The soils were alkaline with a total absence of carbon and nitrogen. The Soldhar samples had higher amounts of copper, iron and manganese, whereas the Ringidad soil was devoid of copper but had higher phosphate (Kumar *et al.,* 2004).

In a study looking at desert soils, Nagy *et al.,* (2005) studied prokaryotic community structure and compostion in biological soil crusts (BSCS) from the Sonoran Desert with culture-independent techniques involving 16S rRNA genes. The Sonoran Desert samples contained traces of *Gemmatinodetes*, *Chloroflexi* and

Deinococcus/Thermus, which were not present in the Colorado Plateau BSCS, arid soils or agricultural soils.

1.2.2 Thermophiles

Temperature is one of the main factors in determining where Archaea and Bacteria live. These prokaryotes can be classified into one of three groups; psychrophiles, mesophiles and thermophiles. Thermophiles can be classified into obligate or facultative. Obligate thermophiles require high temperatures to survive, whereas facultative thermophiles can withstand high or low or temperatures (below 50°C). Hyperthermophiles are found in environments above 80°C. These organisms are usually studied to understand their thermo-stable proteins.

Thermophilic bacteria thrive between 55°C to 65°C and occur naturally in hydrothermal vents (Slobodkin *et al.,* 2001; Sako *et al.,* 2003), hot springs (Brock *et al.,* 1972; Kumar *et al.,* 2004), solfataric fields (Goorissen *et al.,* 2003), compost heaps (Strom, 1985; Bae *et al.,* 2005), and in hot water heaters (Brock and Boylen, 1973). They were first isolated in 1879 where they were found living at 72°C (Kim *et al.,* 2001). Recently the studies of thermophilic Bacteria have been overshadowed by the increasing interest in thermophilic Archaea, which were thought to only occur at high temperatures of 100°C and above. The majority of Archaea being isolated from extreme environments or specialized ecological niches such as hydrothermal vents (Canganella, 1998; González *et. al.,* 1998; Takai *et al.,* 2001;) and hot springs (Barns *et al.,* 1994; González *et al.,* 1999). However, distant relatives have recently been found in terrestrial environments (Jurgens *et al.,* 1997; McMullan *et al.,* 2004), marine and lake sediments (DeLong, 1992; Schleper *et al.,* 1997), temperate ocean water (Vetriani *et al.,* 1999), polar seas (DeLong *et al.,* 1994) and oil reservoirs (Miroshnichenko *et al.,* 2004). They are reported to survive for long periods at low temperatures without being active or may grow slowly (Rahman *et al.,* 2003). Of the two sub-domains of Archaea, the Crearchaeota are thermophilic, while the Euryarchaeota, comprise extreme halophiles, sulphur reducers, sulphate reducers, thermophilic

heterotrophs, and methanogens. Recent molecular phylogenetic studies have indicated that Archaea may be more diverse and widespread than what is represented by cultured members of the Domain. Two new phylums, Koryarchaeota and Nanoarchaeota have recently been identified (Bintrim, 1997; Brochier, *et al.,* 2005; Auchtung *et al.,* 2006).

For many years, the evolutionary relationships of thermophiles have been debated with some scientists believing that the last common ancestor of thermophilic Archaea and Bacteria may have been a hyperthermophile, as thermophiles tend to be found in the deep branches of phylogenetic trees. Of the Archaea, all crenarcheota cultured to date are thermophiles and the deepest euryarchaeal branchings are thermophiles. Within the Bacteria, the deepest branches are also thermophiles with thermophily scattered throughout the domain. Other scientists are sceptical and believe that the earth cooled rapidly and that heat-loving lifestyles may be a secondary adaptation (Olsen *et al.,* 1994; Reysenbach *et al.,* 1999; Islas *et al.,* 2003).

1.2.3 The effect of geochemistry and pH and how it affects thermophillic microbial communities

Geochemistry and pH are also important environmental variables that control the distribution of Bacteria and Archaea. Acidophiles are found in habitats of pH 1-5 in areas like sulphuric pools, geysers and acid mine drains. Neutrophiles are found between pH 6-8 in areas such as the human body, whereas alkalophiles can be found at pH 9-11 and are usually associated with areas where carbonate is found, such as soda lakes and alkaline soil. Cytoplasmic pH is an important aspect of bacterial cell physiology, as it controls permeability of the cell membrane to protons by controlling the activity of ion transport systems that facilitate proton entry (Booth, 1985).

Understanding the relationship between geochemistry and microorganisms is important because production and consumption processes in soils contribute to the global cycles of many trace gases that are relevant for atmospheric chemistry and

climate. Soil microbial processes contribute to the budgets of atmospheric trace gases (Conrad, 1996). Microbes are important in the biogeochemical cycles of carbon, oxygen, nitrogen, hydrogen, phosphorous, sulfur, potassium, magnesium, calcium and iron.

1.3 Antarctica

Antarctica is considered the most extreme continent on Earth due to its windy and cold climate with average temperatures of between -40°C and -50°C. It is also the world's largest desert with the polar plateau having snowfall of less than 5 cm per year. Due to its isolation from human activity and pollution, geographical position and physical characteristics, Antarctica remains one of the most pristine places in the world. For these reasons, Antarctica is worthy of scientific study as little is known about this extreme, isolated continent (Hansom and Gordon, 1998).

The geology of Antarctica is fundamental to understanding the formation of the continents in the southern hemisphere, with super-continent Gondwanaland breaking up into fragments about 180 million years ago. Antarctica was at the centre of this giant land mass with the other continents surrounding it (South America, India, Australia and New Zealand). The final break-up phase was the rifting of New Zealand from the Marie Byrd sector, which occurred 84 million years ago (Hansom and Gordon, 1998).

Surprisingly, about 2% of Antarctica is ice-free and is primarily comprised of the Antarctic Peninsula, the Transantarctic Mountains and the scattered coastal oases. Despite being extremely restricted areas, the ice-free zones are of immense importance not only as habitats and breeding grounds for the flora and fauna of the Antarctic but also in providing evidence for Antarctic geological and glacial history. Other minor ice-free areas include the steaming ground found in a number of circumpolar islands, South Sandwich Islands and Marie Byrd Land; and on the continent itself, Mount Erebus (Ross Island), Mount Melbourne and Mount Rittmann (northern Victoria Land) (Logan *et al.,* 2000).

1.3.1 Hot mineral soils on Antarctica as an environment and a habitat

Geothermal habitats occur worldwide but the best known are found in Iceland, North America (Yellowstone National Park), New Zealand, Japan, Italy, and Russia (Kristjansson, 1992). These geothermal habitats are associated primarily with tectonically active zones where major crustal movements of the Earth occur. In these areas, deep-seated magmatic materials are thrust close to the Earth's surface and serve as heat sources. The groundwater is highly heated but does not boil because of lithostatic pressure. When the fluid reaches a sufficiently high temperature, the pressure generated forces the fluid through pores and fissures back to the surface of the Earth, where it produces thermal springs and geysers. If the water supply is low, such as that found on the mountains of Antarctica, steam rather than hot water arises to the surface producing fumaroles, as seen on Mt. Erebus. As the hot fluid passes up through the Earth's surface, minerals dissolve and account for the extensive soil mineralization. The exact composition of the thermal waters depends on the chemistry of the underlying rock. The two main buffering systems of thermal springs are sulphuric acid with a pK of 1.8 and carbonate/bicarbonate systems with pK values of 6.3 and 10.2. The acidic buffering system is found in high temperature fields whereas the alkali buffering system is found in low temperature fields (Brock, 1986).

One of the most well-known geothermal habitats, is found on volcanoes. Volcanic activity has occurred on Earth since the early outgassing phase about 4 billion years ago. There are few volcanic areas left today but some can be found in Antarctic, on Mount Melbourne, Mount Rittman and Mount Erebus. Because water is present on these three mountains, they may represent some of the oldest habitats for cellular life (Brock, 1986).

A number of studies have investigated the bacterial and archaeal communities living on the steaming grounds of volcanoes in Antarctica. Endospore-forming bacteria were isolated from active fumaroles on Mt. Rittman and Mt. Melbourne, and from active and inactive fumaroles on Candlemas Island, South Sandwich

archipelago and Deception Island (Cameron & Benoit, 1970). Cameron and Benoit (1970) found specimens of *Corynebacterium*, *Mycobacterium*, and *Nocardia spp*., as well as *Pseudomonas sp*., and *Bacillus sp*. on Deception Island. On Candlemas Island, Logan *et al.,* 2000, found a dominant, moderately thermophilic and acidophilic, aerobic endospore-forming bacteria and performed genetic fingerprinting and routine phenotypic tests. They found that the Candlemas Island isolates were not distinguishable from the Mt. Rittman strains, although the two sites were 5600 km apart. A similar study isolated thirteen strains of endospore-forming bacteria from geothermal soils at Cryptogram Ridge, Mt. Melbourne, and at the vents and summit of Mt. Rittman in northern Victoria Land. They used 16S rRNA gene sequencing, SDS-PAGE and routine phenotypic characterisation tests and found novel species of *Aneurinibacillus* and *Brevibacillus* (Allan *et al.,* 2005). In 2004, a study to determine physico-chemical properties and isolate existing strains of heterotrophic microorganisms through molecular genetic techniques on Mt. Melbourne, found that physico-chemical features of geothermal grounds might affect the colonisation history and dispersal of microorganisms (Bergagli *et al.,* 2004). Five strains of thermophilic bacteria from Cryptogram Ridge, Mt. Melbourne were found, including a novel thermophilic Gram-positive bacillus, *B. thermoantarcticus* (Nicolaus *et al.,* 1991; Nicolaus *et al.,* 1996).

1.3.2 Mount Erebus and Tramway Ridge

Volcanic activity in Antarctica has continued sporadically throughout the Cenzoic period resulting in the formation of rift volcanoes. Present-day volcanism has only been observed in a few locations. The most well known thermal zones of Mt. Melbourne and the most prominent on Mount Erebus (3794 m), produce fumerolic activity on a daily basis. Erebus, constantly active since 1972, is the only active volcano with an anorthoclase phonolite lava lake in its crater and is the southernmost volcano in the world (Rowe *et al.,* 2000). Mt. Erebus is slightly older than 1 million years and its summit is significantly younger than 100,000 years old, this includes the high temperature soil area on Tramway Ridge (Esser, 2004).

Figure 1.1: Tramway Ridge, Mt Erebus, Antarctica

Tramway Ridge is an ice-free area of gently sloping warm ground containing the hottest soils on Mt. Erebus (up to 65°C). Steam and cold Antarctic temperatures released from the soils result in steam condensing and freezing immediately above the vents, with the ice enclosing warm ground within ice hummocks and ice caves and at the bases of ice towers (Hudson and Daniel, 1988; Broady, 1993). The ridge is located at an elevation of 3 350 m above sea level and is approximately 1.5 km northwest from the main crater. Tramway Ridge is defined by Antarctica NZ as an elongated ice-free patch of approximately 10 000 m^2. The ice-free area is contained within a 40 000 m^2 Antarctic Specially Protected Area 130 (ASPA), in which the top half is a restricted zone where entry is prohibited and the bottom half is entry by permit. It has been designated an ASPA because of its unusual ice-free, high temperature ecosystem (Management plan, 2002) produced by condensation of steam from the supply of geothermal heat and the moist fumaroles (Allan *et al.,* 2005). The site offers a unique opportunity to study the microbiology of probably the most southern high-temperature soils on Earth, separated from other global volcanic features for over 30 million years.

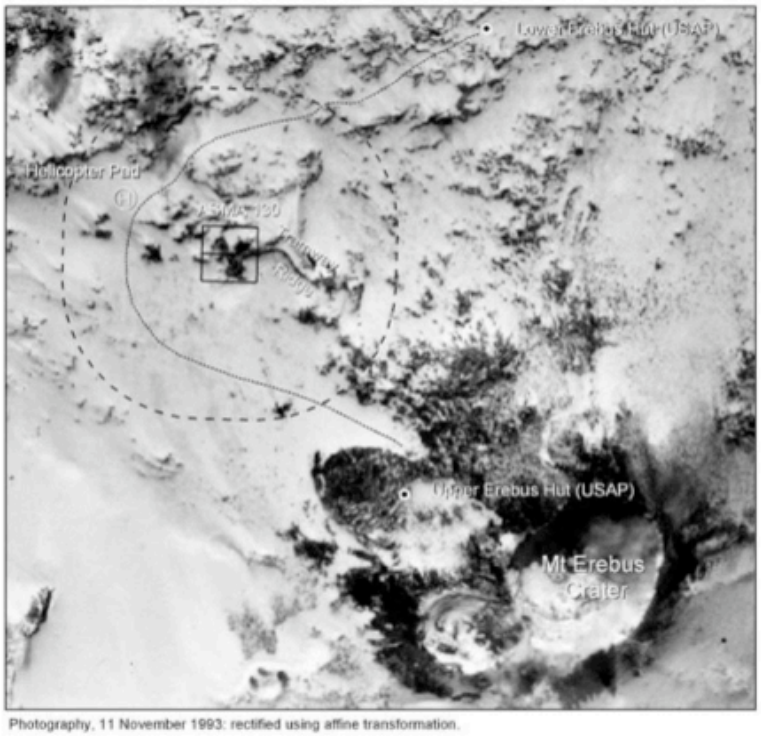

Figure 1.2: Tramway Ridge, ASPA 130, Mt. Erebus, Antarctica

On Tramway Ridge, different components of the biota colonise the ground at different temperatures. The innermost zones, closest to the steam emissions have surface temperatures of 40 to 60°C and are coated by a black mat dominated by a thermophilic strain of *Mastigocladus laminosus*. The cooler zone of 30 to 40°C is coated with a reddish-brown algal mat and in the outer-most zone, which is 0 to 30°C is dominated by coccoid chlorophytes and moss protonema which form green crusts (Broady, 1993).

The microbiology of fumerole areas, such as Tramway Ridge and other high temperature sites is unique because they contain organisms that are found nowhere else in Antarctica (Broady, 1993). They have also been poorly researched using only classical cultivation approaches. An early expedition by Ugolini and Starkey (1966), detected various bacteria and fungi but no actinomycetes in samples collected by an active fumerole. Approximately 9 x 10^6 bacteria per unit area, were found by plating on albumin agar and 6,500 fungi by plating on peptone-dextrose novobiocin agar. Among the fungi were species of *Penicillium*, *Aspergillus* and *Neurospora* and a yeast, as well as a fungus that failed to produce spores. They recorded that the bacteria and fungi densities

equalled many fertile cultivated soils of temperate regions. Another study looked at microorganisms with acridine orange direct (AOD) and most probable number (MPN) counts, as well as preliminary studies using culture-dependent methods to observe thermophiles on Tramway Ridge. They found members of both *Clostridium* and *Bacillus*, which demonstrates that thermophilic bacteria are found on Mt. Erebus, in steam-warmed soil (Hudson and Daniel, 1988). A closer investigation was directed at *Bacillus schlegeli*, a CO and H_2 oxidizer, which could be used as a carbon and energy source for bacteria living on Tramway Ridge, as there was no trace of sulfur, as seen at Yellowstone National Park and other geothermal areas (Hudson *et al.,* 1988).

As only culturing techniques have been used to look at microbial populations previously on Tramway Ridge, I have used both culture-independent techniques and a preliminary culture-dependent study to describe the microbial communities and observed the geochemistry of the soil to understand the molecular ecology at Tramway Ridge.

1.4 Approaches used to investigate the microbial ecology of geothermal habitats

Two different approaches can be used to investigate microbial ecology of geothermal habitats. One approach is culture-dependent, which involves growing bacteria and isolating them. The other is culture-independent, which relies on PCR-based methods to amplify the 16S rRNA gene which is then used to identify the bacteria.

1.4.1 Culture-dependent approaches

Culturing involves preparing a solid or liquid growth medium containing a suitable carbon source, energy source and electron acceptor depending on the physiological type of organism being isolated. The medium is inoculated with the microorganisms and left to incubate at a desired growth temperature until the

organisms multiply so that the media becomes turbid. The microoganisms can then be isolated by several methods, such as sample dilution (Janssen *et al.,* 2002), micromanipulators and optical tweezers (Fröhlich and König, 2000), the construction of stimulated natural environments (Kaeberlein *et al.,* 2000), cell encapsulation in gel microdroplents (Zengler *et al.,* 2002), density-gradient centrifugation (Kvist *et al.,* 2007), and cell sorting using flow cytometry (Hugenholtz, 2002). The past 25 years have seen many advances in culturing work, for example, the isolation of diverse hyperthermophilic bacteria and Crenarchaeota and the sequencing of the first archaeal genome. Most recently, there has been a change from using nutrient rich media to minimal nutrient media to mimic the environment. These simple methods are resulting in previously uncultured bacteria being isolated. Joseph *et al.,* (2003) cultured a collection of 350 isolates from soil by using simple solid media in petri dishes. The isolates were assigned to 60 family-level groups and 27% of the isolates belonged to 20 as-yet-unnamed family-level groupings, many from poorly studied bacterial classes and phyla.

Problems have arisen with culturing techniques because the growth media are selective for different types of bacteria. Therefore, cultivation in the laboratory may not be truly representative. In addition, some bacterial cells are not accessible for cultivation because they have unknown media growth requirements or are bound to soil sediments and are not detected by conventional microscopy (Muyzer *et al.,* 1993). It has been shown that enrichment in liquid batch culture yields only a few strains of bacteria with a specific phenotype. Another limitation is that liquid media usually select for fast-growing organisms, although some researchers have shown that both fast- and slow-growing organisms possessing similar genotypes are found in natural communities. However, slow-growing organisms are often out-competed by the faster growing species in culture (Dunbar, White and Forney, 1997). Plating studies have shown that less than 1% of the total bacterial population can be cultivated on standard media. Recently, culture-independent approaches have been used increasingly for assessing biodiversity (Øvreås and Torsvik, 1998).

1.4.2 Culture-independent approaches

It is now well accepted that an accurate picture of the diversity of Bacteria and Archaea can rarely be obtained using classical cultivation approaches because a high proportion of representatives cannot be cultivated under the limited enrichment provided. In 1987 Woese described 12 bacterial domains that could be identified through cultivation, but today the number of identifiable bacterial divisions has more than tripled to about 40 due mainly to culture-independent techniques. A combination of various genetic culture-independent techniques are used to better describe the diversity and structure of microbial communities (Hugenholtz *et al.,* 1998).

Cultivation-independent methods bypass the need to cultivate a microorganism in order to determine its identity. In cultivation-independent methods, the bulk nucleic acids are extracted directly from the environmental samples and the specific phylogenetic marker genes (16S ribosomal) DNA is amplified (PCR). The DNA varies among species and is either viewed using DNA fingerprinting tools, cloning or both. The 16S gene can then be sequenced to infer phylogenetic relationships among microorganisms.

The 16S rRNA gene is used because most of the nucleotide sequences are conserved between species and it provides sufficient sequence to produce robust phylogenetic trees (Olsen *et al.,* 1986). Other markers that can be used include genes coding for other rRNA molecules, such as 5S rRNA or the 16S-23S intergenic spacer region (Fisher and Triplett, 1999; Ranjard *et al.,* 2001) or phospholipid fatty acids (Frostegård *et al.,* 1997; Jahnke *et al.,* 2001). These biomarkers can be cloned into plasmids and inserted into cells such as *Escheria coli* to produce clone libraries.

In order to obtain the 16S rRNA gene, nucleic acids need to be extracted. To date, two different extraction methods have been used to extract DNA from soil, a direct and an indirect method. Torsvik and Goksoyr describe an indirect DNA extraction method that produces high quality DNA and involves the separation of

bacterial cells from soil particles by differential centrifugation, followed by cell lysis, DNA recovery and several DNA purification steps. The direct DNA extraction method, which produces higher yields of DNA, involves the release of DNA from cells by physical disruption without separating the cells from the soil matrix, followed by alkaline extraction and a series of purification steps (Ikeda *et al.,* 2004). Physical disruption methods include bead mill homogenization, French press, boiling, microwave treatment, freeze-thaw cycles and sonication (Pettit, 2004). Once the total community DNA is extracted, it can then be amplified and either cloned into plasmids or identified using fingerprinting methods.

DNA fingerprinting tools have been developed to describe diverse microbial communities in various environments by providing a pattern or profile of the genetic diversity in a microbial community. These tools include Denaturing Gradient Gel Electrophoresis (DGGE) (Fischer and Lerman, 1979), temperature-gradient gel electrophoresis (TGGE) (Rosenbaum and Riesner, 1987; Felske *et al.,* 1999), Restriction Fragment Length Polymorphism/amplified rDNA-restriction analysis (RFLP/ARDRA), terminal restriction fragment length polymorphism (tRFLP), Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.,* 1995; Keto-Timonen, 2005), Random Amplified Polymorphic DNA analysis (RAPD) (Welsh and McClelland, 1990) and Automated Ribosomal Intergenic Spacer Analysis (ARISA) (Ranjard *et al.,* 2001).

A recent addition to the suite of DNA fingerprinting tools, Automated rRNA Intergenic Spacer Analysis (ARISA) (Fisher and Triplett, 1999) uses the 16S-23S intergenic region of the total Archaeal, Bacterial or Cyanobacterial community DNA, which is amplified with a fluorescently labeled primer. An automated capillary electrophoresis system is then used to provide laser detection producing an electrophoregram with the peaks corresponding to the fluorescent DNA fragments, 400 to 1400bp in length (Fisher and Triplett, 1999). Unlike the conservative 16S and 23S genes, the intergenic region is heterogenous in length and nucleotide sequence due to the presence of several functional units, such as tRNA genes, therefore each taxa is represented by the corresponding peaks (Ranjard *et al.,* 2000; González *et al.,* 2003). ARISA has proven to be rapid, sensitive and provides highly reproducible community-specific profiles (Fisher

and Triplett, 1999; Cardinale *et al.,* 2004). It has been used to look at bacterial and fungal communities in freshwater (Fisher and Triplett, 1999), marine (Hewson *et al.,* 2004; Brown *et al.,* 2005; Leuko *et al.,* 2007) and soil (Ranjard *et al.,* 2001; Hewson *et al.,* 2003; Cardinale *et al.,* 2004).

Like other PCR-based techniques, ARISA has its pitfalls in that there is only a small database available for the ITS region, Ribosomal Internal Spacer Sequence Collection (RISSC) (http://egg.umh.es/rissc/). Therefore ARISA can be coupled with 16S cloning library, RFLP and sequencing to determine the different organisms in a community.

Other DNA fingerprinting techniques such as colony hybridization and restriction fragment length polymorphism (RFLP) of cloned rRNA inserts have been applied to screen clone libraries. In RFLP, DNA is digested with a 4bp cutter restriction endonuclease to produce different patterns to allow the user to differentiate between species. tRFLP is differentiated by adding a fluorescent dye to label the end of the PCR product and amplifying it. In AFLP, a 4-cutter and a 6-cutter restriction endonuclease are used with the fragments ligated to end-specific adaptor molecules. A PCR amplification is performed using primers complementary to each of the two adaptor sequences. In RAPD, several arbitrary short primers (8-12nts) are created and then fragments are amplified from a large template of genomic DNA, producing patterns.

Once DNA fingerprints are obtained, pattern matching analysis can be performed on the restricted fragments using software, such as Gelcompare (Applied Maths, Kortrijk, Belgium) or the BioImage System (Bioimage Corporation, Ann Arbor, Mich, USA). These comparison software programmes perform identity searches of new DNA fingerprinting patterns in existing databases. The software packages can also perform similarity calculations and cluster analyses of the patterns in the database (Liu *et al.,* 1997; Gerner-Smidt *et al.,* 1998). Each different profile can be used to define an operational taxonomic unit (OTU), with clones sharing the same profile, having the same OTU. Therefore a representative from each OTU can be sequenced to give an overview of the bacterial community.

The problems that arise with culture-independent techniques are with the extraction of intact nucleic acids from bacterial cells present in the sample and the removal of humic acids and bacterial exopolysaccharides which may inhibit the RFLP. Addition of acetamide and cosolvents such as glycerol and dimethylsulfoxide (DMSO) have been used to stop the reannealing of the template DNA which can inhibit primer binding. Also culture-independent techniques rely on PCR-based techniques which can result in biases due to primer selection for certain rRNA genes, therefore missing others. Cloning can also produce problems with different cloning efficiencies for different cloning vectors with different primer pairs (Muyzer and Smalla, 1998).

1.4.3 Culture-dependent and culture-independent approaches used to study microbial ecology and Phylogenetics

From culture-dependent and culture-independent approaches, DNA sequences can be obtained and analysed using a range of tools, such as DNAStar, Clustal and MacClade (Nishimura, 2000; Chenna *et al.,* 2003; Maddison and Maddison, 2005). A problem that arises in the use of PCR to amplify mixed target DNA is the formation of chimeric molecules. Computer algorithms, such as the CHECK_CHIMERA in the RDP and Belepheron can been used to detect chimeric sequences (Larsen *et al.,* 1993).

One DNA sequence programme, SeqMan II is a module of DNAstar that assembles overlapping DNA sequence fragments into a stretch of continuous sequence called a contig. SeqMan II allows the user to remove poor quality data and trim vectors. The sequences can then be exported in DNASTAR, GenBank or FASTA file formats for use in other modules or analysis programmes (Nishimura, 2000).

Once the sequences have been edited, they can be placed into NCBI BLAST (http://130.14.29.110/BLAST/), an online tool that finds regions of local

similarity between sequences. The programme compares nucleotide or protein sequences and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships among sequences as well as help to identify members of gene families (Altschul *et al.,* 1990). The RDP (http://rdp.cme.msu.edu/) is a curated database that offers ribosome-related data, analysis services and associated computer programmes. The offerings include phylogenetically ordered alignments of ribosomal RNA (rRNA) sequences, derived phylogenetic trees, rRNA secondary structure diagrams and various software for handling, analyzing and displaying alignments and trees (Maidak *et al.,* 1996).

Once the sequences have been compared they can then be placed into Greengenes. An online tool, Greengenes, (http://greengenes.lbl.gov/) has been developed to provide features such as a standardized set of descriptive fields, taxonomic assignment using five different independent curators, chimera screening and ARB compatibility, which a filter updates weekly from Genbank. Greengenes also can align thousands of 16S rRNA genes via the NAST (Nearest Alignment Space Termination) algorithm which creates multiple sequence alignments (MSA) of 7,682 characters. The NAST files can then be imported into ARB to produce phylogenetic trees and infer bacterial and archaeal evolution (DeSantis *et al.,* 2006a; DeSantis *et al.,* 2006b).

Phylogenetic programmes, such as ARB, (http://www.arb-home.de/) can be used for local alignment optimization for rRNA data analysis and can be integrated with DNAML PHYLIP (the PHYLogeny Inference Package) which is used to infer phylogenies (evolutionary trees) using parsimony, distance matrix, and likelihood methods, including bootstrapping and consensus trees (Feselstein, 1995; Ludwig *et al.,* 2004). An alternative phylogeny package is Phylogenetic analysis using parsimony (PAUP) which can interact with MacClade (Maddison and Maddison, 2002; Swofford, 2002) and Molecular Evolutionary Genetics Analysis (MEGA) contains distance based and maximum parsimony (Kumar *et al.,* 2004).

1.5 Aims, approach and outline of Thesis

This project examines the soil microbiology distributed along a steep thermal gradient in Tramway Ridge, Mt. Erebus, Antarctica. For the first time, culture-independent molecular genetic tools were used to assess the biodiversity, composition and structure of the microbial communities (Archaea and Bacteria) in these unique soils. Soils were sampled along a predetermined physico-chemical gradient (temperature-pH) to assess how the local environmental conditions drive the microbial community composition and structure.

Using a DNA fingerprinting approach coupled with a preliminary culture-dependent study, I have assessed the diversity of the communities on Tramway Ridge.

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Chapter 2 : Microbial Biodiversity of Thermophilic Communities in Hot Mineral Soils of Tramway Ridge

2.1. Abstract

Only a few studies have looked at microbial biogeography in soils and whether microorganisms are endemic to an area is still debatable. Tramway Ridge, a geothermal area on Mount Erebus, Antarctica, provides a unique opportunity due to its isolation and extreme conditions to explore the possibilities of microbial endemism and to identify novel Bacteria and Archaea. This site was chosen for a culture-independent study with a preliminary culturing survey for bacterial communities along three temperature gradients (65°C – 2.5ºC). In addition, a physico-chemical analysis was undertaken to identify which environmental factors were driving the different diversity along the transects. An automated rRNA intergenic spacer analysis (ARISA) was used to assess the diversity across the transects using Bacteria and Cyanobacteria-specific primers and results showed that temperature and pH were the main drivers for these communities. Due to its unique physico-chemical and ARISA profile, a hot temperature site (T-3A, 65ºC) was chosen for further investigation by bacterial and archaeal 16S rDNA clone libraries. Unique rDNA types among the 78 bacterial and 83 archaeal clones were identified by restriction fragment length polymophisms and 18 bacterial and 5 archaeal operational taxonomic units (>97% identity) were observed. All of the bacterial sequences were loosely affiliated with other recognised bacterial divisions, with 40% of the sequences not affiliated to any genus. The archaeal clones were found to be deep-branching and sequences clustered together within the Crenarcaheota. In addition, two strains of Bacilli were isolated. The novel microorganisms show that the Tramway Ridge communities are unique from organisms found in other environments and show that “Everything is (not) everywhere”.

2.2 Introduction

Organisms that survive in extreme thermal environments have challenged our understanding of the physical and chemical constraints on life and stimulated new theories on how life originated on earth and the possible existence of life on other planets (Rothschild and Mancinelli, 2001; Cavicchiolli, 2002; Sakon *et al.,* 2006). Harsh habitats including hot springs and geysers (Kimble *et al.,* 1994; Norris *et al.,* 2002; Kumar *et al.,* 2004), deep-sea hydrothermal vents (Takai *et al.,* 2001; Sako *et al.,* 2003; McCliment *et al.,* 2006), cold and hot deserts (Peters and Conrad, 1995; Nagy *et al.,* 2005) and polar ice (Staley and Gosnik, 1999; Junge *et al.,* 2002) continue to provide a rich resource of novel microorganisms that appear uniquely suited for these extreme environments. One extreme environment that has received little attention is high temperature soil systems such as those found on geothermally heated volcanic grounds (Broady *et al.,* 1987; Nicolaus *et al.,* 1991). It has been suggested that these areas may provide a portal into Earth's subsurface environment, allowing us possible access to ancient and unique microbial lineages (Pepi *et al.,* 2005; Romano *et al.,* 2005).

Antarctica is considered the most extreme continent on Earth due to its severe katabatic winds and intensely dry and cold climate, with average temperatures ranging between -40°C and -50°C (Parish and Bromwich, 1991; Hansom and Gordon, 1998). With more than 1 000 km separating Antarctica from South America and 4 000 km from South Africa and New Zealand (Kennedy, 1993), Antarctica's geographic isolation and climatic severity have allowed it to remain one of the most pristine and least studied continents on Earth (Hansom and Gordon, 1998). While only 2% of the Antarctic is ice-free, mostly found in the Antarctic Peninsula, Transantarctic Mountains and scattered coastal oases (Logan *et al.,* 2000), these ice-free areas are immensely important as exposed habitats and breeding grounds for the limited flora and fauna of the Antarctic (Broady, 1993). In contrast to the predominant cold habitats, Antarctica and its near-by islands also contain small isolated geothermal areas associated with regions of current volcanic activity. These areas include several circumpolar islands including Deception Island, the South Sandwich Islands, Bouvetøya, Marion Island and Îles Kerguelen and continental volcanoes including Mount Erebus, Mount Melbourne

and Mount Rittmann (Logan *et al.,* 2000). Soil surface temperatures at these sites can reach up to 65°C, providing resident microflora with a stable high temperature environment with constant free water from the condensation of steam and melting of snow, and often shelter from wind by ice hummocks (Bargagli *et al.,* 1996).

Mount Erebus is located on Ross Island (77°32'S, 167°10'E, elevation 3 794 m) and is the largest and most active volcano in Antarctica. It is a unique stratovolcano with a convecting anorthoclase phonolite lava lake and is central to the most extensive alkali volcanic province in the world (Esser, 2004). Mount Erebus has been active for the past 1.3 million years, producing frequent strombolian eruptions with less frequent ash production (Rowe, 2000). Located 1.5 km northwest of the main crater, is a unique, ice-free gently sloping geothermal area known as Tramway Ridge (77°31'05"S, 167°06'35"E, elevation 3 350 to 3 400 m, Broady, 1993). This area contains many isolated steam fumaroles that produce moist, hot soils which can reach up to 65°C (Hudson and Daniel, 1988). Steam released from the hot soils condenses and freezes immediately above the vents, often enclosing the warm ground within ice hummocks (Hudson and Daniel, 1988; Broady, 1993). Earlier studies (Ugolini and Starkey, 1966; Hudson and Daniel, 1988; Melick *et al.,* 1991) that investigated bacterial communities on Tramway Ridge using classical cultivation approaches were only able to successfully cultivate a number of novel thermophilic members of *Clostridia* and *Bacillus*, which have been described in many other environments.

It is now well accepted that classical cultivation approaches do not provide an accurate assessment of the diversity of Bacteria and Archaea in a given sample because a high proportion of these organisms cannot be cultivated under the limited enrichment provided (Hugenholtz *et al.,* 1998; Ovreas and Torsvik, 1998; Marchant *et al.,* 2002). Genetic-based culture-independent methods bypass the need to cultivate a microorganism in order to determine its identity and have been used to describe diverse microbial communities in various environments (Giovannoni, 1991; Muyzer, 1993; Hugenholtz *et al.,* 1998). DNA fingerprinting tools, such as automated ribosomal intergenic spacer analysis (ARISA) (Fisher and Triplett, 1999) coupled with 16S rRNA gene library characterization, can be

used to rapidly assess the relative microbial diversity and community structure in a given environmental sample (Fisher and Triplett, 1999). The combination of both culture-independent and culture-dependent tools allows us to identify the communities as well as to understand the physiology and characteristics of these individuals.

For the first time, an analysis and description of the physiochemical environment and associated microbial community living along a steep thermal gradient on the warm grounds of Tramway Ridge site were conducted. We employed both culture-independent approaches with a preliminary culturing study to characterize microbial diversity along a steep thermal gradient in the site. Composition and structure in concert with physico-chemical environmental measurements allowed us to assess and predict which parameters may be essential in driving the microbiology at the Tramway Ridge site. From our analysis, we identified an extraordinary number of deeply branching novel clades in both Bacteria and Archaea whose distribution appear driven by temperature and pH. These observations suggest that this geographically isolated and environmentally extreme site contains unique lineages that may be localized to the site (Staley and Gosnik, 1999).

2.3 Methods and Materials

2.3.1 Soil collection and physico-chemical analysis

Our hypothesis was that temperature was the main environmental factor that was driving the diversity of the bacterial communities on Tramway Ridge, Mt Erebus, therefore four different temperature measurements were taken; along three transects using a temperature probe, a depth measurement over 20 hrs, measurements along one transect for 20 hrs and a measurement at one site for 10 months. Soil temperatures were measured along three replicated transects (T-1, T-2, T-3) on Tramway Ridge, Mt Erebus (167°06’40’E, 77°31’06’S, elevation 3 376 m) in January 2006. Five or six samples (A-E or F) were measured along each

temperature gradient using a calibrated Digi-sense thermocouple probe (Cole Palmer, Illinois, USA). Sample A was always measured at the highest temperature (~65°C) at an active fumarole site in each transect and the subsequent remaining samples were measured at decreasing 10°C intervals to a nearby low temperature area (~5°C). Temperature and relative humidity i-button loggers (Maxim/Dallas Semiconductor Corp, Texas, USA) were placed at 2 cm depth at the hottest site on two transects (T-2A and T-3A) for 20 hrs. Temperature loggers were also placed at a depth of 4 cm for each of the five sample sites on transect 3 (T-3A to T-3E) for 20 hrs. Additionally, one temperature i-button logger was deployed at 4 cm depth at T-3A for 11 months and recovered the following season (Nov. 2006).

Soil was collected from 2-4 cm depth at each location in the transect with a sterile spatula and stored in both sterile Whirlpaks® (Fisher Scientific Ltd., Ontario, Canada) and 50-ml sterile polypropylene tubes. Samples were stored at -20°C until further analysis. In addition, a soil sample (S171), from a low temperature, ice-free arid area in the Dry Valleys (Miers Valley - 163°48'58'E, 78°06'05'S) was collected in Nov 2006 for comparative purposes.

Geochemical analyses were undertaken on each sample in an attempt to identify the major parameters driving microbial community composition and structure in the soil across the intense thermal gradient. pH was determined by slurry technique (1:2.5 mass ratio of samples and deionized water) using a Eutech instrument PC5500 (Eutech, Illinois, USA) according to Edmeades *et al.,* (1985). Moisture content was estimated gravimetrically by drying a subsample (15-45 g) at 105°C until constant weight was achieved. Total organic carbon and total organic nitrogen of the soil were measured with a Leco TruSpec CN analyser (Leco, Minnesota, USA). Soil organic matter was determined by loss on ignition (Heiri *et al.,* 2001). Soil particle size was analyzed by a Malvern Mastersizer 'S' laser diffraction particle size analyzer (Malvern Instruments Ltd, Worcestershire, UK) after a pretreatment with H_2O_2 and $Na_4P_2O_7.10H_2O$ (Konert and Vandenberghe, 1997). Elemental compositional analysis was undertaken to assess the contribution of 27 different elements in the soil samples using ICP-OES/AA with a Perkin-Elmer Elan DRC11 Inductively Coupled Plasma - Mass Spectrometer (GE Healthcare, Auckland, New Zealand) after digestion with

diluted HCl and HNO_3 (Chen and Ma, 2001). Elements assessed were: Li^{7}, B^{10}, Na^{23}, Mg^{24}, P^{31}, K^{39}, Ca^{43}, V^{51}, Cr^{52}, Fe^{54}, Mn^{55}, Co^{59}, Ni^{60}, Cu^{63}, Zn^{68}, Ga^{71}, As^{75}, Se^{82}, Sr^{88}, Ag^{109}, Cd^{111}, In^{115}, Ba^{137}, Tl^{205}, Pb^{207}, Bi^{209}, U^{238}. Due to the samples being replicates along the transects, all sixteen samples from the three transects were grouped based on temperature (for example, Group 1 contained the hottest samples, T-1A, T-2A and T-3A through to group 6, which contained the coldest sample, T-1F). A univariate analysis of variance (ANOVA) was performed with the PRIMER 6 software (Primer-E Ltd, Plymouth, UK) to identify if there were any significant differences in the physico-chemical properties between these groups.

2.3.2 Extraction of genomic DNA and DNA fingerprinting analysis

Genomic DNA was extracted from 0.6 g of soil from each transect and Dry Valley sample, by a CTAB/bead-beating method as described by Coyne *et al*., (2001). DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Inc., Delaware, USA). DNA samples were electrophoresed on a 1% agarose gel at 100V for 20 mins, stained for 20 mins with ethidium bromide, destained for 10 minutes and the DNA was visualised by transillumination and recorded using an Alpha Innotech Imaging System (Alpha Innotech, California, USA).

ARISA is a rapid and reproducible method of microbial community fingerprint analysis (Fisher and Triplett, 1999; Cardinale *et al.,* 2004; Brown *et al.,* 2005), which can be used to identify differences between transect sites. Bacterial 16S-23S rRNA intergenic spacer region (ITS) was amplified by PCR as described in Cardinale *et al.,* (2004) from each of the 17 samples. Each reaction contained; 0.25 μM of each bacterial primer, ITSF (5'-HEX-GTCGTAACAAGGTAGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3'), 1 X Taq PCR buffer with $MgCl_2$ (Roche Diagnostics, New Zealand), 1.5 U of Taq DNA polymerase, 0.2 mM dNTPs and 20 to 100 ng of gDNA. The mixture was held at 94°C for 5 min, followed by 30 cycles of 94°C for 45 sec, 55°C for 1 min, 72°C for 2 min, and a final extension at

72°C for 7 min. PCR for cyanobacterial ARISA was performed as previously described in Wood *et al.*, (2007). Each reaction contained 0.6 μM Cy-ARISA-F (5'-FAM-GYCAYRCCCGAAGTCRTTAC-3') and 23S30R (5'-CHTCGCCTCTG TGTGCCWAGGT-3') (Wood *et al.,* 2007), 1 X Taq PCR buffer, 4 mM $MgCl_2$, 4 mM BSA, 1 U of Taq DNA polymerase (Roche Diagnostics, New Zealand), 0.2 mM dNTP. The mixture was held at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 2 min, and a final extension at 72°C for 7 min. All PCR reactions were run on a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Masachusetts, USA). Triplicate PCR reactions were pooled and cleaned using a QuickClean PCR Purification Kit (Genscript Corporation, New Jersey, USA). Amplicons were diluted 1:10 with sterile water and 2 μL of diluted product was mixed with 0.25 μL of ROX-labeled genotyping internal size standard ETR900R (GE Healthcare) and the balance of the sample was made to 10 μL with 0.2 v/v tween-20 in sterile water. Intergenic spacer lengths were determined by capillary electrophoresis using the MegaBACE 500 DNA Analysis system (GE Healthcare). Electrophoresis was conducted with a 44°C separation temperature, 10kV voltage and 120 min separation time.

2.3.3 Statistical analysis of ARISA fingerprints

ARISA fragment lengths (AFL) were processed using Genetic Profiler V.2 (GE Healthcare) and then transferred to Microsoft Excel for further analysis. AFLs were aligned using an Excel Macro. AFL that were less than 2 bp different in length were considered identical and only the AFL with the highest florescence was maintained. AFL below 250 Fluorescence Units were discarded as they were deemed to be background noise. Any AFL shorter than 300 bp were considered artifacts and removed. AFL were transposed to presence/absence data for all further analysis. A similarity-ranking test was performed to identify which AFLs were similar between each of the samples from each transect for bacterial and cyanobacterial community structure.

To examine which subset of physico-chemical parameters may best explain the difference in AFL profiles (i.e. biotic structure) the BEST procedure of the

PRIMER 6 software was used (Primer-E Ltd., Clarke and Gorley 2006). The BEST procedure calculates the value of Spearman's rank correlation coefficient (ρ) using every possible combination of variables until it finds the 'best' fit (i.e. the combination of parameters whose Euclidean distance matrix gives the highest value ρ). To visualize the relative similarity of AFL profiles in each sample, nonmetric multidimensional scaling (MDS) based on the Bray-Curtis similarity index was used. The relative distances between sites in these ordination plots indicate the relative similarity of the AFL profiles. Plots with a stress value less than 0.20 provide interpretable information (Clarke 1993). MDS was undertaken with 100 random restarts and the results plotted in two-dimensions. Agglomerative, hierarchical clustering of the Bray-Curtis similarities was carried out using the CLUSTER method of the PRIMER 6 software (Primer-E Ltd) and the results were plotted onto the two-dimensional MDS at similarity levels of 20% and 40%.

2.3.4 16S rRNA clone library construction and analysis

The 16S rRNA genes of Bacteria were amplified in triplicate using the universal bacterial primers 27F (5'-AGAGTTTGATC(AC)TGGCTCAG-3') and 1518R (5'-AAGGAGGTGATCCA(ACGT)CC(AG)CA-3') (Giovannoni, 1991). Each 25 µL amplification reaction contained 10 ng of template, 1 X Taq PCR buffer, 25 mM $MgCl_2$, 1 U of Taq DNA polymerase (Roche), 0.25 mM dNTPs, 1% DMSO and 0.5 µM of each primer with sterile water to 25 µL. The following thermal cycling conditions were used; 2 min denaturing step at 94°C was followed by 34 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min with the annealing time being gradually increased by 2 sec per cycle, with a final extension at 72°C for 7 min. The 16S rRNA genes of the resident Archaea were amplified in triplicate using the universal archaeal primers 21F (5'-TTCCGGTTGATCCY GCCGGA-3') and 958R (5'-YCCGGCGTTGAMTCCAATT-3') according to DeLong, 1992, Bano, (2004) The following thermal cycling conditions were used; 5 min denaturating step at 94°C was followed by 34 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 2 min. Final extension was at 72°C for 5 min. The

success of each amplification reaction was determined by resolving on an agarose gel following the procedures above.

The separate triplicate amplifications from Bacteria (27F/1518R) and Archaea (21F/958R) were pooled and cleaned using a QuickClean PCR Purification Kit (Genscript Corporation). Aliquots of the pooled products were cloned using TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Plasmid DNA was isolated from 5 ml overnight cultures using a standard alkaline lysis miniprep protocol (Ausubel *et al.,* 1995). Plasmid DNA from 150 bacterial clones and 87 archaeal clones were re-amplified using the TA vector-specific primers M13F/M13R (Invitrogen) and screened for the correct inserts, then visualized by agarose gel electrophoresis as above. Aliquots from each of the successful clone amplifications (5 μL) were incubated for 1.5 hrs with the restriction enzyme *Hae*III (Roche) according to the manufacturer's specifications. Restriction patterns were visualized on an ethidium bromide stained 2% agarose gels, as above and analysed using Gelcompare II (Applied Maths, Keistraat, Belgium) to distinguish restriction patterns.

A representative of each unique restriction pattern for Bacteria were sequenced unidirectionally using primer 27F (5'-) and for Archaea, bidirectional sequencing with the TOPO M13F and M13R primers. Sequences were compared with each other and those that were >97% similar were grouped into operational taxonomic units (OTU). Representatives for each bacterial OTU in the clone library were further sequenced with primers 907R (5-'CCGTCAATTCCTTTGAGTTT-3') (Lane, 1991) and 1518R to obtain the full 16S rRNA gene sequence. Sequencing was undertaken using the DYEnamic ET Dye Terminator Kit using an ABI 3130XL DNA sequencer or Big Dye v3.1 using a MegaBACE 500 DNA analysis system (Amersham Pharmacia Biotech).

2.3.5 16S rRNA gene library analysis

Sequences were edited and analysed using Seqman II Version 5.5.1 software (DNASTAR Inc., USA). They were compared with the GenBank database using

Basic Local Alignment Search Tool (BLASTn) (Altschul *et al.,* 1997). The sequences were checked for chimera formation using Bellerophon (Huber *et al.,* 2004) and the CHECK_CHIMERA tool of the Ribosomal Database Project (Maidak *et al.,* 2001). Sequences sharing >97% similarity were considered the same OTU. A single representative of each OTU was used in subsequent phylogenetic analyses. Sequences were aligned using Greengenes (DeSantis *et al.,* 2006), imported into ARB (Ludwig *et al.,* 2004) where they were manually aligned. Phylogenetic inferences were made using the PHYLIP package and DNADIST, DNAML, DNAPARS, FITCH, NEIGHBOR and SEQBOOT (Felsenstein, 1993). Pairwise evolutionary distances were computed from percent similarities by the correction of Jukes and Cantor (1969) and the phylogenetic tree was constructed by the Neighbour-joining method (Saitou and Nei, 1987). The support for each node was determined by assembling a consensus tree of 100 bootstrap replicates.

2.3.6 Estimation of soil bacterial and archaeal richness

Rarefaction, richness and diversity were calculated using the DOTUR package, including the nonparametric richness estimators ACE (Chao and Lee, 1992), Chao1 (Chao, 1984) and the Shannon-Weaver diversity index (Schloss and Handelsman, 2005). In all instances, the default settings and the furthest neighbour with a 3% cutoff in DOTUR was used (Schloss and Handelsman, 2005). The distance matrix required for DOTUR was calculated from 16S rRNA gene alignments using DNADIST with a Jukes-Cantor distance correction in PHYLIP 3.66 (Felsenstein, 2006). Clones that were not sequenced but shared the same RFLP pattern were duplicated in the gene alignments.

2.3.7 Aerobic enrichment cultures and isolation

Sterile media of trypticase soy broth (TSB) with 0.2% potato starch and Castenholz Medium D (CMD) (Hudson and Daniel, 1988) were prepared and transported to Tramway Ridge where the pH was adjusted to 8.6 with NaOH. Approximately 0.1 g of soil from sample site T-1A and T-2A was mixed with 5

mls of PBS and then 1 ml was inoculated into the media. Cultures were incubated in the ground at 65°C for 3 days and subsequently transferred to a 65°C incubator at New Zealand's Scott Base (77°51' S, 166°45'). All samples were transferred into fresh media after 14 days and returned to New Zealand for analysis. Cultures were centrifuged for 5 mins at *13 200* x g and media was removed to provide DNA pellets, which were then stored in a -80ºC freezer. gDNA was extracted with a PureLink Genomic DNA Mini Kit™ (Invitrogen) and ARISA analysis was performed on cultures using the bacterial primers, ITSF and ITSReub using the same PCR conditions as above. AFLs were aligned with an excel macro as in section 2.3.3 with the same binning process and compared with the AFLs from the extracted DNA from soil to identify if community structures was identical.

For isolation, batch cultures of CMD inoculated with soil microbes from T-2A were serially diluted. After four transfers to fresh media, 200μL of each sample was streaked on sterile CMD agar plates and incubated at 65°C. Colonies that grew after 48 hrs were streaked onto sterile CMD plates and incubated another 48 hrs then rinsed with 1 ml of sterile CMD broth to collect cells for molecular analysis and cryopreserved at -80°C.

2.4 Results

2.4.1 Physico-chemical analysis

Our hypothesis was that temperature was the main driver of the bacterial diversity at Tramway Ridge. Our intention was to measure temperatures along three transects over a 20 hr period but due to the extreme weather, we were only able to take measurements along one transect, T-3. In addition, the other three temperature measurements were; taken along the three transects using a temperature probe, a depth measurement at site T-3A over 20 hrs and a measurement at site T-3A for 10 months. Temperatures obtained with a temperature probe at 4 cm depth along each of the transects (245 - 549 cm) ranged

from 65°C next to the fumaroles (A), to 2.5-5°C at the cooler sites (E or F) (Table 2.1). Each fumarole was surrounded by a pronounced surface ring of cyanobacterial mat (~35-40°C) with a second outer ring of moss (~25°C). At site T-3A there was less variation in the mean temperatures recorded at 4 cm depth (54.3°C, SD 3.78) than at the near surface position at 2 cm (16.2°C, SD 9.56) over a 20 hr period (see Appendix). Temperatures across transect 3 ranged from 54.3°C to 3.1°C (Table 2.1). Temperatures recorded at the T-3A fumarole site over 10 months, on the whole remained remarkably constant (61.5ºC, SD 3.3). However, 55 pronounced downward spikes in temperature (up to 24 hours) occurred during the 10 months falling to as low as 17°C (Fig. 2.1). Relative humidity measurements at the fumarole vents (T-2A, T-3A) were consistent over a 10 hr period and showed little variability between the sites (see Appendix). The moisture content within and between the transects were not correlated to the temperature gradient.

The soil pH was near neutral (7.4 to 6.8) directly at the fumarole sites, and more acidic (4.68 to 3.48) at sampling sites with temperatures 50°C and lower (Table 2.1). Total carbon at all sites was low (0.06 to 0.24%), with higher amounts found at the fumarole vent sites (Table 2.1). Total nitrogen (0.18% to 0.48%) were also low. C:N ratios (0.2 to 1.5) were higher in the hotter soil sites compared with the lower termperature sites, except for site T-3A, which had the highest total nitrogen measurement (Table 2.1). Moisture content ranged from 15.9% at the cooler sites to 56.69% at the hotter soil sites, with the most moisture found at site T-3C with 76.87%. In general the soil texture along the transects were graded from a sandy loam at the fumarole vent to a clay soil in the colder sites (Table 1). The element analysis showed that aluminium, sodium and potassium were high, with low levels of trace elements in the Tramway Ridge soils.

Table 2.1. Physico-chemical data of the soils from Tramway Ridge, Mt. Erebus, Antarctica sampled in January 2006.

	Physical characteristics				Organic chemistry				Particle Size (%)			Soil
Sample	Temperature (°C)[a]	Distance (cm)[b]	pH	Moisture (%)[c]	% C	% N	C:N	% OM	Sand	Silt	Clay	texture[d]
T-1A	65.0	0	7.40	48.30	0.24	0.19	1.3	0.84	69.3	28.1	2.6	SDL
T-1B	41.3	37	3.88	37.61	0.06	0.18	0.3	1.09	36.8	54.9	8.3	STL
T-1C	24.9	171	3.80	60.28	0.09	0.33	0.3	1.94	33.2	62.5	4.3	STL
T-1D	18.6	177	3.92	20.75	0.04	0.15	0.3	0.72	62.1	33.8	4.1	SDL
T-1E	10.0	417	4.15	22.86	0.07	0.28	0.3	0.76	55.9	40.7	3.4	SDL
T-1F	2.5	549	4.52	17.06	0.04	0.17	0.2	1.15	3.5	28.1	68.4	C
T-2A	65.0	0	7.38	56.69	0.24	0.16	1.5	0.77	48.9	46.7	4.4	SDL
T-2B	50.0	54	5.00	50.09	0.15	0.22	0.7	1.24	45.2	49.3	5.5	SDL
T-2C	34.0	67	3.48	57.03	0.08	0.27	0.3	1.89	30.8	62.6	6.6	STL
T-2D	26.5	92	3.69	44..22	0.07	0.22	0.3	0.76	37.3	55.5	7.2	STL
T-2E	14.0	245	4.15	56.41	0.10	0.31	0.3	1.25	4.1	48.6	47.3	STC
T-3A	62.5 (54.3 ± 3.8)	0	6.80	53.66	0.18	0.48	0.4	0.58	70.2	26.9	2.9	SDL
T-3B	50.3 (30.9 ± 3.5)	49	5.00	44.94	0.10	0.20	0.5	1.52	52.8	42.8	4.4	SDL
T-3C	28.0 (21.2 ± 4.6)	72	3.54	76.87	0.08	0.26	0.3	1.54	25.2	51.5	23.3	STL
T-3D	16.3 (9.6 ± 2.7)	124	4.05	44.76	0.06	0.24	0.3	1.77	39.6	56.3	4.1	STL
T-3E	5.1 (-3.1 ± 4.6)	294	4.68	15.9	0.06	0.17	0.4	0.67	65.7	30.2	4.1	SDL

a. Temperatures were taken at a 10 cm depth using a temperature probe. Numbers in brackets represent the mean and standard deviation of temperature over a 10 month period at 4cm depth along transect 3.

b. Distance is reported from fumarole site (A).

c. Moisture is measured in g of H_2O [dry weight].

d. SDL sandy loam, STL silt loam, STC silt and C clay.

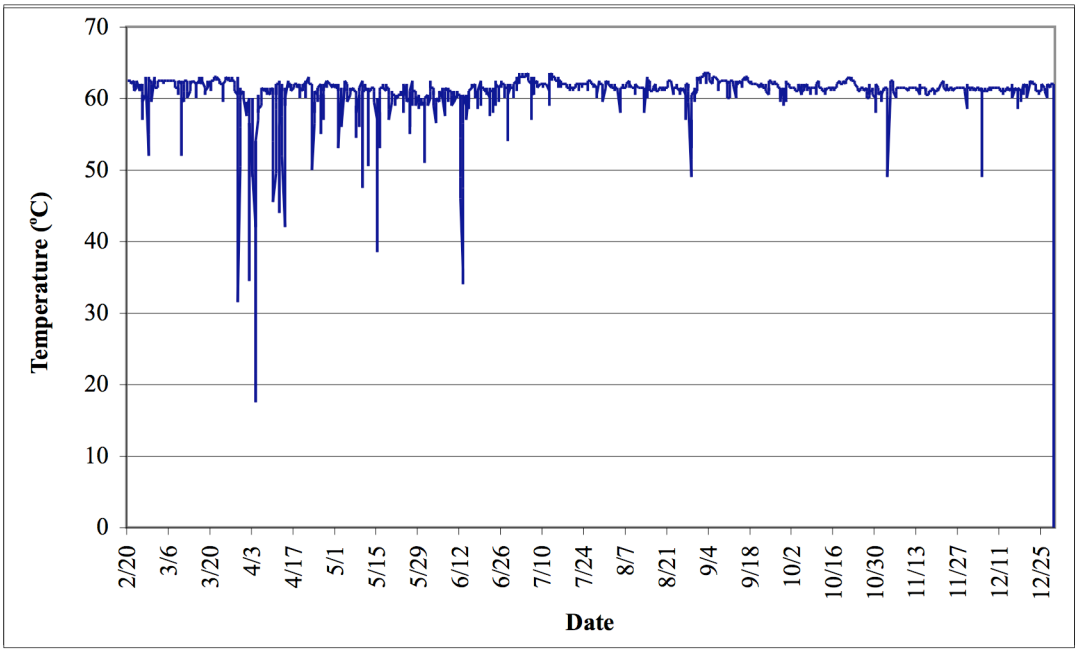

Fig. 2.1. Temperature for 10 months beginning 20 February 2006 for sampling site T-3A at 4cm depth at Tramway Ridge, Mt Erebus, Antarctica.

To identify which environmental factors were significantly different between the temperature groups, an ANOVA was performed. Results showed that temperature ($F = 67.13$, $P < 0.0001$), pH ($F = 35.77$, $P < 0.0001$), total percentage of carbon ($F = 13.91$, $P = 0.0003$), Cr^{52} ($F = 75.29$, $P < 0.0001$) and Ni^{60} ($F = 7.66$, $P = 0.0034$) were the most significant between the groups (see Appendix).

2.4.2 DNA fingerprinting analysis

Analysis of the bacterial ARISA data for all samples identified a total of 125 distinct ARISA fragment lengths (AFL) (i.e. peaks). AFL size ranged from 305 bp to 957 bp. The highest number of bacterial AFLs were observed in T-1E (42), followed by samples T-1F (37) and T-2E (37) (Fig. 2.2A). In general, less bacterial diversity was shown in the hotter samples with an increase in bacterial diversity, as temperatures decreased (Fig 2.2B). Sample T-3B contained only one AFL. Analysis of the

cyanobacterial ARISA data for all samples identified a total of 64 distinct AFLs. These AFL ranged in size from 301 bp to 954 bp. The highest number of AFLs were observed in T-2E (16) followed by samples T-1E (15) and T-1B (15) (Fig. 2.2B). There was no correlation with the number of AFLs observed and the temperature for cyanobacteria. Two-dimensional multidimensional scaling (MDS) shows the relative values of similarity between samples based on the ARISA data. For all 16 transect samples for bacteria, the three highest temperature sites (T-1A, T-2A and T-3A) formed a tight cluster (40% similarity). The other samples along the temperature gradient from Tramway Ridge formed a loose cluster (20% similarity), with the exception of T-2B, T-3B and T-3D, which showed less than 20% similarity with the other samples (Fig. 2.3A). The data for the cyanobacteria again showed tight clustering for the high temperature samples (20% similarity). With the exception of T-2C (less than 20% similarity) all other samples clustered loosely together (20% similarity) (Fig. 2.3B).

To identify which environmental factors may be driving the structure and diversity of the Bacteria and Cyanobacteria communities, a BEST analysis was performed based on the AFLs. Results for the bacteria BEST analysis, showed that the highest Spearman's rho (ρ) correlation of 0.516($P < 0.05$) was due to a combination of temperature, pH and moisture. In the BEST analysis of the Cyanobacteria data the highest correlation ($\rho = 0.668$, $P < 0.05$) involved temperature, pH, %C, Li^7 and Ca^{43} (see Appendix).

To assess the uniqueness of the Tramway Ridge microbial communities samples, an ARISA analysis was undertaken on samples collected from dry soil in Miers Valley (S171) for comparison. The MDS analysis showed that there was no similarity in the bacterial and cyanobacterial communities between the two locations (Fig 2.3A). Sample S171 is not plotted on the cyanobacterial MDS as the major differences in the AFL profile prevented visual assessment of variation in community structure within the Tramway Ridge samples.

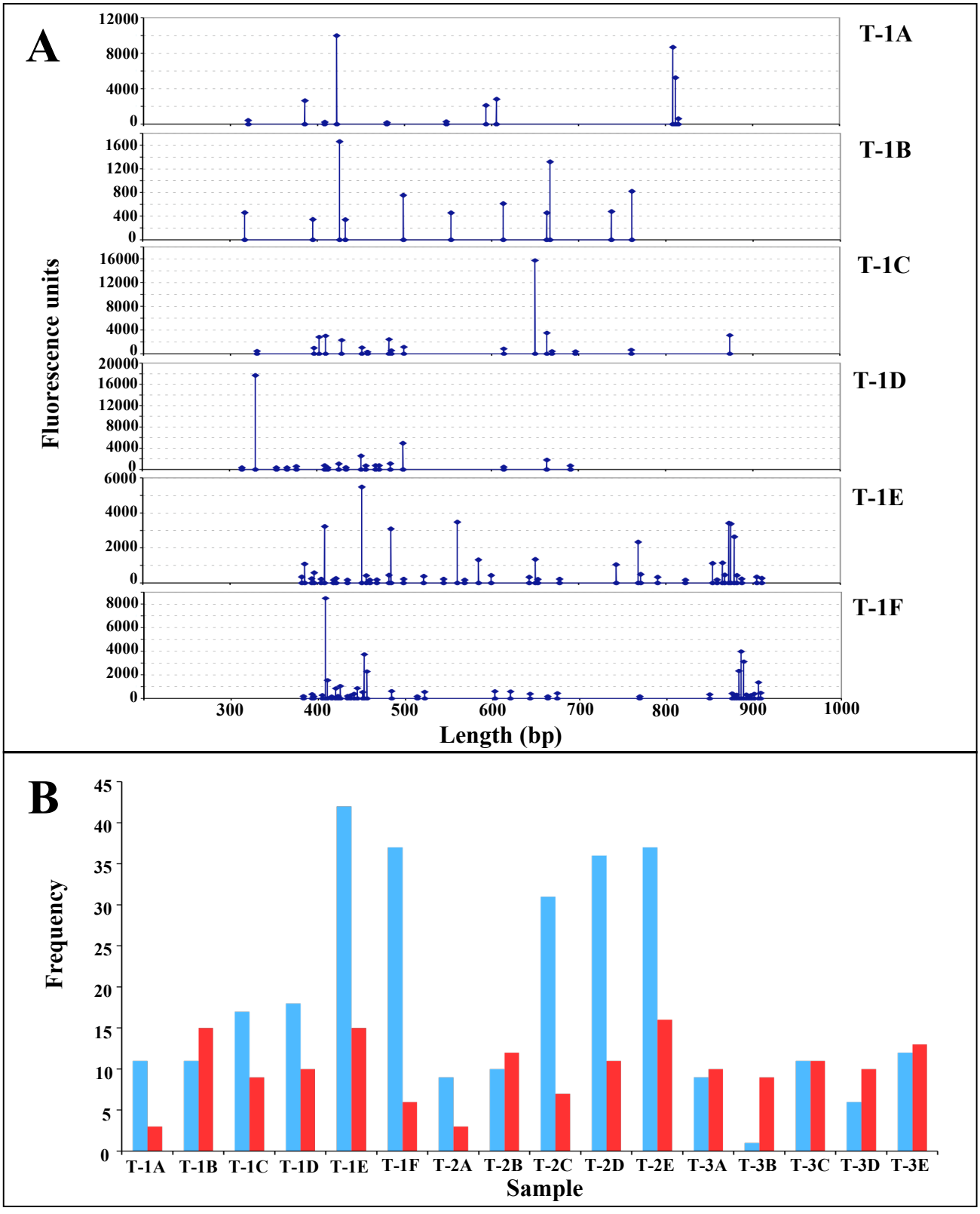

Fig. 2.2. ARISA Fragment Lengths (AFL) for Bacteria and Cyanobacteria. (A) ARISA profiles for samples on transect 3. (B) Number of AFLs for Bacteria and Cyanobacteria. Blue bars represent Bacteria, red bars represent Cyanobacteria.

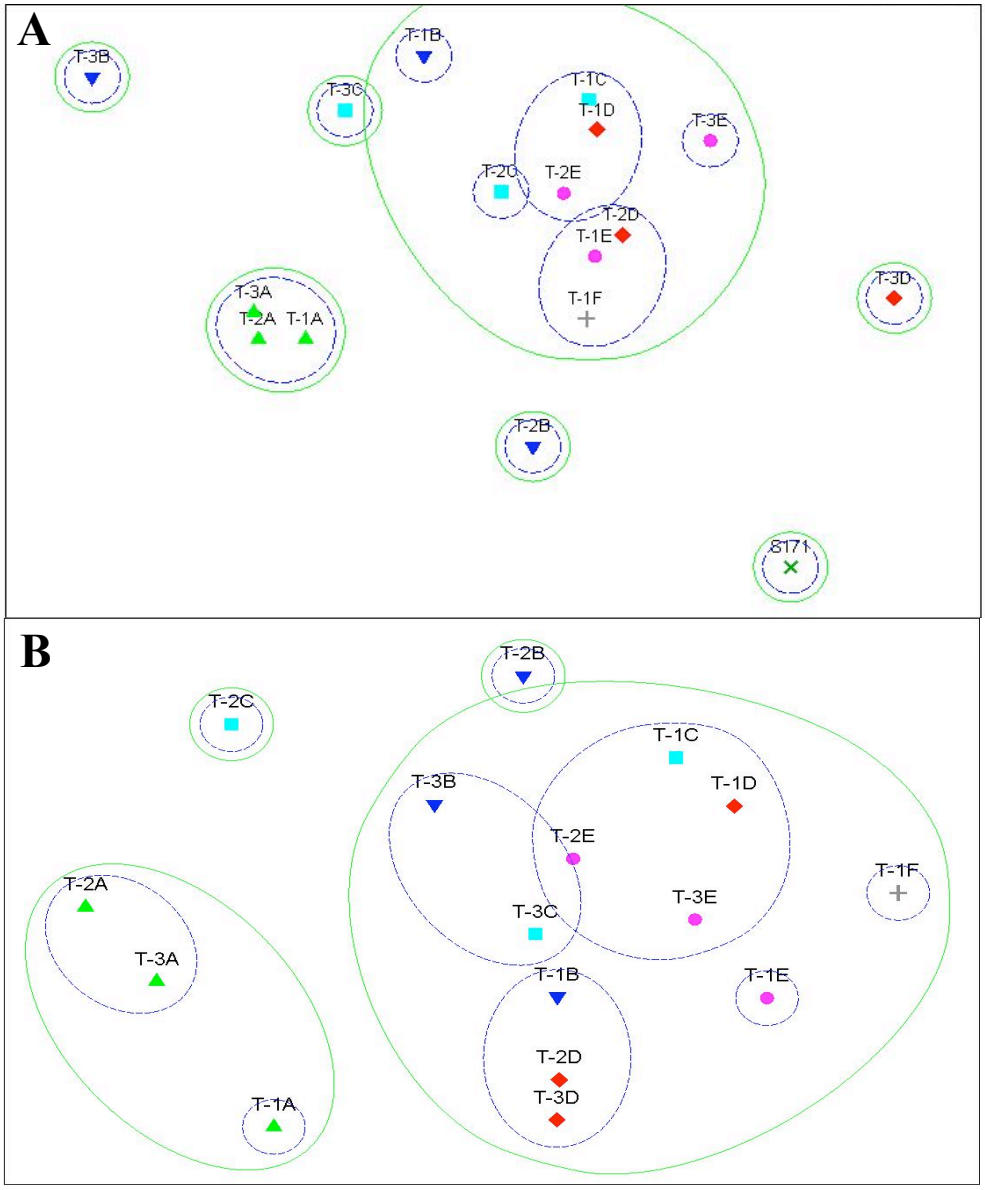

Fig. 2.3. Nonmetric multidimensional scaling (MDS) based on the Bray-Curtis similarity index for Bacteria (A, stress = 0.11) and Cyanobacteria (B, stress = 0.14). ARISA profiles from transects 1, 2 and 3 on Tramway Ridge, Antarctica, 2006 and S171 (bacterial MDS only) from Miers Valley, Antarctica, 2006. Points enclosed by solid lines cluster at 20% similarity, points enclosed by dashed line cluster at 40% similarity. ▲ 65-60°C, ▼ 50-40°C, ■ 34-24°C, ◆ 26-16°C, ● 14-5°C, + 2.5°C and × S171.

2.4.3 16S rRNA gene library analysis

The physico-chemical data and ARISA analysis was used to identify one sample to do further analysis on. The hotter temperature sites showed similar values and because there was more temperature data on T-3A, this sample was used for cloning and phylogeny. A total of 78 bacterial and 83 archaeal clones containing inserts of the correct size were subjected to RFLP analysis. The RFLP analysis identified 61 unique banding patterns for Bacteria and 16 for the Archaea. Samples with unique patterns were sequenced and those >97% similarity were grouped together into operational taxonomic units (OTU). Eighteen OTUs were identified from the bacterial library and 5 from the archaeal library. The dominant OTU were A (16 clones), L (12) and M (12) for Bacteria and U (36) and S (26) for Archaea (Table 2.2).

2.4.4 Phylogenetic analysis

All bacterial sequences recovered from the soil sample T-3A, showed less than 92% similarity, with the majority (14 of 18) having less than 90% identity with their closest known relatives (NCBI BLASTn). Archaeal sequences showed less than 98% sequence identity with other clones (Table 2.2). All the bacterial and archaeal sequences were less than 92% similar to cultured isolates.

The largest clade within the Bacteria (31 sequences, 40% of total), containing clones F6, D2, C9, F11 and B10, branched within an unknown group between candidate division OP10 and *Chloroflexi* (Fig. 2.4). However, the similarity between these clones was very low (<90%), and may represent several unknown groups of Bacteria, which have not been previously detected in any other environments. Likewise, clone G1 had no close similarity (<88%) with its closest neighbour, an environmental clone and grouped in the candidate division OP10. Clones C3 and F7, (<88%) grouped within the *Acidobacteria*. All three sequences were supported by high bootstrap values. Clone B12 lacked any similarity (<85% BLASTn) to known organisms and

Table 2.2. 16S rRNA clones identified in a soil sample from site T-3A, Mt Erebus, Antarctica 2006.

Bacterial OTU	No. of clones	Closest sequence match with blastn, origin (accession number)	% similarity	Closest cultured match with blastn, genus (accession number)	% similarity
A – (C3)	16	Bacterium clone, soil (AY493926)	88	*Bacillus thermocatenulatus*, Firmicutes (Z26926)	80
B – (C9)	6	Bacterium clone, Yellowstone National Park (DQ324877)	89	*Thermoanaerobacter tengcongensis*, Firmicutes (AF209708)	80
C – (F6)	9	Bacterium clone, Yellowstone National Park (DQ324886)	86	*Thermoanaerobacter mathranii*, Firmicutes (Y11279)	83
D – (A9)	4	Thermus Tok3 A.1, Tokaanu hot spring (L10069)	88	*Thermus brockianus*, Thermus (Y18409)	86
E – (B2)	1	Bacterium S119, thermophilic methanol-utilizing bacteria (AY040675)	91	*Thermus Silvanus*, Thermus (X84211)	91
F – (B6)	1	Bacterium clone, sandy carbonate sediment (EF208698)	86	*Anaerolinea thermophila*, Chloroflexi (AB046413)	83
G – (B10)	1	Bacterium clone, hot spring, Navada (DQ490008)	89	*Thermoanaerobacter mathranii*, Firmicutes (Y11279)	84
H – (B12)	1	OP10 bacterium clone, hot springs, Alvord Desert Basin (DQ645244)	85	*Caldilinea aerophila*, Chloroflexi (AB067647)	81
I – (C11)	1	Fischerella sp. MV11 (DQ786171)	85	*Mastigocladus laminosus*, Cyanobacteria (DQ431003)	85
J – (D2)	3	Bacterium clone, Yellowstone National Park (DQ324877)	89	*Desulfovirgula thermocuniculi*, Firmicutes (DQ208689)	84
K – (D6)	1	Fischerella sp. MV11 (DQ786171)	85	*Mastigocladus laminosus*, Cyanobacteria (DQ431003)	85
L – (D10)	12	Bacterium clone, California grassland (EF516153)	86	*Gemmata obscuriglobus*, Planctomycete (X81957)	85
M – (F11)	12	Bacterium clone, Yellowstone National Park (DQ324877)	88	*Gemmata obscuriglobus*, Planctomycete (X81957)	85
N – (E4)	1	Bacterium clone, poplar tree microcosm (AJ863234)	93	*Conexibacter woesei*, Actinobacteria (AJ871305)	85
O – (E5)	1	Bacterium clone, pasture soil (DQ083302)	90	*Caldilinea aerophila*, Chloroflexi (AB067647)	83
P – (F7)	2	Bacterium clone, soil (AY493926)	87	*Desulfotomaculum thermobenzoicum*, Firmicutes (Y11574)	83
Q – (G1)	5	Bacterium clone, Australian arid soils (AF234118)	88	*Rubrobacter xylanophilus*, Actinobacteria (CP000386)	83
R – (182)	1	Bacterium clone, Guaymas Basin (AF419667)	85	*Anaerolinea thermophila*, Chloroflexi (AB046413)	81
Archaeal OTU					
S – (A2)	26	Clone, subsurface water (DQ336957)	97	*Candidatus Nitrosopumilus maritimus* (DQ085097)	83
T – (E7)	7	Crenarchaeon clone, subsurface thermal spring, Austrian Alps (AM039531)	96	*Candidatus Nitrosopumilus maritimus* (DQ085097)	85
U – (C1)	36	Crenarchaeon clone, subsurface thermal spring, Austrian Alps (AM039531)	97	*Candidatus Nitrosopumilus maritimus* (DQ085097)	84
V – (C6)	7	Crenarchaeon clone, subsurface thermal spring, Austrian Alps (AM039531)	95	*Candidatus Nitrosopumilus maritimus* (DQ085097)	80
W– (D10)	7	Crenarchaeon clone, subsurface thermal spring, Austrian Alps (AM039531)	96	*Candidatus Nitrosopumilus maritimus* (DQ085097)	85

a. Phylotypes are defined as < 97% difference. Clones in brackets represent sequences ≤1200 bp for Bacteria ≤900 bp for Archaea

branched deeply within the candidate division OP10 (Fig. 2.4). Clone D10 was deep branching in the *Planctomycetes* (only 85% similarity with a cultured *Planctomycete*). Clones C11 and D6 were deep branching in the *Cyanobacteria* (only 85% similarity to a cultured *Cyanobacteria*, and clones A9 and B2 were deep branching in the *Deinococcus/Thermus* group (86% and 91% similarity to cultured *Thermus* sp.). All these groupings were supported by high bootstrap values. Clone E4 grouped within the *Actinobacteria* as supported by high bootstrap values but again only had low similarity to cultured representatives of the *Actinobacteria* (85% with *Conexibacter woesei*). Finally three clones, E5, B6 and 182, grouped within the *Chloroflexi* with long branch lengths and only had low similarity with cultured members of the group (less than 84% similarity).

All of the archaeal sequences grouped together in one clade, supported by high bootstrap values, within the soil-seawater Crenarchaeota group (Fig. 2.5). These sequences also grouped with clones from soil and a subsurface thermal spring from the Austrian Alps. The closest cultured representative of the Crenarchaeotes is *Candidatus Nitrosopumilus maritimus* with 85% similarity to the closest clone, which was isolated from a tropical seawater tank from an aquarium.

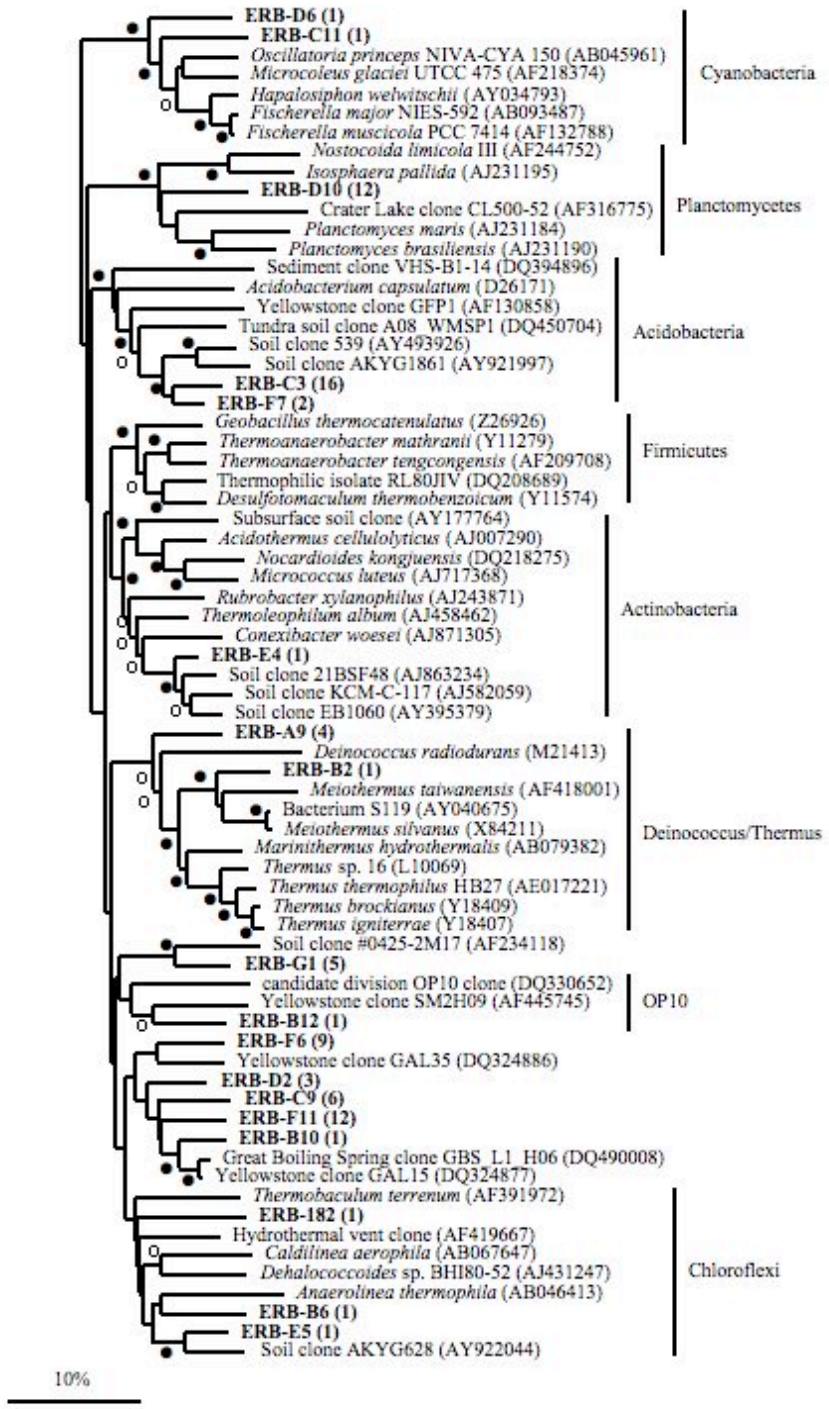

Fig. 2.4. Phylogenetic tree showing the placement of 16S rDNA gene clones from Tramway Ridge, T-3A, within the Bacteria. Branch points with support (bootstrap values of >90%) are indicated by filled circles; open circles with marginal support (bootstrap values of 70 to 90%). Branch points without circles are not resolved (bootstrap values <70%).

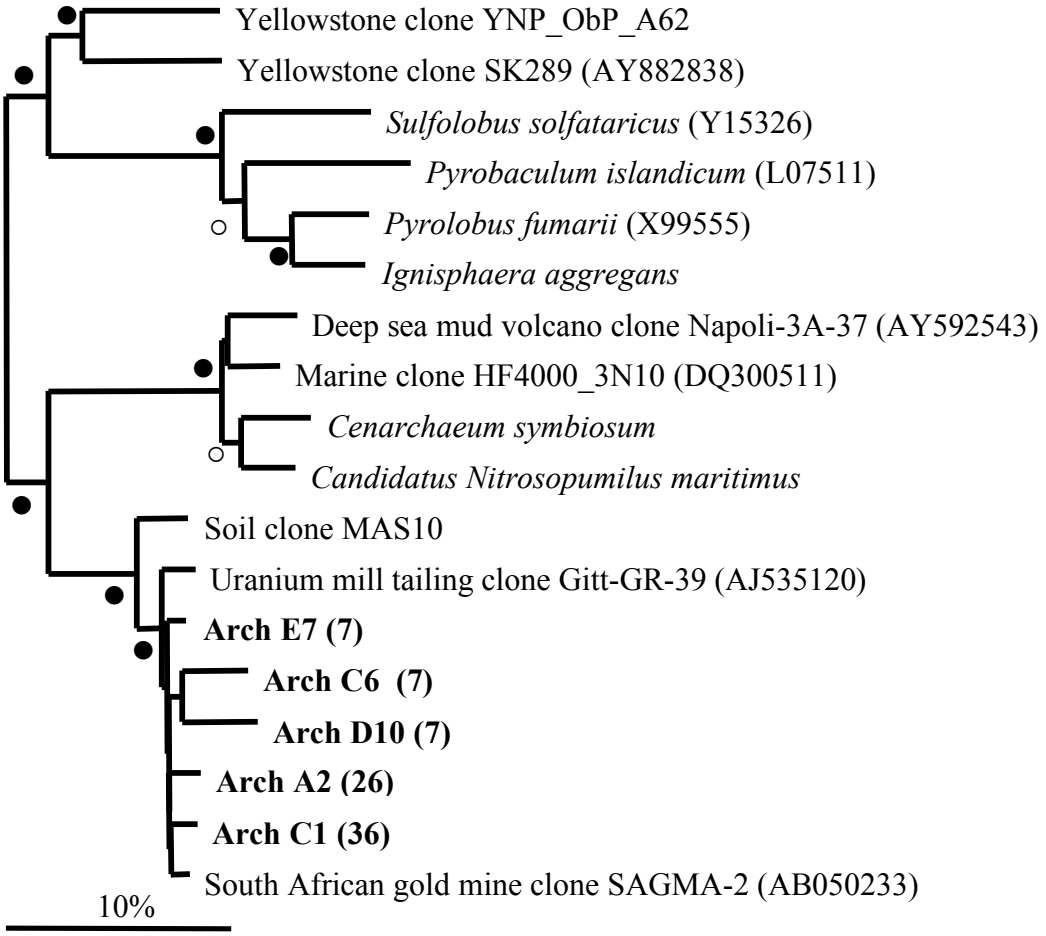

Fig. 2.5. Phylogenetic tree showing the placement of 16S rDNA gene clones from Tramway Ridge, T-3A, within the Archaea. Branch points with support (bootstrap values of >90%) are indicated by filled circles; open circles with marginal support (bootstrap values of 70 to 90%). Branch points without circles are not resolved (bootstrap values <70%)

The Shannon-Weaver index was used to assess the diversity within the 16S rRNA clone libraries. The analysis indicated a high biodiversity for Bacteria (2.39, 95% confidence interval 2.14 - 2.64). In contrast, the index value was much lower for Archaea (1.35, 95% confidence interval 1.20 - 1.50). Rarefaction curves for the bacterial library did not level off demonstrating that the diversity plateau had not been reached. Whereas for Archaea, the rarefaction curves leveled off, indicating sufficient

sampling had been undertaken and that archaeal diversity in this sample was relatively limited and well represented in the library (Fig. 2.6).

The Chao1 estimate of species richness for Bacteria was 45.5 (95% confidence interval, 24.66 - 131.50) which is markedly higher than the observed number of OTU, suggesting that more sequences should have been obtained to allow a stable estimate of species richness. The ACE estimator was comparable to the Chao1 estimate with a value of 41.34 (95% confidence interval 24.55 -101.23). The Chao1 and ACE values for the Archaea were 5 (95% confidence interval of 5). This was identical to the observed number of OTUs indicating that the diversity of T3-A sample was well sampled for Archaea.

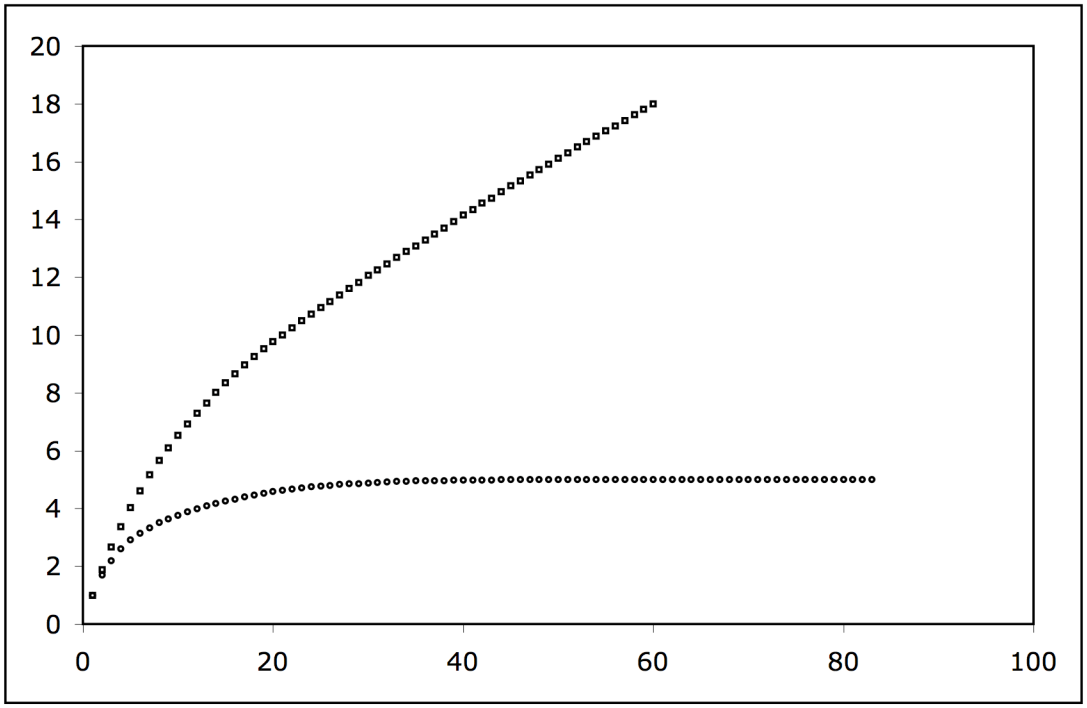

Fig. 2.6. Rarefaction curve using partial 16S rRNA Bacteria and Archaea sequences from Tramway Ridge (T-3A) using 97% cut-off (species-level). ◇ Bacteria **O** Archaea.

2.4.5 Analysis of cultures and isolates

In the culture sample from site T-1A, there was no observable growth in CMD or TSB media. Likewise there was no observable growth in the TSB media from sample site T-2A. ARISA analysis on the cultures showed that 10 AFLs were identified from site T-1A in CMD media, three AFLs from site T-2A in CMD media, 13 AFLs from site T-1A in TSB media and one AFL from site T-2A in TSB media. There was less AFLs present in the cultures compared with the number of AFLs observed from the gDNA extracted from the soil samples, except for T-1A TSB. The ARISA showed that the community composition was different in the media compared with the community composition from the DNA extractions. Five AFLs found in T-1A TSB were identical to T-1A CMD.

Two subcultures isolated from site T-2A in CMD media were serially diluted to isolate single cultures for sequencing. The first isolate was 97% similar to *Geobacillus* from a volcanic island (AY603072) and the second subculture was 99% similar to *Anoxybacillus* isolated from Mt. Rittman (AJ6189).

2.5 Discussion

Located 1.5 km northwest of the main crater of Mount Erebus, is a unique, ice-free gently sloping geothermal area known as Tramway Ridge (77°31’05”S, 167°06’35”E, elevation 3 350 to 3 400 m, Broady, 1993). This area contains many isolated steam fumaroles that produce moist, hot soils which can reach up to 65°C (Hudson and Daniel, 1988). Tramway Ridge is one of the most isolated geothermal environments on the Earth, surrounded by hundreds of kms of ice and massive thermal differentials. Tramway Ridge supports prokaryotic thermophilic communities that thrive in the warm and moist conditions (Broady *et al.,* 1984). This pristine area provides a unique opportunity to explore the possibilities of microbial endemism.

Geothermal environments similar to Tramway Ridge, have been the focus of significant attention because of their use as analogues for early Earth environments (Cavichiolli, 2002; Weidler *et al.,* 2007). Studies in these areas have led to the discovery of novel organisms and have provided a new understanding of chance versus necessity in evolutionary pathways (Rothschild and Mancenelli, 2001). With the advent of cuture-independent methods, new microbial taxa have been identified at an increasing rate but still little is known about the genetic affinities and evolution of these organisms (Vincent, 2000).

This is the first study to analyse physico-chemical properties along a temperature gradient in Tramway Ridge and also the first culture-independent study in Antarctic geothermal soils. This study provides us with an overview of the composition and diversity of bacterial communities along the temperature gradients. Other studies have shown that temperature is a major driver of bacterial diversity (Norris *et al.,* 2002, Purcell *et al.,* 2006). Therefore, our hypothesis was that temperature would be driving the bacterial diversity along the gradients at Tramway Ridge and novel deep-branching Bacteria and Archaea would be identified.

2.5.1 Physico-chemical analysis

Our results showed that over 10 months, temperatures were surprisingly stable at the high temperature site (T-3A), with short intermittant periods of time (less than 24 hours) where temperatures decreased by up to 40°C (Fig. 2.1). This is likely to be due to ice falling on the fumarolic soils because of heavy snow-fall. Along the gradient, we found that pH at temperatures of >50°C were essentially neutral, whereas temperatures <50°C were extremely acidic, which was also noted by Hudson and Daniel (1988). As seen at Yellowstone National Park, neutral pH at the hotter temperature sites may be due to the release of CO_2 from below the surface producing carbonate. As the steam is released, the state of oxidation can change and the pH rises during hydrolysis (Nordstrom *et al.,* 2005). Our results showed that soils along the gradient changed from sandy loam to clay soils, which could be due to the acid

breaking down the sediments to produce clay at the sites further from the fumarole. Our research and previous Antarctic studies, have found low amounts of carbon, nitrogen and soil organic matter, which is a consistent trait of Antarctic soils and could be due to the absence of vascular plants on this extreme dry continent (Parsons *et al.,* 2004; Aislabie *et al.,* 2006a, Aislabie *et al.,* 2006b). Low traces have also been found in other volcanic soils in Hawaii (Gomez-Alvarez *et al.,* 2007). Surprisingly, soil organic matter was higher than expected compared with carbon levels. This could be due to other factors in the soil, e.g. carbonates. Moisture content along the gradients were stable with the highest moisture content found at site T-3C (76.87%) and the lowest at site T-3E (15.9%). High concentrations of aluminium, sodium and potassium were observed which is characteristic of volcanic soils and have been detected at elevated concentrations at the summit of Mount Erebus (Rosenberg, 1988). A univariate analysis of variance (ANOVA) was performed to identify if there were any significant differences in the physico-chemical properties between the temperature groups. The ANOVA showed that the geochemistry along the transects were similar, except for Cr^{52} and Ni^{60} which had slightly higher concentrations in the cooler site (T-1F), but due to these elements being close to detection levels, they were not shown to be significant.

2.5.2 DNA fingerprinting

It has been proposed that only 1% of microorganisms can be cultured (Torsvik *et al.,* 1996; Hugenholtz, 1998). The advent of culture-independent DNA fingerprinting techniques (e.g., automated rRNA intergenic spacer analysis (ARISA) and restriction fragment length polymorphism (RFLP)), has enabled microbiologists to efficiently describe and compare microbial communities without the need to cultivate (Walker and Pace, 2007). Despite well-documented limitations of these methods, e.g. biases due to DNA extraction (de Lipthay *et al.,* 2004) and PCR primers (Hongoh *et al.,* 2003), the methods are extremely powerful in that they can allow us to examine even the most minor members of the community. Automated rRNA intergenic spacer analysis (ARISA) is a rapid and reproducible community fingerprinting method,

which can be used to identify differences in microbial diversity among many samples (Fisher and Triplett, 1999; Cardinale *et al.,* 2004; Brown *et al.,* 2005). The ARISA results showed that the number of ARISA fragment lengths (AFLs) for Bacteria decreased with an increase in temperature along the gradient. This trend has also been seen in other studies that have observed low bacterial diversity in hot soils (Norris *et al.,* 2002, Purcell *et al.,* 2006). With only one exception, the ARISA profiles showed that the Tramway Ridge samples supported diverse bacterial communities. Interestingly only one AFL was observed in the bacterial ARISA from sample T-3B. It is plausible that one species dominated this sample and this resulted in the suppression of other bacterial signals or that the universal primers used did not amplify all Bacteria in the sample. The Cyanobacteria showed no change in diversity across the thermal gradient, which implies that temperature may not have an effect on these communities. In comparison, another study by Sompong *et al.,* (2005) found that the diversity of cyanobacterial morphotypes fell as temperature increased from 30°C to 80°C in hot springs in northern Thailand. The similarity-ranking test which is based on the same AFL present in each sample, showed that there was no correlation with microbial community structure and temperature along the transects. However, the nonmetric multidimensional scaling (MDS) results which takes into account physico-chemical parameters showed that along the gradient, the hot temperature sites were more similar in AFLs than those found at the cooler sites, suggesting that the Bacteria and Cyanobacteria community structure found in these sites are different from those found at the cooler sites. ARISA, like other culture-independent techniques has its limitations. It can result in overestimation of a community's diversity due to some bacterial species containing more than one length of ITS region. Likewise it may also underestimate diversity due to overlapping intergenic spacer sizes of unrelated species (Fisher and Triplett, 1999).

The ARISA profiles and physio-chemical data were used in a BEST statistical analysis to identify which abiotic factors may be driving the bacterial and cyanobacterial communities. The results indicated that notable differences in temperature and pH were the main factors that best explained the observed

community distribution patterns. Temperature and pH have also shown to be driving the diversity in many other environments. Norris *et al.,* (2002) observed changes in soil microbial diversity across a temperature gradient following a geothermal heating event at Yellowstone National Park. They found that heated soils were less complex than those from cooler soils. Another study by Fierer and Jackson (2005), observed that pH was the best predictor of both soil bacterial diversity and richness when sampling from sites in North and South America.

2.5.3 Phylogeny

The results from the physico-chemical and ARISA analysis indicated that the samples from the hotter sites were less complex and more similar to each other. Only a handful of studies have used culture-independent techniques to identify bacterial communities living in hot thermal soils (Norris *et al.,* 2002), therefore site T-3A was chosen for extensive phylogenetic analysis.

All the sequences obtained from the clone library were deep branching and were affiliated (NCBI BLASTn) with environmental sequences obtained from soil-related or hot spring-related samples. All sequences were not closely related to any cultured microorganisms, showing less than 92% sequence identity with their corresponding closest BLAST relatives. From our phylogenetic study, the largest clade of bacterial clones (40%) from site T-3A composes an unknown group that was deep-branching between candidate division OP10 and the *Chloroflexi* (Fig. 2.5). The most similar sequences from cultured isolates were from the *Firmicutes* and *Planctomycetes* phylum but with very percentages (86 to 89%).

The next most dominant phylum (23%) were deep-branching *Acidobacteria*. This phylum has also been seen in other studies of Antarctic microbial habitats including, soil biotopes (Saul *et al*., 2005; Smith *et al*., 2006; Niederberger *et al*., in press), cryoconite holes (Christner *et al*., 2003) and cryptoendolithic communities (de la

Torre *et al*., 2003). Due to the surrounding acidic soils, we would expect that there would be a large number of these species present.

Representatives of the phylum *Planctomycetes* made up 15% of the library. *Planctomycetes* have been recognized as common inhabitants of soils (Buckley *et al.,* 2006) but only a small number of signatures of *Planctomycetes* have been recovered from Antarctic soil samples in Luther Vale and Miers Valley (Smith *et al.,* 2006; Neiderberger *et al.,* in press). This phylum has become significant in understanding evolutionary biology due to its unique cellular compartmentalisation and absence of peptidoglycan. Therefore, it may form an ancient deep-branching lineage within Bacteria. Some species in this phylum have also shown to perform anaerobic ammonia oxidation (Fuerst, 2005) and it has been suggested that carbohydrate fermentation and sulphur reduction are possible mechanisms employed under anaerobic conditions (Elshahed *et al.,* 2007).

Members of candidate division OP10 are uncommon in clone libraries from soils in Antarctica but two representatives were identified in soils from Miers Valley (Smith *et al.,* 2006). In our library, OP10 represented 8% (7 clones). To date, no representatives of these organisms have been isolated and little information is available. These organisms have been found in hydrothermal (Kanokratna *et al.,* 2004) and geothermal environments (Hugenholtz *et al.,* 1998) and may have a possible role in metal cycling (Stein *et al.,* 2002).

Deinococcus-thermus have been shown to withstand heat, cold, desiccation and high levels of ionizing radiation (Mattimore and Battista, 1996) and have been described in a number of Antarctic microbial habitats (de la Torre *et al*., 2003; Saul *et al*., 2005; Shravage *et al*., 2007; Aislabie *et al*., 2006a). In our library, they represented 6.4%.

Chloroflexi represented 3.8% of the T-3A clone library and have been described in small numbers in Luther Vale (Niederberger *et al.,* in press) and Miers Valley (Smith *et al.,* 2006). Members of this phylum tend to be found in moist areas and have been

identified in mesophilic (Mummey *et al.,* 2006) and thermophilic environments (Botero *et al.,* 2004), still little is known about their function in these environments.

Cyanobacteria distribution is found in many areas of Antarctica but are more abundant in lakes and ponds (Vincent, 2000). Many studies have identified novel species of cyanobacteria, suggesting that these maybe endemic to Antarctica (Casmatta *et al.,* 2005; Jungblut *et al.,* 2005). Surprisingly, only a small number of *Cyanobacteria* were identified in the clone library, although areas of the transects were covered in cyanobacterial mats and a large number of ARISA cyanbacterial AFLs were obtained. This may be due to the samples being obtained from below the mats and cyanobacterial primers being specific for that phylum.

Actinobacteria have been identified in many bacterial libraries and are a common feature of soils. Surprisingly, only one representative was identified, although they have been found to make up 13% to 26% in other libraries (Smith *et al.,* 2006; Niederberger *et al.,* in press).

No members of the *Firmicutes* were found in the clone library, although we were able to culture representatives from this phylum. With only a small number of *Firmicutes* present in the soil, primers may be biased towards amplifying other Bacteria that are more dominant, whereas the culturing media could be biased towards *Firmicutes*. In addition, Janssen (2006) suggested that members of the *Firmicutes*, may be underrepresented because clls or spores may be difficult to lyse and so are difficult to detect with PCR techniques that rely on DNA extraction. Also, interesting to note is that there were no members of the Proteobacteria phylum present, although these have found to be dominant in many soil habitats in Antarctica (Saul *et al.,* 2005; Niederberger *et al.,* in press). The rarefaction analysis, ChaoI and Ace estimator indicated that further sampling of clones should be undertaken to obtain a full overview of the bacterial diversity present in the T-3A site.

In contrast the 16S rRNA gene sequences libraries appear to have captured the full extent of the archaeal diversity (Fig. 2.4). The presence of Crenarchaeotes at

Tramway Ridge were not surprising, although these clones were very deep branching, forming a tight clade within the soil-freshwater-subsurface group (Fig. 2.6; Weidler *et al.,* 2007). The Tramway clones were 95 to 97% similar with known groups from Austrian thermal springs, where the average temperature is 45.6°C and pH is approximately 8.0 (Weidler *et al.,* 2007) but were not similar to cultured Crenarchaeotes. These Archaea are found in numerous thermal and mesophilic habitats worldwide including thermal springs (Weidler *et al.,* 2007), soils (Jurgens *et al.,* 1997), marine sponges (Preston *et al.,* 1996) and marine planktonic environments (DeLong *et al.,* 1998). Some recent reports have suggested that these organisms may be important in the nitrogen cycle (Francis *et al.,* 2005; Treusch *et al.,* 2005). No Euryarchaeotes were found in the clone library, despite the use of universal archaeal PCR primers that have shown to identify these Archaea in other clone libraries (Newberry *et al.,* 2004; Kim *et al.,* 2005).

2.5.4 Culturing and isolation studies

The ARISA results from the cultures showed that there was significantly less diversity (fewer AFLs) when compared with an ARISA of genomic DNA extracted directly from the soils, except for site T-1A from TSB media, which had 13 AFLs. The ARISA also showed that community composition was slightly different in the culture media compared with community composition found in the DNA extractions from the soils, with 36% of the AFLs found in the cultures being the same as those found in the extracted DNA from soil.

From our isolate study, we obtained two strains of *Bacillus*, which are closely related to those found in previous studies. One isolate was 97% related to *Geobacillus amylolyticus* from a volcanic soil in Santorini, Greece, where soil temperatures range between 85 and 95°C (Meintanis *et al.,* 2005). The second isolate was 99% related to *Anoxybacillus amylolyticus* from Mt. Rittman, Antarctica. (Poli *et al.,* 2006). Previous cultivation studies on Tramway Ridge soils also used CMD media to isolate neutrophilic strains of *Bacillus* (Hudson and Daniel, 1988). Most of their samples

had positive enrichments for strains of *Bacillus acidocaldarius*, *Bacillus schegelii* and *Clostridium thermohydrosulfuricum* (Hudson *et al.,* 1988). Other isolates obtained from Mt. Erebus include four *Cyanobacteria* species, including *Mastigocladus laminosus* and *Phormidium fragile*, as well as three species of *Actinomycetes* (Ugolini and Starkey, 1966; Broady, 1984; Melick *et al.,* 1991).

2.5.5 Conclusion

One of the longstanding theories of microbial biogeography is the paradigm that "everything is everywhere, the environment selects" (Baas-Becking 1934, cited in Staley and Gosnik, 1999). However, only a small number of studies have tried to verify this, especially in soil ecotypes (Frieer and Jackson, 2005). Antarctica is highly important to the study of evolutionary biology and endemism due to its isolation and extreme environmental pressures (Vincent, 2000). The application of 16S rDNA genetic analysis to Antarctic bacteria implies the presence of not only unusual species, but also novel taxa at the genus, family, and higher levels, as has been shown by many Antarctic microbial studies (Bowman *et al.,* 2000; de la Torre *et al.,* 2003, Van Trappen *et al.,* 2004). The thermal soil from sample T-3A of Mt. Erebus may represent a novel and unique habitat for microbial life most likely due to its geographic isolation and extreme conditions (both, environmental and elevation). Identification of deep-branching clades of novel Bacteria and Archaea from Tramway Ridge, which may represent new genera adds further evidence to indicate that "everything is not everywhere". Although this study is far from exhaustive it indicates that high specieds diverity, the majority of which are exceptionally deep branching, inhabit these harsh envrionments. Representatives from the culture-independent study have not been previously cultured from Tramway Ridge and more phylogenetic characterisation is needed. Many of these organisms appear to be unique and further isolation or metagenomics studies may provide insight into physiology, taxonomy and the discovery of evolutionary metabolic pathways.

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Chapter 3 : General conclusion

This final chapter is intended to give a brief conclusion to the preceeding chapters and a discussion of future considerations.

The aim of this study was to determine whether there was a difference in microbial communities along a temperature gradient and to identify if the bacterial and archaeal communities were different from those found in other environments. Methodologies were successfully used to identify the diversity of the microbial communities along three transects on Tramway Ridge, Mt. Erebus, Antarctica (as described in Chapter 2).

For the first time, physico-chemical properties were determined and a culture-independent study was performed on soils from the Tramway Ridge site along a temperature gradient. ARISA analysis provided a quick method to identify the microbial community diversity of extracted DNA from soil samples. As was predicted, ARISA analysis showed that temperature gradients determine the microbial diversity in the soils. ANOVA and BEST analysis also showed that pH was important. From the physico-chemical and ARISA analysis, a hot temperature site (T-3A) was chosen for further phylogenetic analysis. To analyze the sample, restriction endonucleases were used to identify the different OTUs in the 16S rRNA gene clone library, which were then subsequently sequenced and analyzed. From this analysis, we identified deep-branching novel Archaea and Bacteria, suggesting that the microbial community at Tramway Ridge maybe endemic. A preliminary culturing attempt was performed and identified that the majority of the community found in the cultures were different from the communities found in the extracted DNA. Preliminary culturing enrichments resulted in the isolation of two *Bacillus* sp., which have not been previously observed on Mt. Erebus.

This study provides a foundation for future work on the Tramway Ridge soil samples, which will give a more comprehensive overview of this unique and isolated site. Another visit to the Tramway Ridge site could allow us to perform more culture work and cloning a metagenome from a soil sample, could provide more information on the microbial communities.

A chemical analysis on the soils for sulphur, silica, aluminium and carbonate needs to be carried out. Sulphur is important as it could be the energy source for the bacteria, although it was not seen on the volcano or detected but it may explain the very acidic soils. Aluminium was previously analyzed for in the chemical analysis but due to the large quantities, it was over the detection level for the ICP-MS. Silica is also another element that could be analyzed for, as it is one of the main constituients of anorthoclase phonolite.

DAPI staining would provide an estimation of bacterial cells in the soil samples, as previous studies have observed different counts using plating techniques, AOD and MPN counts (Ugolini and Starkey, 1966; Hudson and Daniel, 1988). Fluorescence *in situ* hybridization (FISH) in combination with epifluorescence and confocal laser scanning microscopy (CLSM) could also allow the detection of targeted species (Moter and Göbel, 2000).

ARISA analysis on cultures at other timeframes would give an indication of bacterial diversity that was gained or lost over time. Our hypothesis is that each inoculation to new media leads to a decrease in bacterial diversity.

Archaeal ARISA could also be performed on the 16 samples. Previously, we had not done this because it has been shown that many Archaea only have one tRNA, therefore the ITS regions are similar in size and hard to distinguish between the different species. Recently it has been shown that halophilic Archaea range in ITS size (Leuko *et al.,* 2007), therefore the ARISA analysis may be used on our samples.

From the physico-chemical analysis the results indicated low C:N ratios, which suggests that there could be a large amount of ammonia present in the soils. A DNA microarray method could be used to detect and quantify functional genes in the environment, including ammonia oxidation genes (Cho and Tiedje, 2002).

To confirm the ARISA study, clone libraries from the other four sample sites along transect 3 will give a more overall insight into the communities. The ARISA study only provides an overview of the diversity but clone libraries would show us whether the community structure and composition were different among the sites.

Also, if there is another expedition to Tramway Ridge, more cultures could be obtained by making media specific for *Acidobacteria*, *Planctomycetes*, candidate division OP10, *Actinobacteria*, *Deinococcus/Thermus*, *Chloroflexi* and *Cyanobacteria*. With the exception of candidate division OP10, media for all members of each domain have been isolated (Janssen *et al.,* 2002; Schoenborn et al., 2004, Cragelund *et al.,* 2007, Damsté *et al.,* 2005, Yeager *et al.,* 2007). A more in-depth study from the cultures could teach us about the evolution of the microorganisms at Tramway Ridge and may even suggest that these novel organisms are from ancient lineages. It would also be beneficial to obtain a core from site T-3A to identify if there are any differences between the communities at the surface and those further down as temperature increases. From the core, a metagenome project could lead to a number of novel organisms being discovered and give us insight into new metabolic pathways.

As outlined in the preface, we hope to submit this work for publishing in Environmental Microbiology.

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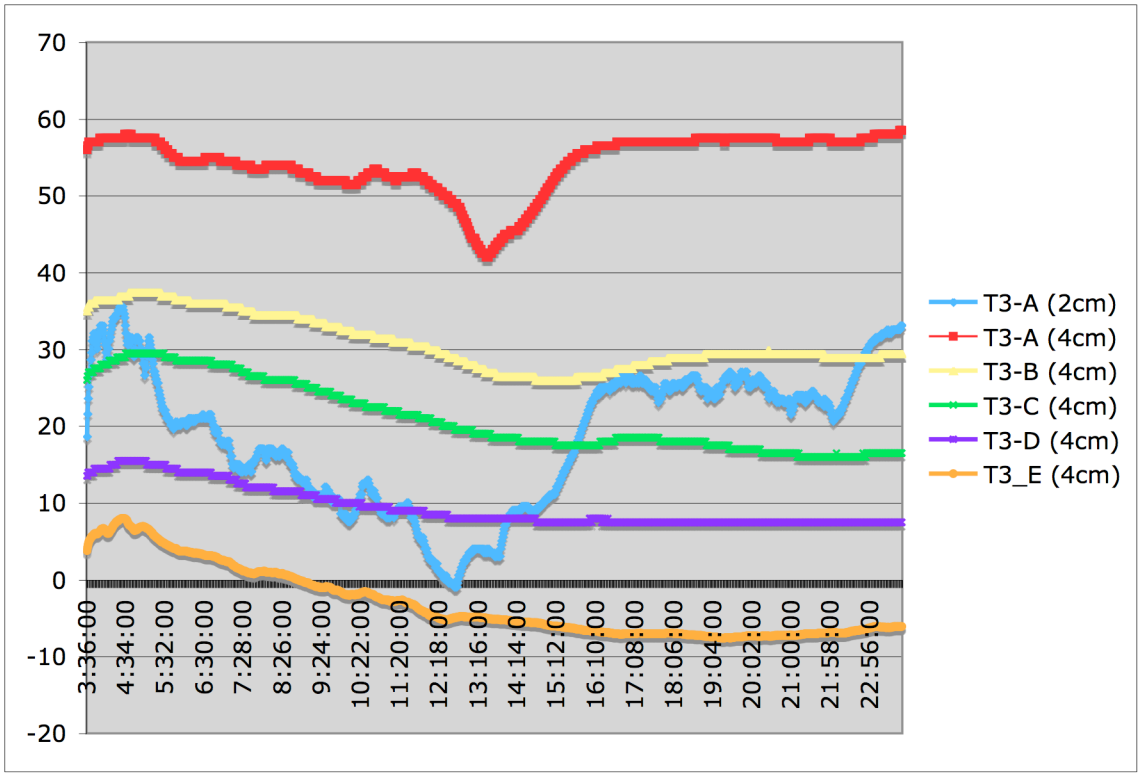
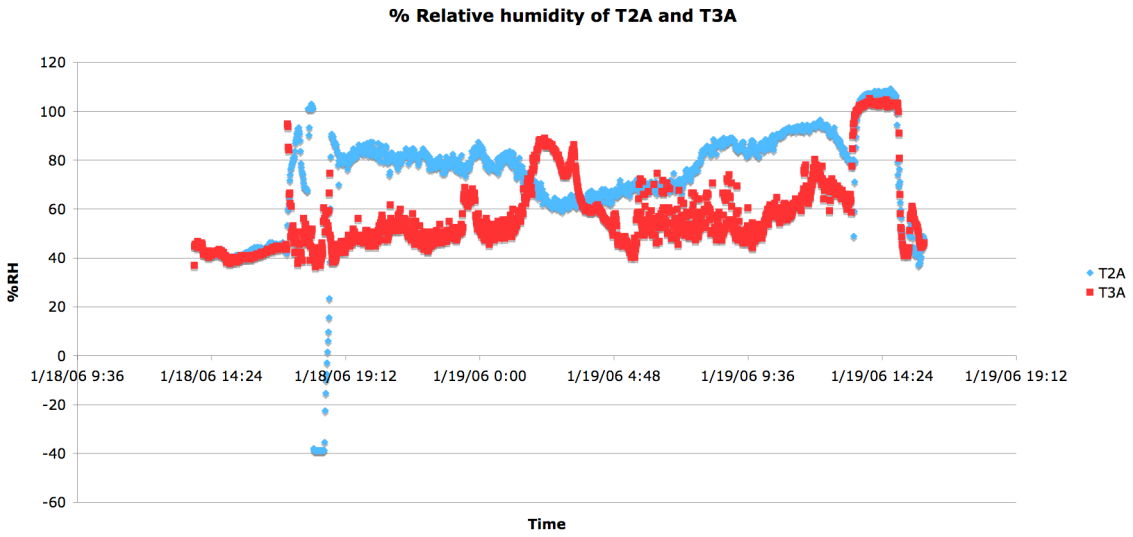
Appendices

Appendix A - Physico-chemical

Temperature logger data from transect 3 over a 20 hour period



% Relative humidity of T-2A and T-3A



Results of ICP-MS for 16 soil samples from Tramway Ridge, Mt. Erebus, Antarctica

Groups	Sample	Li 7	B 10	Na 23	Mg 24	P 31	K 39	Ca 43	V 51
1	**T1A**	3.0	11.6	217.3	611.6	297.2	182.9	788.9	0.0
	T2A	7.3	-9.4	98.3	263.4	126.5	174.6	209.3	0.1
	T3A	9.8	15.0	4648.6	1001.8	648.3	5469.5	1509.2	0.3
	Mean	6.7	5.7	1654.7	625.6	357.3	1942.3	835.8	0.1
	SD	2.8	10.8	2117.5	301.6	217.2	2494.1	531.7	0.1
2	**T1B**	7.7	6.4	834.0	1074.3	1589.6	930.9	492.6	2.6
	T2B	18.9	8.4	600.8	960.1	853.5	778.3	319.2	2.7
	T3B	6.1	14.4	2484.5	439.9	461.1	3291.9	594.9	1.5
	Mean	10.9	9.7	1306.4	824.8	968.0	1667.0	468.9	2.3
	SD	5.7	3.4	838.5	276.1	467.8	1150.6	113.8	0.6
3	**T1C**	5.8	25.0	1540.8	950.2	2381.2	1710.1	646.5	5.2
	T2C	26.0	11.7	337.4	866.2	1080.3	505.0	274.0	4.0
	T3C	2.7	-3.2	177.5	345.2	501.5	238.1	168.1	0.2
	Mean	11.5	11.2	685.2	720.5	1321.0	817.8	362.9	3.1
	SD	10.3	11.5	608.5	267.6	786.0	640.3	205.2	2.1

Groups	Sample	Li 7	B 10	Na 23	Mg 24	P 31	K 39	Ca 43	V 51
4	**T1D**	2.3	-4.9	902.8	495.6	892.0	1314.1	865.8	-0.2
	T2D	16.8	7.8	413.2	989.8	1344.0	558.0	321.9	3.5
	T3D	5.6	13.3	276.6	847.9	779.9	612.9	322.1	0.5
	Mean	8.2	5.4	530.9	777.8	1005.3	828.3	503.3	1.3
	SD	6.2	7.6	268.9	207.7	243.9	344.2	256.3	1.6
5	**T1E**	11.2	12.3	1956.6	1895.9	1530.4	2393.4	1236.6	2.0
	T2E	8.5	4.9	507.4	923.5	1385.3	977.6	474.9	7.2
	T3E	6.1	19.8	3985.0	883.2	1082.2	3996.6	1317.9	0.1
	Mean	8.6	12.3	2149.6	1234.2	1332.6	2455.9	1009.8	3.1
	SD	2.1	6.1	1426.3	468.2	186.7	1233.3	379.7	3.0
6	**T1F**	9.3	9.5	5193.9	2151.9	1532.9	5746.8	1988.1	3.8

Sample	Cr 52	Cr 53	Fe 54	Mn 55	Co 59	Ni 60	Cu 63	Cu 65
T1A	-0.3	-12.1	12739.8	500.0	0.8	0.3	2.3	2.9
T2A	-0.5	-18.8	16875.8	1236.5	1.1	0.0	0.8	1.9
T3A	-0.1	-33.5	19446.4	1586.2	1.4	0.0	1.3	1.9
Mean	-0.3	-21.5	16354.0	1107.6	1.1	0.1	1.5	2.2
SD	0.2	8.9	2762.7	452.7	0.2	0.2	0.7	0.5
T1B	-0.4	-28.2	40470.2	1491.1	2.1	0.2	5.7	8.1
T2B	-0.4	-21.1	44877.2	1838.3	2.6	0.3	3.9	6.6
T3B	-0.1	-5.9	17306.0	978.4	1.1	0.1	2.0	2.4
Mean	-0.3	-18.4	34217.8	1435.9	1.9	0.2	3.9	5.7
SD	0.1	9.3	12093.0	353.2	0.6	0.1	1.5	2.4
T1C	0.1	-30.6	52147.6	1658.0	2.6	0.2	7.6	11.8
T2C	-0.1	-11.2	44711.1	2042.4	2.6	0.2	4.9	7.8
T3C	-0.5	-12.3	20640.5	921.3	1.3	0.0	0.2	0.9
Mean	-0.2	-18.0	39166.4	1540.6	2.2	0.1	4.2	6.8
SD	0.2	8.9	13447.0	465.2	0.6	0.1	3.0	4.5

Sample	Cr 52	Cr 53	Fe 54	Mn 55	Co 59	Ni 60	Cu 63	Cu 65
T1D	0.0	1.5	8963.2	526.9	0.6	0.1	0.7	1.0
T2D	-0.5	-29.3	46780.2	1748.3	2.3	0.0	4.4	7.4
T3D	-0.3	-24.5	22659.7	1208.3	1.5	0.0	1.2	2.3
Mean	-0.3	-17.4	26134.4	1161.1	1.5	0.1	2.1	3.6
SD	0.2	13.6	15633.0	499.7	0.7	0.1	1.6	2.8
T1E	-0.1	-20.3	36826.8	2121.5	2.8	0.4	4.5	6.8
T2E	-0.2	-12.6	39387.5	1776.2	2.5	0.1	6.2	8.8
T3E	0.2	-19.7	12786.1	778.7	0.9	0.1	0.1	0.5
Mean	0.0	-17.5	29666.8	1558.8	2.1	0.2	3.6	5.4
SD	0.2	3.5	11982.1	569.3	0.8	0.1	2.6	3.5
T1F	1.0	-10.5	26247.0	1253.1	2.2	0.9	8.3	9.6

Sample	Zn 66	Zn 68	Ga 71	As 75	Se 77	Se 82	Sr 88	Ag 109
T1A	79.0	84.6	9.3	0.7	-5.8	0.5	35.7	0.1
T2A	87.2	106.0	8.7	1.2	-3.5	3.6	19.6	0.1
T3A	105.0	111.6	9.7	6.1	-8.0	1.3	48.2	0.3
Mean	90.4	100.7	9.3	2.7	-5.8	1.8	34.5	0.2
SD	10.8	11.6	0.4	2.5	1.8	1.3	11.7	0.1
T1B	180.7	173.1	27.3	10.5	-12.1	0.7	18.2	0.7
T2B	218.9	211.6	32.4	6.1	-6.8	1.5	115.6	0.6
T3B	85.3	86.8	10.2	5.0	-3.2	1.0	19.3	0.2
Mean	161.6	157.2	23.3	7.2	-7.4	1.0	51.0	0.5
SD	56.2	52.2	9.5	2.4	3.6	0.3	45.7	0.2
T1C	240.4	223.0	36.9	9.5	-11.0	0.8	20.3	1.0
T2C	215.8	207.2	34.1	8.5	-4.1	2.1	143.4	0.7
T3C	67.5	63.4	12.3	4.7	-4.9	0.4	9.4	0.2
Mean	174.6	164.5	27.7	7.6	-6.7	1.1	57.7	0.6
SD	76.4	71.8	11.0	2.1	3.1	0.7	60.8	0.3

Sample	Zn 66	Zn 68	Ga 71	As 75	Se 77	Se 82	Sr 88	Ag 109
T1D	66.8	64.2	5.3	4.5	-3.7	0.4	10.9	0.1
T2D	191.3	183.1	36.0	6.6	-10.2	1.7	107.9	0.8
T3D	115.2	121.7	12.2	3.4	-8.3	0.5	14.8	0.2
Mean	124.4	123.0	17.8	4.8	-7.4	0.9	44.5	0.4
SD	51.3	48.5	13.1	1.3	2.7	0.6	44.8	0.3
T1E	177.8	173.2	26.1	27.7	-5.9	1.2	49.6	0.6
T2E	236.9	232.2	30.7	8.0	-5.2	1.3	277.9	1.2
T3E	56.5	56.1	7.4	7.3	-2.4	0.7	29.3	0.2
Mean	157.0	153.8	21.4	14.4	-4.5	1.1	118.9	0.6
SD	75.1	73.2	10.1	9.5	1.5	0.3	112.7	0.4
T-1F	139.3	132.0	15.8	16.7	-1.9	1.0	53.0	0.6

Sample	Cd 111	In 115	Ba 137	Tl 205	Pb 206	Pb 207	Pb 208	Bi 209
T1A	0.1	0.1	227.2	0.1	2.7	2.6	2.6	0.0
T2A	10.8	0.1	570.6	0.0	3.8	3.6	3.8	0.0
T3A	2.4	0.5	313.8	0.1	19.9	18.6	19.6	0.2
Mean	4.4	0.2	370.6	0.1	8.8	8.2	8.6	0.1
SD	4.6	0.2	145.8	0.0	7.9	7.3	7.7	0.1

T1B	0.4	0.5	192.1	0.1	19.4	18.2	19.0	0.2
T2B	1.9	0.4	260.2	0.1	14.0	13.2	13.5	0.1
T3B	1.0	0.4	163.1	0.0	15.9	15.1	15.5	0.2
Mean	1.1	0.4	205.1	0.1	16.4	15.5	16.0	0.2
SD	0.6	0.0	40.7	0.0	2.2	2.0	2.2	0.0

T1C	0.8	0.5	230.1	0.1	16.1	15.4	15.6	0.3
T2C	2.1	0.5	284.4	0.1	16.5	15.5	16.0	0.2
T3C	0.6	0.3	53.6	0.0	12.4	11.8	12.1	0.1
Mean	1.1	0.4	189.4	0.0	15.0	14.2	14.6	0.2
SD	0.7	0.1	98.5	0.0	1.9	1.7	1.8	0.1

Sample	Cd 111	In 115	Ba 137	Tl 205	Pb 206	Pb 207	Pb 208	Bi 209
T1D	0.5	0.2	47.9	0.1	7.3	6.8	7.1	0.1
T2D	1.8	0.4	222.5	0.1	13.3	12.6	12.9	0.1
T3D	0.3	0.5	328.4	0.1	10.8	10.2	10.6	0.1
Mean	0.8	0.4	199.6	0.1	10.4	9.9	10.2	0.1
SD	0.7	0.1	115.7	0.0	2.5	2.4	2.4	0.0
T1E	1.2	1.4	233.7	0.1	48.4	46.0	47.0	0.5
T2E	1.2	0.7	408.2	0.1	12.2	11.5	11.9	0.1
T3E	0.7	0.3	109.1	0.0	13.4	12.5	13.2	0.2
Mean	1.0	0.8	250.3	0.1	24.6	23.3	24.0	0.3
SD	0.2	0.5	122.7	0.0	16.8	16.1	16.2	0.2
T1F	0.6	0.7	155.1	0.1	24.7	23.0	24.3	0.3

Sample	U 235	U 238
T1A	3.4	1.2
T2A	4.6	1.6
T3A	6.3	2.2
Mean	4.8	1.7
SD	1.2	0.4

T1B	6.8	2.4
T2B	7.8	2.5
T3B	4.1	1.5
Mean	6.2	2.1
SD	1.6	0.5

T1C	12.1	3.9
T2C	8.6	2.7
T3C	3.2	1.1
Mean	7.9	2.6
SD	3.7	1.1

Sample	U 235	U 238
T1D	2.6	0.8
T2D	7.8	2.8
T3D	4.8	1.7
Mean	5.1	1.8
SD	2.1	0.8

T1E	12.8	4.4
T2E	6.2	2.0
T3E	5.4	1.7
Mean	8.1	2.7
SD	3.3	1.2

T1F	6.6	2.7

Mean soil physical and chemical properties for the 16 soil samples based on temperaure; 60 - 65°C (T-1A, T-2A and T-3A), 40 - 50°C (T-1B, T-2B and T-3B), 24 - 34°C (T-1C, T-2C and T-3C), 16 - 26°C (T-1D, T-2D and T-3D) and 2.5°C (T-1F). All given element concentrations are in mg/kg of dried weight.

	60 - 65°C	40 - 50°C	24 - 34°C	16 - 26°C	14 - 5°C	2.5°C	**F**	**P**
Temp	64.2	47.2	29	20.5	9.7	2.5	67.13	<0.0001
pH	7.2	4.6	3.6	3.9	4.3	4.5	35.77	<0.0001
% Moisture	27.6	34.3	27.1	23.1	19.4	6.5	1.47	0.283
%C	0.2	0.1	0.08	0.06	0.08	0.04	13.91	0.000
%N	0.3	0.2	0.3	0.2	0.3	0.2	0.61	0.695
%OM	10.2	10.8	8.5	10.3	7.6	13.9	0.70	0.636
Li 7	6.7	10.9	11.5	8.2	8.6	9.3	0.17	0.969
B 10	5.7	9.7	11.1	5.4	12.3	9.5	0.18	0.963
Na 23	1654.7	1306.4	685.2	530.9	2149.6	5193.9	1.73	0.215
Mg 24	625.6	824.8	720.6	777.8	1234.2	2151.9	3.03	0.064
P 31	357.3	968	1321	1005.3	1332.6	1532.9	1.47	0.283
K 39	1942.3	1667	817.8	828.3	2455.9	5746.8	1.58	0.252
Ca 43	835.8	468.9	362.9	503.3	1009.8	1988.1	3.18	0.056
V 51	0.1	2.3	3.1	1.3	3.1	3.8	0.93	0.501
Cr 52	-0.3	-0.3	-0.2	-0.3	-0.04	1	75.29	<0.0001
Fe 54	16354.1	34217.8	39166.4	26134.4	16550.8	26247	0.83	0.554
Mn 55	1107.6	1435.9	1540.6	1161.1	1558.8	1253.1	0.33	0.884
Co 59	1.1	1.9	2.2	1.5	2.1	2.2	0.92	0.504
Ni 60	0.1	0.2	0.1	0.07	0.2	0.9	7.66	0.003
Cu 63	1.5	3.9	4.2	2.1	3.6	8.3	1.38	0.310
Zn 68	100.7	157.2	164.5	123	153.8	132	0.37	0.856
Ga 71	9.3	23.3	27.7	17.8	21.4	15.8	0.82	0.565
As 75	2.7	7.2	7.6	4.8	14.4	16.7	1.95	0.172
Se 82	1.8	1	1.1	0.9	1.1	1	0.38	0.851
Sr 88	34.5	51	57.7	44.5	118.9	53	0.43	0.816
Ag 109	0.2	0.5	0.6	0.4	0.6	0.6	0.85	0.547
Cd 111	4.4	1.1	1.1	0.8	1	0.6	0.86	0.541
In 115	0.2	0.4	0.4	0.4	0.8	0.7	1.16	0.394

Ba 137	370.6	205.1	189.4	199.6	250.3	155.1	0.82	0.564
Tl 205	0.05	0.05	0.04	0.08	0.08	0.1	1.49	0.276
Pb 207	8.8	16.4	15	9.9	23.3	23	1.01	0.460
Bi 209	0.09	0.19	0.2	0.1	0.3	0.3	0.77	0.593
U 238	1.7	2.1	2.6	1.8	2.7	2.4	0.47	0.792

Results of a one-way ANOVA followed by a tukey test ($\propto = 0.05$). Define here what groups 1-6 are temperature groups with 1 being the hottest and 6 being the coolest. 60 - 65°C (T-1A, T-2A and T-3A), 40 - 50°C (T-1B, T-2B and T-3B), 24 - 34°C (T-1C, T-2C and T-3C), 16 - 26°C (T-1D, T-2D and T-3D) and 2.5°C (T-1F)

	F	p	Tukey test
Temp	67.13	<0.0001	1 2 3 4 5 6
pH	35.77	<0.0001	1 2 3 4 5 6
% Moisture	1.47	0.2830	
%C	13.91	0.0003	1 2 3 4 5 6
%N	0.61	0.6954	
%OM	0.70	0.6361	
Li 7	0.17	0.9688	
B 10	0.18	0.9628	
Na 23	1.73	0.2151	
Mg 24	3.03	0.0637	
P 31	1.47	0.2829	
K 39	1.58	0.2519	
Ca 43	3.18	0.0563	
V 51	0.93	0.5013	
Cr 52	75.29	<0.0001	1 2 3 4 5 6
Fe 54	0.83	0.5538	
Mn 55	0.33	0.8836	
Co 59	0.92	0.5037	
Ni 60	7.66	0.0034	1 2 3 4 5 6
Cu 63	1.38	0.3096	
Zn 68	0.37	0.8556	
Ga 71	0.82	0.5645	
As 75	1.95	0.1723	
Se 82	0.38	0.8511	
Sr 88	0.43	0.8163	
Ag 109	0.85	0.5470	
Cd 111	0.86	0.5407	
In 115	1.16	0.3937	
Ba 137	0.82	0.5643	
Tl 205	1.49	0.2756	
Pb 207	1.01	0.4603	
Bi 209	0.77	0.5932	
U 238	0.47	0.7923	

BEST analysis for Bacteria

Biota and/or Environment matching

Data worksheet
Name: Data2
Data type: Environmental
Sample selection: All
Variable selection: All

Resemblance worksheet
Name: Resem1
Data type: Similarity
Selection: All

Parameters
Rank correlation method: Spearman
Method: BIOENV
Maximum number of variables: 5
Resemblance:
Analyse between: Samples
Resemblance measure: D1 Euclidean distance

Variables
1 Temp
2 pH
3 % Moisture
4 %C
5 %N
6 %OM
7 Li 7
8 B 10
9 Na 23
10 Mg 24
11 P 31
12 K 39
13 Ca 43
14 V 51
15 Fe 54
16 Mn 55
17 Co 59
18 Ni 60
19 Cu 63
20 Zn 68
21 Ga 71
22 As 75
23 Se 82
24 Sr 88
25 Ag 109
26 Log(Cd 111)

27 In 115
28 Ba 137
29 Tl 205
30 Pb 207
31 Bi 209
32 U 238

Global Test
Sample statistic (Rho): 0.516
Significance level of sample statistic: 5.3%
Number of permutations: 999 (Random sample)
Number of permuted statistics greater than or equal to Rho: 52

Best results

No.Vars	Corr.	Selections
3	0.516	1-3
2	0.512	1,2
4	0.512	1-4
5	0.502	1-4,11
5	0.501	1-4,29
4	0.499	1,2,4,11
4	0.497	1-3,11
3	0.495	1,2,11
3	0.495	1,2,4
3	0.494	1,3,4

Outputs
Plot: Graph11

BEST analysis for Cyanobacteria

Biota and/or Environment matching

Data worksheet
Name: Data1
Data type: Other
Sample selection: All
Variable selection: All

Resemblance worksheet
Name: Resem1
Data type: Similarity
Selection: All

Parameters
Rank correlation method: Spearman
Method: BIOENV
Maximum number of variables: 5
Resemblance:
Analyse between: Samples
Resemblance measure: D1 Euclidean distance

Variables
1 Temp
2 pH
3 % Moisture
4 %C
5 %N
6 %OM
7 Li 7
8 B 10
9 Na 23
10 Mg 24
11 P 31
12 K 39
13 Ca 43
14 V 51
15 Fe 54
16 Mn 55
17 Co 59
18 Ni 60
19 Cu 63
20 Zn 68
21 Ga 71
22 As 75
23 Se 82
24 Sr 88
25 Ag 109
26 Cd 111

27 In 115
28 Ba 137
29 Tl 205
30 Pb 207
31 Bi 209
32 U 238

Global Test
Sample statistic (Rho): 0.668
Significance level of sample statistic: 0.2%
Number of permutations: 999 (Random sample)
Number of permuted statistics greater than or equal to Rho: 1

Best results

No.Vars	Corr.	Selections
5	0.668	1,2,4,7,13
5	0.663	1,2,4,7,9
5	0.663	1,2,4,7,12
4	0.654	1,2,4,7
4	0.647	2,4,7,13
5	0.646	1,2,4,5,7
4	0.640	2,4,7,12
4	0.637	2,4,7,9
5	0.635	1,2,4,7,18
5	0.634	1,2,4,7,10

Outputs
Plot: Graph8

Appendix B - Phylogeny

Bacterial sequences

Brackets indicate the OTU and then the clone name

(A) - C3

CGACTTAGTCCCATCAGGAGTCTCGCCTTCGGCACCTACCTCCCTTGCG
GGTTGGATCGGCGACTTCGGGCGCTCCTCCCTTTGGTGGCTTGACGGG
CGGTGTGTACAAGGCTCAGGAACACATTCACCGCGGCATTGCTGATCC
GCGATTACTAGCGATTCCGGCTTCATGGAGGCGAGTTGCAGCCTCCAA
TCTGAACTGAGCGGCCTTTTTTGGGATTGGCTCCCCCTCGCGGGTTGGC
GTCCCTTTGTCGGCCGCATTGTAGCACGTGTGCAGCCCTGGGCATAAG
GGCCATGAGGACTTGACGTCATCCCCACCTTCCTCCGTTTTATCAACGG
CAGTCCCAACAGAGTGCCCGGCCGAACCGCTGGCAACTGTTGGCAAG
GGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTG
ACGACAGCCATGCAGCACCTTGCACCCTGTCCGGTTTTACCCGGAAGA
CCCCATCTCTGGGGCTGTCAGGGGCATTCTAGCCCAGGTAAGGTTCTT
CGCGTTGCGTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGTCCC
CGTCAATTCCTTTGAGTTTCAGCCTTGCGACCGTACTCCCAGGCGGGAT
GCTTAACGCGTTAGCTGCGGCACTCGGGGACTAGAAGACCCCGAACA
CCAAGCATCCATCGTTTAGGGCTAGGACTACCAGGGTATCTAATCCTG
TTTGCTCCCCTAGCTTTCGCGCCTCAGCGTCAGTGTCGGCCCAGCAGCC
CGCCTTCGCCACCGGTGTTCCTCTCGATATCTACGCATTTCACCGCTAC
ACCGAGAATTCCAGCTGCCCCTTCCGCACTCTAGCTCTGCAGTATCACT
TGGCCCCTACCGAGTTAAGCCCGGAGATTTCACAAGTGACTTGCAGTG
CCGCCTACGCGCGCTTTACGCCCAGTAAATCCGAACAACGCTTGCCCC
CTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTACAAA
GGGTACCGTCATCGCACGGGGTATTAGCCCGTGCTTATTCGTCCCCTTC
TTTCGGAGTTTACATCCCGAAGGACTTCATCCTCCACGCGGCGTTGCTG
GGTCAGGCTTGCGCCCATTGCCCAAGATTCCCGACTGCTGCCTCCCGT
AGGAGTCTGGCCCGTGTGTCAGTGCCAGTGTGGCCGACCGCCCTCTCA
GGCCGGCTACGGATCGTCGCCTTGGTGAGCCGTTACCTCACCAACTAG
CTAATCCGCTGCAGGCTCCTCCCCGGGCGCGAGGCCTTGTTGCCAAGG
TCCCCCGCTTTCACGACGCAGCCCGCAGGCCGCGCCGCTGCATGCGGT
ATTAGCCCGGGTTTCCCCGGGTTATCCCCCACCCAGAGGCAGATTACC
CACGTGTTACTCACCCGGACGCCACTCTACTCGAGAGCCGAAGCCCTC
TTTCGCGTACGACTGCATGT

(B)- C9

TCAGGAGTCTCGCCTTCGGCACCTACCTCCCTTGCGGGTTGGATCGGC
GACTTCGGGCGCTCCTCCCTTTGGTGGCTTGACGGGCGGTGTGTACAA
GGCTCAGGAACACATTCACCGCGGCATTGCTGATCCGCGATTACTAGC
GATTCCGGCTTCATGGAGGCGAGTTGCAGCCTCCAATCTGAACTGAGC
GGCCTTTTTTGGGATTGGCTCCCCCTCGCGGGTTGGCGTCCCTTTGTCG
GCCGCATTGTAGCACGTGTGCAGCCCTGGGCATAAGGGCCATGATGAC
TTGACGTCGTCCCCACCTTCCTCCGGCTTAACGCCGGCAGTCCCCTTAG
AGTGCTCGGCATGACCCGGTGGCAACTAAGGGCAAGGGTTCCGCTCGT

TATGGGACTTAACCCGACATCTCACGACACGAGCTGACGACAGCCATG
CAGCACCTGTGCCGGGTTCCGGACTGGATACCGTTGGCAACCCCTTTC
GGGGCGCTACTTCCCGGCGCTTTCGCACATGTCAAGCCCAGGATAAGG
TTCTTCGCGTAGCCTCGAATTAAGCCACATGCTCCACCGCTTGTGTGAG
CCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGACCATACTCCCCAGGC
GGAGGACTTAGCACTTTTGCTTCGGCAGCGAGCCCATGTCAAGCCCAC
TACCTAGTCCTCATCGTTTAGGGCTAGGACTACCGGGGTATCTAATCC
CGTTTGCTCCCCCAGCTTTCGGGTCTGAGCGTCAGGGACAGCCCAGGT
GGCCGCCTTCGCCACTGGCGTTCCTCCCGATCTCTACGCATTTCACCGC
TACACCGGGAATTCCACCACCCTCTGCTGCCCTCAAGCCGGGCAGTTT
CCCGCAACGTCCCACGGTTGAGCCGTGGGCTTTCACACGGGACTTACC
CGGCCGCCTACACCCGCTTTACGCCCAGTAAATCCGGGCAACGCTCGC
CCCCTACGTCTTACCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTCC
TCTGGAGGTACCGTCACCCACCCGGAATGGGTGGGCTTCGTCCCTCCT
GACAGGGGTTTACGACCCGAAGGCCTTCATCCCCCACGCGGCGTCGCT
GGGTCAGGCTTGCGCCCATTGCCCAAGATTCCCAACTGCTGCCTCCCG
TAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGACCACCCTCTC
AGGCCAGCTACCCGTCATCGCCTTGGTGGGCCGTTACCCCACCAACCA
GCTGATAGGCCGCGGGCCCATCCCGAAGCAGAAGGGCTTGCGCCCAA
CCGTTTCCTCCTCCGGGGATGCCCCCAGAGGGGGACATCGGGTATTAG
CCCCGGTTTCCCGGGGTTATCCCCGTCTTCGGGGTAGGTTACCCACGTG
TTACTCACCCGTTCGCCGCTAGAGCTGAAACCGCGGGTTGCCCCGCGG
CCAACCCCCGCTCGACTTGCAT

(C) - F6

CAGCCCCGCCCTCGACGCCTGCCTCCCCTCGCGGGGTTGGCCCGGCGG
CTTCAGGCGTTGCCACCTCCCATGACGTGACGGGCGGTGTGTACAAGA
CCCGGGAACGTATTCACCGCCGTGTGGCTGACCGGCGGTTACTAGCGA
TTCCGGCTTCACGCAGGCGAGTTGCAGCCTGCGATCCGAACTGGGACC
GGGTTTTTGGGATTGGCTCCCCCTCGCGGGTTCGCAACCCATTGTCCCG
GCCATTGTAGCATGTGTGTCGCCCAGGCCATCAGGGGCATGATGACTT
GACGTCATCCCCACCTTCCTCCGGCTCATCGCCGGCAGTCCCTCTAGA
GTGCCCGGCCGAACCGGTGGCAACTAGAGGCGGGGGTTGCGCTCGTT
GCGGGACTTAACCCAACACCTCACGGCACGAGCTGACGACAGCCATG
CACCACCTGTGCCGGCTCCCGACTTTACGGGTCCCTCCCCTTTCGGTTC
GGTACTTCCGGCATGTCAAGGCCTGGTAAGGTTCTTCGCTTTGCATCG
AATTAAACCACATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTT
GAGTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGCGCACTTAACGCGT
TAGCTTCGGCACGGAAGGGGTTCAGACCTCCCACACCTAGTGCGCATC
GTTTAGGGCGTGGACTACCCCGGGTATCTAATCCGGTTTGCTCCCCAC
GCTTTCGCGCCTCAGTGTCAGGAACGAGCCAGGTGGCCGGCTTCCCCA
CTGGTGTTCCTCCCGATCTCTACGCATTTCACCGCTCCACCGGGAATTC
CACGTCCCTCTCCGGTGCTCAAGCCCGACGGTTTCGGACGGCCTCCGC
CAGTTGAGCCAGGGGCTTTCACGCCCGACTTAAGGCCCCACCTACGCG
CGCTTTACGCCCAGTAAATCCGGGTAACGCTTGGCCCCTACGTTTTACC
GCGGCTGCTGGCACGTAGTTGGCCGGGCCTTATTCGCTGGGTACCGTC
CTTGCTCGTCCCCGGCAAAAGCGGTTTACACCCCGAAGGGCTTCTTCC
CGCACGCGGCGTCGCTGGGTCAGGCTTTCGCCCATTGCGCAAGATTCC
TCACTGCTGCCTCCCGTAGGAGTCGGGCCCGTGTGTCAGTGCCCGTGT

GGGGGACCACCCTCTCAGGCCCCCTACCCGTCTTCGCCTTGGTAGGCC
ATTACCCTACCAACAAGCTGATGGGCCGCAGGCCCCTCCCTGGACGCC
TGTTGCCAGGCTTTCCTCTCGGCTATGACCCCGAGAGCGTATGCGGTA
TTAGCGCGCCTTTCGGCACGTTATCCCCCATCCAGGGGCAGGTCACCT
ACGTGTTACGCCCCCGGACGCCACTGAGGGACCAGGGCAAGCCCTGCT
CCCTCCGTACGACTGCATGTGTAG

(D) - A9

GTTCGACTTCGTCCAGTCACCGACCCCACCTTCGACGGCTCCCTCCCTT
GCGGGTTGGGCCACCGGCTTCGGGTGGAGCCAGCTCCCATGACGTGAC
GGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGCCGTGTGGCTGA
CCGGCGGTTACTAGCGATTCCGGCTTCACGCAGGCGAGTTGCAGCCTG
CGATCCGAACTGGGACCGGGTTTTTGGGATTGGCTCCCCCTCGCGGGT
TCGCAACCCATTGTCCCGGCCATTGTAGCATGTGTGTCGCCCAGGCCA
TCAGGGGCATGATGACTTGACGTCATCCCCACCTTCCTCCGGCTCATC
GCCGGCAGTCCCTCTAGAGTGCCCGGCCGAACCGGTGGCAACTAGAG
GCGGGGGTTGCGCTCGTTGCGGGACTTAACCCAACACCTCACGGCACG
AGCTGACGACAGCCATGCACCACCTGTGCCGGCTCCCGACTTTACGGG
TCCCTCCCCTTTCGGTTCGGTACTTCCGGCATGTCAAGGCCTGGTAAGG
TTCTTCGCTTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGG
TCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGC
GGCTCACTTAATGCGTTAGCTTCGGCACCGAGGGAGGAACCCCCCGAC
ACCTAGTGAGCATCGTTTAGGGCTGGGACTACCGGGGTATCTAATCCC
GTTCGCTCCCCCAGCTTTCGGGTCTGAGCGTCACAAACTGGCCAGGTG
GCTGCCTTCGCTATCGGCGTTCCTCCCGGTATCTGCGCATTTCACCGCT
ACTCCGGGAATTCCACCACCCCCTCCAGCCGTCTAGCTTGGCCGTATC
CAGCGCACCCCCACGGTTGAGCCGCGGTCTTTAACGCCAGACGCGCCA
AACCGCCTACACGCCCTTTACGCCCAGTAAATCCGGGTAACGCTCGCG
CCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTGGCCGGCGCTATTA
CCTGGGTACCGTCAGACCCCTCATCAGGGATTTCGCCCCCAGTTCAGG
AGTTTACACCCCGAAGGGCTTCATCCTCCAAGCGGCGTCGCTCCGTCA
GGCTTGCGCCCATTGCGGACGATTCCTAACTGCTGCCTCCCGTAGGAG
TGGGGCCCGTGTCTCAGTGCCCCTGTGGCCGGCCATCCTCTCAGACCG
GCTACCCGTCGTCGCCTTGGTGGGCCTTTACCCCACCAACCAGCTGAT
GGGACGCGGGCCCATCCGGAAGCAGGCTTAAACCCTTTGATCGTACCC
CAAAAGATACGACCACATGCGGGATTAGCCCGAGTTTCCCCGAGTTGT
CCCACACTTCCGGGTAGGTCACCCACGCGTTACTCACCCGTCCGCCGC
TGACCATGGAGTAAATCCCCACGGCCCGCACGACTTGCATTCT

(E) - B2

CATCACGAGCCATACCGTCGTGACCTGCCTCCCTTGCGGGTTAGCGCG
GCCACTTCTAGTACAGCCCGCTTTCGTGATGTGACGGGCGGTGTGTAC
AAGACCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAG
CGATTCCAGCTTCATGCAGTCGAGTTGCAGACTGCAATCCGAACTGGG
ACCGGCTTTTTGCGATTAGCTCCCCCTCGCGGGTTTGCAGCGCTTTGTG
CCGGCCATTGTAGCACGTGTGTAGCCCTGGGCATAAGGGCCATGAGGA
CTTGACGTCATCCCCACCTTCCTCCGTTTTATCAACGGCAGTCCCAACA

GAGTGCCCGGCGAACCGCTGGCAACTGTTGGCAAGGGTTGCGCTCGTT
GCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATG
CAGCACCTTGCACCCTGTCCGGTTTTACCCGGAAGACCCCATCTCTGG
GGCTGTCAGGGGCATTCTAGCCCAGGTAAGGTTCTTCGCGTTGCGTCG
AATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTT
GAGTTTCAGTCTTGCGACCGTACTTCCCAGGCGGAGTGCTTAACGCGT
TAGCTTCGGCACCCAGCGAAAGCCGGACACCCAGCACTCATCGTTTAG
GGCGTGGACTACCCGGGTATCTAATCCGGTTTGCTCCCCACGCTTTCGC
GCCTCAGCGTCACGTACTGTCCAGGCAGCTGCCTTCGCTATTGGCGTTC
CTTCCGGTATCTGCGCATTTCACCGCTACTCCGGAAATTCCGCTGCCCC
CTCCAGCCGTCGAGCCCTCCAGTATCCGAGGCAGGTCCAGGGTTGAGC
CCTAGTCTTTAGCCTTGGACTTAGAGGACCGCCTACACGCCCTTTACGC
CCAGTAAATCCGGGTAACGCTCGCACCCTCCGTATTACCGCGGCTGCT
GGCACGGAGTTAGCCGGTGCTATTAGCAGGGTACCGTCATGATCGTCC
CCTGTTCAGAGGTTTACACCCCGAAGGGCTTCGTCCCTCAAGCGGCGT
CGCTCGTTCAGGCTTGCGCCCATTGACGAAGATTCCTAACTGCTGCCTC
CCGTAGGAGTGGAGCCCGTGTCTCAGTGCTCCTGTGGCTGGTCGTCCT
CTCAGACCAGCTATGCGTCGTCGGCTTGGTAGGCCGTTACCCCACCAA
CTACCTGATGCACCGCGGGCTCATCCCAAAGCGATAAATCTTTAGAGA
CCTCACAGGAGAGGGCTCGCCATCCAGGATTAGCTAAGGTTTCCCCTA
GTTGTCCCAGACTTTGGGGTAGATCACCCACGTGTTACTCACCCGTCC
GCCACTGGCCTGACCGAAATCAGGCCCGTTCGACTGCA

(F) - B6

GTTCGACTTCGTCCCAGTCACCGGCCCCACCCTCGACGGCTGCCTCCTT
GCGGTTGGCCCACCGGCTTCAGGTGTTGCCAGCTCCCATGACGTGACG
GGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCACCATGGCTGAT
GCGCGGTTACTAGCAACTCCGCCTTCATGGGGGCGGGTTGCAGCCCCC
AATCCGAACTGAGACCGGCTTTGATGGGATTGGCTCCCCCTCGCGGGT
TGGCTGCCCATTGTACCGGCCATTGTAGCGTGTGTGTCGCCCAGGACA
TACAGGCCATGCTGACTTGACGTCATCCCCACCTTCCTCCCAGTTGCCC
AGGTGGTCCCCCTAGACACGTGTAACTAGGGGCAGGGGTTGCGCTCGT
TACAGGACTTAACCTAACACCTCACGGCACGAGCTGACGACAGCCATG
CAGCACCTGTGCGCGCTCCCTTGCGGGTCGGTCCGGTTTCCCTTCCCTA
CCACGCGCATGTCAAGCCCTGGTAAGGTTCTTCGTGTAGCCTCGAATT
AAACCACACGCTCCGCTGCTTGTGCGGGCCCCCGTCAATTCCTTTGAG
TTTTAACCTTGCGGCCGTAGTCCCCAGGCGGGACACTTAACGCGTTAG
CTTCGGCACGGAAGGGGTTCAGACCTCCCACACCTAGTGTCCATCGTT
TACGGCTTGGACTACCGGGGTTTCTAATCCCGTTCGCTCCCCAAGCTTT
CGTGTCTGAGCGTCAGCAACCGGCCAGGACGCCGCCTGCGCCACTGGT
GTTCCTCCCGATCTCTACGCATTTCACCGCTCCACCGGGAATTCCACGT
CCCTCTCCGGTGCTCAAGCCCGACGGTTTCGGACGGCCTCCCCCAGTT
GAGCCAGGGGCTTTCACGCCCGACCTGCCGAGCCGCCTGCACACGCTT
TACGCCCAGTAAATCCGGGTAACGCTTGCCTCCTACGTTTTACCGCGG
CTGCTGGCACGTAGTTAGCCGAGACTTATTCCTGCCGTACGGTCCTTGC
TCCTCCGGCAGAAAAGGAGTTTACGACCCGAAGGCCGTCATCCTCCAC
GCGGCGTTGCTGCGTCAGGCTTTCGCCCATTGCGCAAGATTCCTCACT
GCTGCCTCCCGTAGGAGTCGGGCCCGTGTGTCAGTGCCCGTGTGGGGG
ACCACCCTCTCAGGCCCCCTACCCGTCTTCGCCTTGGTAGGCCATTACC

CTACCAACAAGCTGATGGGCCGCAGGCCCCTCCCTGGACGCCTGTTGC
CAGGCTTTCCTCTCGGCTATGACCCCGAGAGCGTATGCGGTATTAGCG
CGCCTTTCGGCACGTTATCCCCCATCCAGGGGCAGGTCACCTACGTGT
TACGCACCCGGACGCCACTGAGGGACCAGGGCAAGCCCTGCTCCCTCC
GTACGACTGCA

(G) - B10

GTCACCGACCCCACCTTCGACGGCTCCCTCCCTTGCGGGTTGGGCCAC
CGGCTTCGGGTGGAGCCAGCTCCCATGACGTGACGGGCGGTGTGTACA
AGACCCGGGAACGTATTCACCGCCGTGTGGCTGACCGGCGGTTACTAG
CGATTCCGGCTTCACGCAGGCGAGTTGCAGCCTGCGATCCGAACTGGG
ACCGGGGTTTTTGGGATTGGCTCCCCCTCGCGGGTTCGCAACCCATTGTC
CCGGCCATTGTAGCATGTGTGTCGCCCAGGCCATCAGGGCATGATGAC
TTGACTTCATCCCCACCTTCCTCCGGGCTCATCGCCGGCAGTCCCTCTA
GAGTGCCCGGCCGAACCGGTGGCAACTAGAGGCGGGGGTTGCGCTCG
TTGCGGGACTTAACCCAACACCTCACGGCACGAGCTGACGACAGCCAT
GCACCACCTGTGCCGGCTCCCGACTTTACGGGTCCCTCCCCTTTCGGTT
CGGTACTTCCGGCATGTCAAGGCCTGGTAAGGTTCTTCGCTTTGCATCG
AATTAAACCACATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTT
GAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGCTCACTTAATGCGTT
AGCTTCGGCACCGAGGGAGGAACCCCCCGACACCTAGTGAGCATCGTT
TAGGGCTGGGACTACCGGGGTATCTAATCCCGTTCGCTCCCCCAGCTT
TCGGGTCTGAGCGTCAGGGACAGCCCAGGTGGCCGCCTTCGCCACTGG
CGTTCCTCCCGATCTCTACGCATTTCACCGCTACACCGGGAATTCCACC
ACCCTCTGCTGCCCTCAAGCCGGGCAGTTTCCCGCGACGTCCCACGGT
TGAGCCGTGGGCTTTCACACGGGACTTACCCGGCCGCCTACACCCGCT
TTACGCCCAGTAAATCTGGGCAACGCTCGCCCCCTACGTCTTACCGCG
GCTGCTGGCACGTAGTTAGCCGAGACTTATTCCTGCCGTACGGTCCTT
GCTCCTCCGGCAGAAAAGGAGTTTACGACCCGAAGGCCGTCATCCTCC
ACGCGGCGTTGCTGCGTCAGGCTTTCGCCCATTGCGCAAGATTCCTCA
CTGCTGCCTCCCGTAGGAGTCGGGCCCGTGTGTCAGTGCCCGTGTGGG
GGACCACCCTCTCAGACCCCCTACCCGTCTTCGCCTTGGTAGGCCATTA
CCCTACCAACAAGCTGATGGGCCGCAGGCCCCTCCCTGGACGCCTGTT
GCCAGGCTTTCCTCTCGGCTATGACCCCGAGAGCGTATGCGGTATTAG
CGCGCCTTCCGGCACGTTATCCCCCATCCAGGGGCAGGTCACCTACGT
GTTACGCACCCGGACGCCACTGAGGGACCAGGGCAAGCCCTGCTCCCT
CCGTACGACT

(H) - B12

CTTCGTCCAGTCACCGACCCCACCTTCGACGGCTCCCTCCCTTGCGGGT
TAGCCCACCGCCTTCGGGTGTTGCCGACTTTCGGGACGTGACGGGCGG
TGTGTACAAGGCCCGGGAACGTATTCACCGCAGTGGTGCTGACCTGCG
GTTACTAGCGACTCCGCGTTCATGCAGGCGAGTTGCAGCCTGCAATCC
CAACTGAGGGCCGTTTTGATGGGATTCGCTCCGCCTCGCGGCTTCGCT
GCCCATTGTACCGGCCCATTGTAGCGTGTGTGTAGCCCAGGGCATAAG
GGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGCCTTTCGGCGGC
AGCCCCCCTAGAGAGTGCAACTAGGGGCGGGGGTTGCGCTCGTTCTGG

GACTTAACCCAACACCTCACGGCACGAGCTGACGACAGCCATGCAGC
ACCTGTGCCGGCTCCCGCCTTTACGGGTCGTTCCCCTTTCGGGGTCCTA
CTTCCGGCATGTCAAGCCCTGGTAAGGTTCTTCGCGTAGCATCGAATT
AAACCACACGCTCCGCCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAG
TTTTAGCCTTGCGGCCGTAGTCCCCAGGCGGGGTGCTTAACGGGTTAC
CTTCGGCGCGGACAGGGGTCGATACCTGCCCACACCTAGCACCCACCG
TTTAGGGCGTGGACTACCCGGGTATCTAATCCGGTTCGCTCCCCACGC
TTTCGTCCCTGAGCGTCAGGAACGGGCCAGGAGGCCGCCTTCGCCACC
GGTGTTCCTCCCGGTATCTACGCATTTCACCGCTACACCGGGAGTTCCA
ATCTCCTCTCCTGCCCTCGAGCTCCGCAGTATCCGGTGTCAGCCTCTCG
GTTGAGCCGAGAGATTTCACACCAGACTTACGAAGCCGCCTACGTGCG
CTTTACGCCCAGTAAATCCGGGCAACGCTCGCCCCCTACGTATTACCG
CGGCTGCTGGCACGTAGTTAGCCGGGGCTTATTCACCAGGTACCGTCC
GAGGTCGTCCCTGGCAAAAGGAGTTTACACCCCGAAGGGCTTCGTCCT
CCACGCGGCGTCGCTGGGTCAGGCTTGCGCCCATTGCCCAAGATTCCC
GACTGCTGCCTCCCGTAGGAGTGGGGCCCGTGTCTCAGTGCCCCTCGG
GCCGGCCATCCTCTCAGACCGGCTACCCGTCGCAGGCTTGGTGAGCCA
TTACCTCACCAACTACCTGATAGGCCGCGGGCCCATCCCGAAGCGGAT
CGCTCCTTTAGCTACACAAGCCAAAGCCCATGTAGCCACATCCGGTAT
TAGCCCGAGTTTCCCCGGGTTATCCCCGTCTTCGGGGTAGGTTACCCAC
GTGTTACTCACCCGTTCGCCGCTAGAGCTTAAACCGTCGGTGTCCCTCC
AGCCAACCCCGTTA

(I) – C11

TCAGGAGTCTCGCCTTCGGCACCTACCTCCCTTGCGGGTTGGATCGGC
GACTTCGGGCGCTCCTCCCTTTGGTGGCTTGACGGGCGGTGTGTACAA
GGCCCGGGAACGTATTCACCGCGGCATGGCTGATCCGCGATTACTAGC
GATTCCGGCTTCACGGGGTCGAGTTGCAGACCCCGATCCGAACTGAGC
CCGGATTTAGGCGATTAGCTCCATCTTGCGATGTGGCGACGCTTTGTG
CCGGGCATTGTAGCACGTGTGTCGCCCAGGCCGTAAGGGCCATGCGGA
CTAGACGTCATCCCCGCCTTCCTCCCGCTTTCGCGGGCAGTCTCCCTAG
AGAGCTCGGCTGACCCGTTAGCAACTAAGGACAAGGGTTGCGCTCGTT
GCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATG
CAGCACCTGTCTCTAGGCTCCCTTGCGGGCACCCCCAACTTTCATCGG
GGTTCCTAGGATGTCAAGGCCTGGTAAGGTTCTTCGCGTTCCTTCGAAT
TAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGA
GTTTCAGCCTTGCGACCATACTCCCCAGGCGGGAGGACTTAGCACTTT
TGCTTCGGCAGCGAGCCGCATGTCAAGCCCACTACCTAGTCCTCATCG
TTTAGGGCTAGGACTACCGGGGTATCTAATCCCGTTTGCTCCCCTAGCT
TTCGCGCCTCAGCGTCAGAAGGGACCCAGCCGCGCGCCTTCGCCACCG
GCGTTCTTCCTGATCTCTACCCATTTCACCGCTACACCAGGAATTCCCT
CTGCCCCGAACGCACTCTAGTCGTGTAGTTTCCACTGCCTTTATCATGT
TAAGCATGACCCTTTGACAATAGACACACACAACCACCTGCGGACGCT
TTACGCCCAATCATTCCGGATAACGCTTGCATCCTCCGTATTACCGCGG
CTGCTGGCACGGAGTTAGCCGATGCTGATTCCTCAAGTAAGTACCTTC
AGTTTTATTCCTTGAGAAAAGAGGTTTACAACCCAAGAGCCTTCCTCC
CTCACGCGGTATTGCTCCGTCAGGCTTTCGCCCATTGCGGAAAATTCCC
CACTGCTGCCTCCCGTATGGAGTTTGGACCGTGTCTCAGTTCCAGTTGC
GGCCTGATCAATCCGTTCAGACCAGCTACTGATTGTCAGCCTTGGTAG

TCCCTTACCACCACCAACTAGCTAATCAGACGCGAGCTCCATCCTCAG
GCAAGCTCAGCCTTTCACCTCCTCGGCCACAATCCCGGTATTAGCATC
GGTTTCCCAATGTCGTCCCGAACCCTGAAGCTAGATTCTCTACGCGTTA
CTCACCCGTCCGCCACTAACTTCCTAAGAAGTCCGTCTCGACTTGCAGT
CGTGTAAGCATACCGC

(J) - D2

GCTATACATGCAGTCGAGCGGGGTCCATCCCCGGGCAACCGGGGAGA
AGACCTAGCGGCGAACGGGTGAGTAACACGTGAGTAACCTACCCCGA
ACACCGGGATAACTCTGGGAAACCGGGGCTAATACCGGATACCCACC
AGAGGACGCATGTTCTTTGGTGGAAAGGTCCGCCGGTTCGGGATGGGC
TCGCGGCCTATCAGCTTGTTGGTGGGGTAACGGCCTACCAAGGCGACG
ACGGGTAGCCGGCCTTAGAGGGTGTCCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTGCGCAAT
GGGCGAAAGCCTGACGCAGCGACGCCGCGTGGGGGACGAAGGCCTTC
GGGTTGTAAACCCCTTTCAGCGGGGAAGAAGCGAAAGTGACGGTACC
CGCAGAAGAAGCCCCGGCCAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGGGGCGAGCGTTGTCCGGATTCACTGGGCGTAAAGAGCTCGTA
GGCGGCTACGGAAGTCGGCTGTGAAAGCCCGGGGCTCAACCCCGGGA
GGCCAGTCGATACTCCGTAGCTAGAGGACGGCAGAGGGAGATGGAAT
TCCCAGTGTAGCGGTGAAATGCGCAGATATCGGGAGGAACGCCAGTG
GCGAAGGCGGCCACCTGGGCTGTCCCTGACGCTCAGACCCGAAAGCT
GGGGGAGCGAACGGGATTAGATACCCCGGTAGTCCCAGCCCTAAACG
ATGCTCACTAGGTGTCGGGGGGTTCCTCCCTCGGTGCCGAAGCTAACG
CATTAAGTGAGCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAA
AGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATT
CGATGCAAAGCGAAGAACCTTACCAGGCCTTGACATGCCGGAAGTAC
CGAACCGAAAGGGGAGGGACCCGTAAAGTCGGGAGCCGGCACAGGTG
GTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCCCGCCTCTAGTTGCCACCGGTTCGGCCGGGCA
CTCTAGAGGGACTGCCGGCGATGAGCCGGAGGAAGGTGGGGATGACG
TCAAGTCATCATGCCCCTGATGGCCTGGGCGACACACATGCTACAATG
GCCGGGACAATGGGTTGCGAACCCGCGAGGGGGAGCCAATCCCAAAA
ACCCGGTCCCAGTTCGGATCGCAGGCTGCAACTCGCCTGCGTGAAGCC
GGAATCGCTAGTAACCGCCGGTCGGCCACACGGCGGTGAATACGTTCC
CGGGTCTTGTACACACCGCCCGTCACGTCATGGGAGCTGGCTCCACCC
GAAGCCGGTGGCCCCACCCGCAAGGGAGGGAGCCGTCGAAGGTGGGG
TCGGTGACTGGACGAAGTCGT

(K) - D6

GAGTCTCGCCTTCGGCACCTACCTCCCTTGCGGGTTGGATCGGCGACTT
CGGGCGCTCCTCCCTTTGGTGGCTTGACGGGCGGTGTGTACAAGGCTC
AGGAACACATTCACCGCGGCATTGCTGATCCGCGATTACTAGCGATTC
CGGCTTCATGGAGGCGAGTTGCAGCCTCCAATCTGAACTGAGCGGCCT
TTTTTGGGATTGGCTCCCCCTCGCGGGTTGGCGTCCCTTTGTCGGCCGC
ATTGTAGCACGTGTGCAGCCCTGGGCATAAGGGCCATGATGACTTGAC
GTCGTCCCCACCTTCCTCCGGCTTAACGCCGGCAGTCCCCTTAAAGTGC

TCGGCATGACCCGGTGGCAACTAAGGGCAAGGGTTCCGCTCGTTATGG
GACTTAACCCGACATCTCACGACACGAGCTGAAGACAGCCATGCAGC
ACCTGTGCCGGGTTCCGGACTGGATACCGTTGGCAACCCCTTTCGGGG
CGCTACTTCCCGGCGCTTTCGCACATGTCAAGCCCAGGATAAGGTTCT
TCGCGTAGCCTCGATTTAAACCACTTGCTCCACCGCTTGTGTGAGCCCC
CGTCAATTCCTTTGAGTTTCAGCCTTGCGACCGTACTCCCCAGGCGGCT
CACTTAATGCGTTAGCTTCGGCACCGAGGGAGGAACCCCCCGACACCT
AGTGAGCATCGTTTAGGGCTGGGACTACCGGGGTATCTAATCCCGTTC
GCTCCCCCAGCTTTCGGGTCTGAGCGTCAGGGACAGCCCAGGTGGCCG
CCTTCGCCACTGGCGTTCCTCCCGATCTCTACGCATTTCACCGCTACAC
CAGGAATTCCCTCTGCCCCGAACGCACTCTAGTCGTGTAGTTTCCACTG
CCTTTATCATGTTAAGCATGACCCTTTGACAATAGACACACACAACCA
CCTGCGGACGCTTTACGCCCAATCATTCCGGATAACGCTTGCATCCTCC
GTATTACCGCGGCTGCTGGCACGGAGTTAGCCGATGCTGATTCCTCAA
GTACCTTCAGTTTTATTCCTTGAGAAAAGAGGTTTACAACCCAAGAGC
CTTCCTCCCTCACGCGGTATTGCTCCGTCAGGCTTTCGCCCATTGCGGA
AAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTC
CAGTGTGGCTGATCATCCTCTCAGACCAGCTACTGATCGTCGCCTTGGT
AGTCCCTTACACCACCAACTAGCTAATCAGACGCGAGCTCATCTTCAG
GCAGCTAGCCTTTCACCTCCTCGGCACATCCGGTATTAGCATCGGTTTC
CCAATGTTGTCCCGAACCTGAAGCTAGATTCTCACGCGTTACTCACCC
GTCCGCCACTAACTTCCTAAGAAGTCCGTTCGACTGCATGTGTAAG

(L) - D10

ATCAGGAGTCTCGCCTTCGGCACCTACCTCCCTTGCGGGTTGGATCGG
CGACTTCGGGCGCTCCTCCCTTTGGTGGCTTGACGGGCGGTGTGTACA
AGGCTCAGGAACACATTCACCGCGGCATTGCTGATCCGCGATTACTAG
CGATTCCGGCTTCATGGAGGCGAGTTGTAGCCTCCAATCTGAACTGAG
CGGCCTTTTTTGGGATTGGCTCCCCCTCGCGGGTTGGCGTCCCTTTGTC
GGCCGCATTGTAGCACGTGTGCAGCCCTGGGCATAAGGGCCATGATGA
CTTGACGTCGTCCCCACCTTCCTCCGGCTTAACGCCGGCAGTCCCCTTA
GAGTGCTCGGCATGACCCGGTGGCAACTAAGGGCAAGGGTTCCGCTC
GTTATGGGACTTAACCCGACATCTCACGACACGAGCTGACGACAGCCA
TGCAGCACCTGTGCCGGGTTCCGGACTGGATACCGTTGGCAACCCCTT
TCGGGGCGCTACTTCCCGGCGCTTTCGCACATGTCAAGCCCAGGATAA
GGTTCTTCGCGTAGCCTCGAAATTAAGCCACATGCTCCACCGCTTGTGT
GAGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGACCATACTCCCCA
GGCGGAGGACTTAGCACTTTTGCTTCGGCAGCGAGCCCATGTCAAGCC
CACTACCTAGTCCTCATCGTTTAGGGCTAGGACTACCGGGGTATCTAA
TCCCGTTTGCTCCCCTAGCTTTCGCGCCTCAGCGTCAGAAGGGACCCA
GCCGCGCGCCTTCGCCACCGGCGTTCCTACCGATATCAACGCATTTCA
CCGCTCCACCGGTAGTTCCCGCGGCCCCTTTCCCCCTCGAGCCGACCA
GTATCCACGGCAATTCCCCGGTTGAGCCGGGGGCTTTCACCGAAGACT
TGGCCGACCGCCTACGCGCCCTTTAAGCCCAGTGAATCCGAGTAACGT
TTGCACGGTTCGTCTTACCGCGGCTGCTGGCACGAACTTAGCCCGTGC
TTCCTCTGGGGTTAGGTCACACCCTTGCGGGCTTTCCTCCCCCCTGACA
GCGGTTTACAACCCGAAGGCCTTCATCCCGCACGCGGCGTCGCAGGGT
CAGGCTTGCGCCCATTGCCCAAGATCCTCGACTGCAGCCACCCGTAGG
TGTCTGGCCAGTGTCTCAGTGCCAGTGGCGCGGGCCATGCTCTCACAC

CCGCTAGGCATCTTCGCCTTGGTAGGCCGTTACCCTACCAACTAGCTA
ATACCACGTGGGCCGCTCCCCAGGCGGAAGGCTTTCGGCCAACCCTTT
GGTACCTAAGTCATGCGACCTGAGTACATCGCCCGGTATTAGCCCCAG
TTTCCCGGGGTTATCCCAGTCCTGGGGGTACGTTACCCACGCCTTACTG
CCCCTTACGCCGCTCGGCTCAGCCCCTTGCGGAGCTGTCCTCGCTCGAC
TGCATGC

M - F11

GCGATTCCGGCTTCATGGGGGCGGGTTGCAGCCCCCAATCCGAACTGA
GACCGGCTTTGATGGGATTGGCTCCCCCTCGCGGGTTGGCTGCCCATT
GTACCGGCCATTGTAGCGTGTGTGTCGCCCAGGACATACAGGCCATGC
TGACTTGACGTCATCCCCACCTTCCTCCCAGTTGCCCAGGCGGTCCCCC
TAGACACGTGTAACTAGGGGCAGGGGTTGCGCTCGTTACAGGACTTAA
CCTAACACCTCACGGCACGAGCTGACGACAGCCATGCAGCACCTGTGC
GCGCTCCCTTGCGGGTCGGTCCGGTTTCCCTTCCCTACCACGCGCATGT
CAAGCCCTGGTAAGGTTCTTCGTGTAGCCTCGAATTAAACCACACGCT
CCGCTGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAACCTTGCG
GCCGTAGTCCCCAGGCGGGACACTTAATGCGTTAGCTTCGGCACCGAG
GGAGGAACCCCCCGACACCTATTGAGCATCGTTTAGGGCTGGGACTAC
CGGGGTATCTAATCCCGTTCGCTCCCCCAGCTTTCGGGTCTGAGCGTCA
AGGACAGCCCAGGTGGCCGCCTTCGCCACTGGCGTTCCTCCCGATCTC
TACGCATTTCACCGCTACGCCGGGAATTCCACCACCCTCTGCTGCCCTC
AAGCCGGGCAGTTTCCCGCGACGTCCCACGGTTGAGCCGTGGGCTTTC
ACACGGGACTTACCCGGCCGCCTACACCCGCTTTACGCCCAGTAAATC
CGGGCAACGCTCGCCCCCTACGTCTTACCGCGGCTGCTGGCACGTAGT
TAGCCGGGGCTTCCTCTGGAGGTACCGTCACCCACCCGGAATGGGTGG
GCTTCGTCCCTCCTGACAGGGGTTTACGACCCGAAGGCCTTCATCCCC
CACGCGGCGTCGCTGGGTCAGGCTTGCGCCCATTGCCCAAGATTCCCA
ACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCGGTCTGG
CTGACCACCCTCTCAGGCCAGCTACCCGTCATCGGCTTGGTGGGCCAT
TACCCCACCAACTACCTGATAGGCCGCGGGCCCCTCCCCAAGCGGGCC
CTCGCGGGCCCTTTAGTTACCGGACATCCGTCCGGTAACCACATGCGG
TATTAGCCCCAGTTTCCCAGGGTTATCCCCCACTTGGGGGCAGGTTAC
CCACGTGTTACTCAGCCGTTCGCCGCTGGCTGGGACCTAAGCCCCAGC
CCGCTCGACTGCA

(N) - E4

TCGTCCAGTCGCCAGCCCCACCCTCGACGGCTGCCTCCTTGCGGTTGG
CCCACCGGCTTCAGGTGTTGCCAGCTCCCATGACGTGACGGGCGGTGT
GTGCAAGGCCCGGGAACGTATTCACCGCACCATGGCTGATGCGCGGTT
ACTAGCAACTCCGCCTTCATGCAGGCGGGTTGCAGCCTGCAATCCCAA
CTGAGACCGGCTTTGATGGGATTCGCTCCCCCTCGCGGGTTTGCAGCC
CTTTGTACCGGCCAATGTAGCACGTGTGTAGCCCAGGGCATAAGGGGC
ATGATGACTTGACGTCATCCCCACCTTCCTCCGACTTTTCGCCGGCAGT
CTCCCTAGAGTCCCCGGCATTACCCGCTGGCAACTAGGGACAGGGGTT
GCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGA
CAGCCATGCACCACCTGTGCACGGGCCCCGAAGGGCTGCCGTGTTTCC
ACGACATTCCCGTGCATGTCAAGCCCTGGTAAGGTTCTTCGCGTTGCG

TCGAATTAAACCACATGCTCCGCTGCTTGTGCGGGCCCCCGTCAATTC
CTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGTGCTTAATG
CGTTAGCTTCGGCACGGGGAGAGTCGACACTCCCCACACCTAGCACCC
ATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCA
CGCTTTCGCGTCTCAGCGTCAGTACCGTCCCAGAGAGCCGCCTTCGCC
ACGGGTGTTCTTCCCGATATCTGCGCATTTCACCGCTACACCGGGAATT
CCACTCTCCTCTTCCGGACTCTAGCCAAGTGGTTTCCGCCGACGTCTCG
AGGTTGAGCCTCGAGTTTTCACAGCGGACCTACCTGGCCGCCTACACG
CGCTTTACGCCCAATGAATCCGGACAACGCTAGCCCCCTACGTATTAC
CGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTCTTCTGGGGGTACCGT
CACCCTCTGGGCTGTTCACCCGAGGAGCTTCGTCCCCCCTGAAAGCGG
TTTACAACCCGAAGGCCTTCTTCCCGCACGCGGCGTCGCTGCGTCAGG
CTTTCGCCCATTGCGCAAGATTCCCCACTGCTGCCTCCCGTAGGAGTCT
GGGCCGTGTCTCAGTCCCAGTGTGGCTGGCCATCCTCTCAGACCAGCT
ACGGATCGTCGCCTTGGTAGGCCATTACCCCACCAACGAGCTAATCCG
CCGCAGGCCCCTCCTCGGCCGGAAGCCGAAGCTACCTTTTCTCCGGCG
CCCGCAGGCTCCGGAGGCTATCCGGTATTAATCCGGGTTTCCCCGGGC
TATCCCGGTGCCGAGGGCAGGTTACCCACGTGTTACTCACCCGTTCGC
CGCTTTCCCCGAGCCCGAAGGCTCGGTTCTCGCTCGACTGCA

(O) - E5

CCCCACCCTCGACGGCTGCCTCCTTGCGGTTGGCCACCGGCTTCAGGT
GTTGCCAGCTCCCATGACGTGACGGGCGGTGTGTACAAGGCCCGGGA
ACGTATTCACCGCACCATGGCTGATGCGCGGTTACTAGCAACTCCGCC
TTCATGGGGGCGGGTTGCAGCCCCCAATCCGAACTGAGACCGGCTTTG
ATGGGATTGGCTCCCCCTCGCGGGTTGGCTGCCCATTGTACCGGCCAT
TGTAGCGTGTGTGTCGCCCAGGACATACAGGCCATGCTGACTTGACGT
CATCCCCACCTTCCTCCCAGTTGCCCAGGCGGTCCCCCTAGACACGTGT
AACTAGGGGCAGGGGTTGCGCTCGTTACAGGACTTAACCTAACACCTC
ACGGCACGAGCTGACGACAGCCATGCAGCACCTGTGCGCGCTCCCTTG
CGGGTCGGTCCGGTTTCCCTTCCCTACCACGCGCATGTCAAGCCCTGGT
AAGGTTCTTCGCGTTGCGTCGAATTAAACCCCACGCTCCGCTGCTTGTG
CGGGCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCC
AGGCGGGACACTTAATGCGTTGGCTTCGGCACTGGCGGGGTTGATACC
GCCAACACCTAGTGTCCATCGTTTACGGCTAGGACTACCGGGGTATCT
AATCCCGTTCGCTCCCCTAGCTTTCGCGCCTCAGCGTCAAGAACGGGG
CAGGAAGCCGCTCGCCACTGGTGTTCCTCCCGATATCTACGCATTTCA
CCGCTACACCGGGAATTCCGCTTCCCTCTCCCGCCTTCGAGTCGACCA
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TTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGC
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CCCATTGCGCAAAATTCCCGACTGCTGCCTCCCGTAGGAGTCTGGGCC
GTGTCTCAGTCCAGTCTGGGTGATCGTCCTCTCAGACCACCTACCGATC
ATCGCTTGGTGAGCCGTTACCCCACCAACGAGCTAATCGGACGCAGGC
CCCTCCCGAAGCGCCTCGCGGCTTTGATGACACGACCGAACGTGCCAC
CACATGCGGTATTAGCCACCCTTTCGGGCAGTTATCCCCCACTTCGGG

GCAGGTCACCTACGCGTTACTCAGCCGTTCGCCACTAACGGCCGGAGC
AAGCCCGCCGTCCGT

(P) - F7

AGTCACCAGTCCTACCTTCGGCGTCCCCCTCCTTTGACGGTTAGGGTAA
CGACTTCGGGCGTGACCAGCTTCCATGGTGTGACGGGCGGTGTGTACA
AGGCCCGGGAACGAATTCACTGCAGTATGCTGACCTGCAATTACTAGC
GATTCCAGCTTCATGCAGTCGAGTTGCAGACTGCAATCCGAACTGGGA
CCGGCTTTTTGCGATTAGCTCCCCCTCGCGGGTTTGCAGCGCTTTGTGC
CGGCCATTGTAGCACGTGTGTAGCCCTGGGCATAAGGGCCATGAGGAC
TTGACGTCATCCCCACCTTCCTCCGTTTTATCAACGGCAGTCCCAACAG
AGTGCCCGGCGAACCGCTGGCAACTGTTGGCAAGGGTTGCGCTCGTTG
CGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGC
AGCACCTTGCACCCTGTCCGGTTTTACCCGGAAGACCCCATCTCTGGG
GCTGTCAGGGGCATTCTAGCCCAGGTAAGGTTCTTCGCGTTGCGTCGA
ATTAAACCACATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTG
AGTTTCAGCCTTGCGACCGTACTCTCCAGGCGGGATGCTTAACGCGTT
AGCTGCGGCACTCGGGGACTAGAAGACCCCGAACACCAAGCATCCAT
CGTTTAGGGCTAGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCTAG
CTTTCGCGCCTCAGCGTCAGTGTCGGCCCAGCAGCCCGCCTTCGCCAC
CGGTGTTCCTCTCGATATCTACGCATTTCACCGCTACACCGAGAATTCC
AGCTGCCCCTTCCGCACTCTAGCTCTGCAGTATCACTTGGCCCCTACCG
AGTTAAGCCCGGAGATTTCACAAGTGACTTGCAGTGCCGCCTACGCGC
GCTTTACGCCCAGTAAATCCGAACAACGCTTGCCCCCTACGTATTACC
GCGGCTGCTGGCACGTAGTTAGCCGGGGCTTATTCACCAGGTACCGTC
CGAGGTCGTCCCTGGCAAAAGGAGTTTACATCCCGAAGGACTTCATCC
TCCACGCGGCGTTGCTGGGTCAGGCTTGCGCCCATTGCCCAAGATTCC
CGACTGCTGCCTCCCGTAGGAGTCTGGCCCGTGTGTCAGTGCCAGTGT
GGCCGACCGCCCTCTCAGGCCGGCTACGGATCGTCGCCTTGGTGAGCC
GTTACCTCACCAACTAGCTAATCCGCCGCAGGCTCCTCTCCGGGCGCG
AGGCCTTGTTGCCAAGGTCCCCCGCTTTCACGACGCAGCCCGCAGGCC
GCGCCGCTGCATGCGGTATTAGCCCGGGTTTCCCCGGGTTATCCCCCA
CCCAGAGGCAGATTACCCACGTGTTACTCACCCGGACGCCACTCTACT
CGAGAGCCGAAGCCCTCTTTCGCGTACGACTGCATGT

(Q) - G1

TCACGAGCCATACCGTCGTGACCTGCCTCCCTTGCGGGTTCCCTCGGCC
ACTTCTAGTACAGCCCGCTTTCGTGATGTGACGGGCGGTGTGTACAAG
ACCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGA
TTCCAGCTTCATGCAGTCGAGTTGCAGACTGCAATCCGAACTGGGACC
GGCTTTTTGCGATTGGCTCCCCCTCGCGGGTTTGCAGCGCTTTGTGCCG
GCCATTGTAGCACGTGTGTGGCCCTAGGCATAAGGGCCATGAGGACTT
GACGTCATCCCCACCTTCCTCCGTTTTATCAACGGCAGTCCCCTATGAG
TGCCCGGCATGACCCGCTGGCAACATAGGGCAGGGGTTGCGCTCGTTG
CGGCACTTAAGCCAACACCTCACGGCACGAGCTGACGACAGCCATGC
AACACCTGTGCCGGCTCCCGACTTCACGGGTCGGCCACCCTTTCAGGC
GCCTACTTCCGGCATGTCAAGCCTGGGTAAGGTTCTTCGCTTTGCATCG
AATTATTGCCACGCGCTCCGCCGCTTGTGCGGGCCCCCGTCAATTCCTT

TTGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGCGGACTTAATGC
GTTAGCTTCGGCACAGAGGGCCTAACGCCCCCCACACCTAGTCCCGCA
CCGTTTACGGCCAGGACTACCCGGGTATCTAATCCGGTTCGCTCCTCTG
GCTATCGAGTCGGGTCTCAGCGTCAGGAACGCCAGTCGCCGCCTTCGC
CACTGGTGTTCCTCCCGATATCTGCCCATTTCACCGGTACACCGGGAAT
TCCACCGACCTCTGCCGTCCTCAAGTCCGGCAGTATCGGATGCCATTC
CACGTTGAGCCGTGGGATTTCACATCCGACTTGCCGGACCGCCTACAC
CCGCTTTACGCCCAGTGAATCCGGGTAACGCTAGCCCCCTACGTTTTA
CCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTCTTCTGCAGGTACCG
TCACCACGGTTGCCCGTGGCTTCTTCCCTGCCGAAAGAGGTTTACACC
CCGAAGGGCTTCATCCCTCACGCGGCATCGCTGCGTCAGGCTTTCGCC
CATTGCGCAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCCGGGCCGT
GTCTCAGTCCCGGTGTGGCCGGTCACCCTCTCAGGCCGGCTACCCGTC
ATCGGCTTGGTGGGCCATTACCCCACCAACTACCTGATAGGCCGCGGG
CCCCTCTCGAGGCGGAAGCCCGTTTTCTCCCAAGGCCATGCGACCTCG
TGAGCTTATCCGGTATTAGCCCCAGTTTCCCGGGGTTATCCCGGTCCTC
GAGGCAGGTTACCCACGTGTTACTCACCCGTTCGCCGCTCCGTACTGC
GAGGAGCAAGCTCCCCACAGCCGATCGTAAACTGCGTTTAGCGTGCGC
AGCGTC

(R) – 182

AGTCGCCAGCCCCACCCTCGACGGCTGCCTCCTTGCGGTTGGCCCTTC
GGCTTCAGGTGTTGCCAGCTCCCATGACGTGACGGGCGGTGTGTGCAA
GGCCCGGGAACGTATTCACCGCACCATGGCTGATGCGCGGTTACTAGC
AACTCCGCCTTCATGCAGGCGGGTTGCAGCCTGCAATCCCAACTGAGA
CCGGCTTTGATGGGATTGGCTCCCCCTCGCGGGTTGGCTGCCCATTGC
ACCGGCCATTGTAGCGTGTGTGTCGCCCAGGACATACAGGCCATGCTG
ACTTGACGTCATCCCCACCTTCCTCCAGCTTATCGCTGGCAGTCCCCCT
AGACACGTGTAACTAGGGGCAGGGGTTGCGCTCGTTACAGGACTTAAC
CTAACACCTCACGGCACGAGCTGACGACAGCCATGCAGCACCTGTGCG
CGCTCCCTTGCGGGTCGGTCCGGTTTCCCTTCCCTACCACGCGCATGTC
AAGCCCGGGTAAGGTTCTTCGTGTAGCCTCGAATTAAACCCCACGCTC
CGCTGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGG
CCGTACTCCCCAGGCGGGACACTTAACGCGTTAGCTTCGGCACGGAAG
GTGTAGACAACCCCCCACGCCTAGTGCCCCATCGTTTTACGGCTGGGA
CTACCAGGGTATCAAATCCTGTTCGCTCCCCCAGCTTTCGCTCCTCACC
GTCAGGAACGGCCCAGGAGACCGCCTTCGCCACTGGTGTTCCTCCCGA
TATCTGCGCATTTTCACCGCTACACCGGGATTCCATCTCCCTCTGCCGT
CCTCTAGCCACGGAGTATCGACTGGCCTCCCGGGGTTGAGCCCCGGGC
TTTCAGCAGCCGACTTCCGTAGCCGCTACGAGCTCTTTACGCCCAGTG
AATCCGGACAACGCTCGCCCTCTACGTATTACCGCGGCTGCTGGCACG
TAGTTGGCCGGGGCTTCTTCTGCGGGTACAGTCACTATTCGACTTCTTC
CCCGATGAAAGGGGTTACAACCACGAAGGCTTGCGTCCCCCACGCGG
CGTCGCTGCGTCAGGCTTTCGCCCATTGCGACAAGATTCCTACTGCTGC
CTCCCGTAGGAGTCTGGGCCGTGTCTCAGTACCCAGTGTGGCCGGACA
CCCTCTAAGGCCGGCTACCCGTGGTCGCCTTGGTAGGCCGCTTACCAC
CACCAACAAGCTGATAGACCGCGAGCCCATCACCGAACCGGCGGAGC
CTTTCCACCAAAGAACATGCGTCCTCTGGTGGGTAGTCCGGTATTAGC
CCCGGTTTCTCTAGAGTTAGTCTCCGG

Archaeal sequences

(S) – A2

CCGGCGTTGAATCCAATTGAACCGCAGGCTTCACCCCTTGTGGTGCTC
CCCCGCCAATTCCTTTAAGTTTCAGTCTTGCGACCGTACTCCCCAGGCG
GCAGACTTAACGGCTTCCCTGCGGCACTGGGTTGGCCCGAAGCCAACC
CATCACCGAGTCTGCATCGTTTACAGCTGGGACTACCCGGGTATCTAA
TCCGGTTTGCTCCCCCAGCTTTCATCCCTCACCGTCGAGCGCGTTCTGG
CAAGCCGCCTTCGCCACTGGTGGTCTTCAGTGGATCAGAGGATTTTAC
CCCTACCCACTGAGTACCGCTTGCCTCTCCCGCCTCCTAGCCCTGTAGT
ATCCCCCGCAGCCCATCTGTTGAGCAGGTGGATTTAACGGAAGACTTG
CAGAACCGGCTACGGATGCTTTAGGCCCAATAATCGTCCCGACCACTC
GGGGTGCTGGTATTACCGCGGCGGCTGACACCAGACTTGCCCACCCCT
TATTCTGCACCATTTTTAGAGGTGCCAAAAGATGCCCTTAACGGACAT
CACTCAGAGTAACCCTGTCAAGCTTTCGCCTATTGCAGAGGTTTCGCG
CCTGCTGCGCCCCATAGGGCCTGGGCCCTTGTCTCAGTGCCCATCTCCG
GGCTCCTCCTCTCAGAGCCCGTACCGGTACAGTTTTGGTGGGCCATTA
CCTCACAACAGCTGATCGGCGCAGTCCATCAGGCATATAGGTTTCAGC
ATGATCCATTCCAGAA

(T) – E7

CGAATTGAATTTAGCGGCCGCGAATTCGCCCTTTCCGGCGTTGAATCC
AATTGAACCGCAGGCTTCACCCCCTGTGGTGCTCCCCCGCCAATTCCTT
TAAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGCAGACTTAACGGC
TTCCCTGCGGCACTGGGTTGGCTCGAAGCCAACCCATCACCGAGTCTG
CATCGTTTACAGCTGGGACTACCCGGGTATCTAATCCGGTTTGCTCCCC
CAGCTTTCATCCCTCACCGTCGAGCGCGTTCTGGCAAGCCGCCTTCGCC
ACTGGTGGTCTTCAGTGGATCAGAGGATTTTACCCCTACCCACTGAGT
ACCGGTTGCCTCTCCCGCCTCCTAGCCCTGTAGTATCCCCCGCAGCCCA
TCTGTTGAGCAGGTGGATTTAACGGAAGACTTGCAGAACCGGCTACGG
ATGCTTTAGGCCCAATAATCGTCCCGACCACTCGGGGTGCTGGTATTA
CCGGGCGGCTGACACCAGACTTGCCCACCCCTTATTCTGCACCATTTA
AGAGGGGCCAAGAGATGCCCTTACCGGACATCACTCAGAGTAACCCT
GTCAAGCTTCGCCTATTGCAGAGGTATCGCGCCTGCTGAGCCCCATAG
GGCCTGGGCCCTTGTTCTTCAGTGCCCATCTCCGGGCTCCTCCTCTCAG
AGCCCGTACCGGTTACAGGTTTTTGGTGGGCCATTACCTCACCAACAG
CCTGATCGGCCGCAGTCCAATCCAGGGGCCATATAGGTTTCAGCCATG
ATCCATTCCAGAAACCTTGGCCTATCGCGGTTTATCCTCAGTTTCCCGA
GGTTATCCACGTCCCCTGGGCATGTTGACTACGTGTTACTGAGCTCGTA
TGCCACGCATTGTCTGCGTTGACTCCACATGTGGCTTAGTCCCACTCTG
ATAGCACGTCAGGGTCCGGCATGG

(U) – C1

TATAGTCCTGCAGGTTTAACGAATTCGCCCTTTCCGGCGTTGAATCCAA
TTGAACCGCAGGCTTCACCCCTTGTGGTGCTCCCCCGCCAATTCCTTTA
AGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGCAGACTTAACGGCTT

CCCTGCGGCACTGGGTTGGCTCGAAGCCAACCCATCACCGAGTCTGCA
TCGTTTACAGCTGGGACTACCCGGGTATCTAATCCGGTTTGCTCCCCCA
GCTTTCATCCCTCACCGTCGAGCGCGTTCTGGCAAGCCGCCTTCGCCAC
TGGTGGTCTTCAGTGGATCAGAGGATTTTACCCCTACCCACTGAGTAC
CGCTTGCCTCTCCCGCCTCTAGCCTGTAGTATCCCCCGCAGCCCATCTG
TGACAGGTGATTTAACGGAAGACTTGCAGAACCGGCTCGGATGCTTTA
GGCCCAATTATCGTCCCGACCACTCGGGGTGCTGGTATTACCGCGGCG
GCTGACACCAGACTTGCCCACCCCTTATTCTGCACCATTTTTAGAGGTG
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TCGCCTATTGCAGAGGTTTCGCGCCTGCTGCGCCCCATAGGGCCTGGG
CCTTGTCTCAGTGCCCATTCCGGCTCCTCCTCTCAGAGCCCGTACGGTT
ACAGGTTTGGTGGGCCATTACCTCACCAACAGCCTGATCGGCCGCAGT
CCAATCCAGGGGCCATATAGGTTTCAGCCATGATCCATTCCAGAAACT
TGGCCTATCGCGGTTTATCCTCAGTTTCCCGAGGTTATCCACGTCCCTG
GGCATGTTGACTACGTGTTACTGAGCCGTATGCCACGCATTGCTGCGT
TGACTCGCATGGCTTAGTCCCACTCTGATAGCAGTCGGGTCCGGCAGG
ATCAACCGGAAAGGGCGATTCGCGCCGCTATTCATTCGCCCTATAGTG
GTCGTATACAGTC

(V) – C6

CTTGTGGTGCTCCCCCGCCAATTCCTTTAAGTTCAGTCTTGCGACCGTA
CTCCCCAGGCAGCAGACTTAACGGCTTCCCTGCGGCACTGGGTTGGCT
CGAAGCCAACCCATCACCGAGTCTGCATCGTTTACAGCTGGGACTACC
CGGGTATCTAATCCGGTTTGCTCCCCCAGCTTTCATCCCTCACCGTCGA
GCGCGTTCTGGCAAGCCGCTTCGCCACTGGTGGCCTTCAGTGGATCAG
AGGATTTTACCCCTACCCACTGAGTACCGCTTGCCTCTCCCGCCTCCTA
GCCCTGTAGTATCTCCGCAGCCCATCTTGTTGAGCAGTTGGATTTAACG
GAAGACTGCTAGACCGTTCGGATGCTTTAGGCTCCAATAATCGTCCCG
ACCACTCGGTGTTTTGGTATTACCGCGGCGGCTGACACCAGACTTGCC
CACCCACTTATTCTGCACCATTTTTAGAGGTGCCTAAAAGATGCCCTTA
ACGGACATCACCCAGAGTAACCCTGTCAAGCTTTCTGCGCTATTGCAG
AGGTTTCGCGTCCTTCTGCTGCCCCATAGGGCCTGGGCCCTTGTCTCAG
TGCCCATCTCCGGGCTCCTCCTCTCAGAGCCCGTACCGGTTACAGGTTT
GGTGGGCCATTACCCCACCAACAGCCTGATCGGCACGCAGTCCAATCC
AGGGGCCATATAGGTTTCAGCCATGATCCATTCCAGAAACCTTGGCCT
ATCGCGGTTTATCCTCAGTTTCCCGAGGTTATCCACAGTCCCCTGGGCA
TGTTGACTACGTGTTACTGAGCCGTATGCCGCATATTGCTATGTTGACT
CGCATGGCTTAGTCCCACTCTGATAGCAGTCGGGTCCGGCGGGATCAA
CCGGAAAAGGGCGAATTGCGCAGGCCAGCTAAATTCAATTCGTCACTA
TAGTGAGTCGTATTAC

(W) – D10

CGAATTGAATTTAGCGGCCGCGAATTCGCCCTTTCCGGCGTTGACTCC
AATTGAACCGCAGGCTTCACCCCTTTGTGGTGCTCCCCCGCCAATTCCT
TTAAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGCAGACTTAACGG
CTTCCCTGCGGCACTGGGTTGGCTCGAATGCCAACCCATCACCGAGTC
TGCATCGTTTACAGCTGGGACTACCCGGGTATCTAATCCGGTTTGCTCC
CCCAGCTTTCATCCCTCACCGTCAGAGCGCGTTCTGGCAAGCCAGCCT

TCGCCACTGGTGGTCTTCAGTGGATCAGAGGATTTTACCCCTACCCACT
GAGTACCGTTGCCTCTCCCGCCTCCTAGCCCTGTAGTATCCCCCGCAGC
CCATCTGTTGAGCAGGTGGATTTAACGGAAGACTTGCAGAACCGGCTA
CGGATGCTTTAGGCCCAATAATCGTCCCGACCAATCGGGGTGCTGGTA
TTACCGCGGCGGCTGACACCAGACTTGCCCACCCCTTATTCTGACACC
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CAGAGTAACCCTGTCAAGCTTTCGCCTATTGCAGAGGTTTCGCGCCTG
CTGCGCCCCATAGGGCCTGGGCCCTTGTCTCAGTGCCCATTCCGGGCT
CCTCCTTCAGAGCCCGTACCGGTTACAGTTTGGTGGGCCATTACCTCAC
CACAGCCTGATCGGCCGCAGTCCAATCCAGGGGCCATATAGGTTTCAG
CCATGATCCATTCCAGAAACCTTGGCCTATCGCGGTTTATCCTCAGTTT
CCCGAGGTTATCCACGTCCCTGGGCATGTTGACTACGTGTTACTGAGC
CGTATGCCACGCATTGCTGCGTTGACTCGCATGGCTTAGTCCCACTCTG
ATAGCAGTCGGGTCCGGCGGGATCAACCGGATGGGCGAGCGTTAAAC
ACTGCAGATATCCCTTAGGAGGTTATTCTGGCTGC

Appendix C - DGGE

DGGE analysis was also performed on the 16 samples but changed to ARISA to look at diversity of Archaea, bacteria and cyanobacteria due to ARISA being more sensitive. Below is the procedure that was used and the results.

Methods and Materials

For analysis by DGGE, archaeal DNA was amplified using primer pairs 915F (5'-AGGAATTGGCGGGGGAGCAC-3') and 1335R(GC) (5'CGCCCGCCGCGC CCCGCGCCCGGCCCGCCGCCCCCGCCCCTGTGCAAGGAGCAGGGAC G-3') (Niederberger *et al.,* 2006) under the conditions: an initial 5 minute denaturing step at 94ºC was followed by 22 cycles of 30 sec at 94ºC, 1 min at 60ºC, 1 min at 72ºC and 14 cycles of 1 min at 94ºC, 1 min at 60ºC, 1 min at 72ºC and a final extension of 72ºC for 5 min. *Thermococcus zilligii* was used as a positive control for archaea. For analysis by DGGE for bacteria, DNA was amplified using primers 338F(GC) (5'-CGCCCGCCGCGCCCCGCGCCCGT CCCGCCGCCCCCGCCCTCCTACGGGAGGCAGCAG-3') and 519R G(AT)ATTACCGCGGC(GT)GCTG (Muyzer, 1993, Baker, Smith and Cowan, 2003) under the conditions: an initial 2 min denaturating step at 94°C was followed by 19 cycles of 1 min at 94°C, 1 min at 65°C minus 0.5°C per cycle and 72°C for 3 min, followed by 14 cycles of 1 min at 94°C, 1 min at 55°C, 3 mins at 72°C and a final annealing of 72°C for 7 min. *Fervidobacterium nodosum* was used as a positive control for bacteria. All PCR amplifications were performed in 50µL reactions on a PTC-200 (MJ Research, Inc., Waltham, MA) gradient thermocycler with hot lid using *Thermus aquaticus* DNA polymerase (Roche Diagnostics GmBH, Mannheim, Germany) at 1 unit per reaction, 10x reaction buffer ($MgCl_2$ free), 25mM $MgCl_2$, 0.25mM dNTP mixture, 1% DMSO and 0.5µM of each primer. Two µL of template was added to each reaction. The PCR products were separated in a 1% gel in SBA 1x buffer and visualised by ethidium bromide staining.

DGGE was performed using a DCode Universal Mutation Detection System (Bio-Rad, Richmond, Calif, USA) according to the manufacturer's instructions. Gels

were prepared as described previously (Walter *et al.,* 2000) containing a linear gradient of 30-70% denaturant for bacteria and 25-55% denaturant for archaea [with 100% denaturing solution defined as 40% formamide (v/v) and 7M urea (42% w/v)] for bacteria. Gels were analysed using the software Gelcompare II, Version 3.5 (Applied Maths, St. Martens-Latem, Belgium) as previously described (Grossart *et al.,* 2005). DGGE bands for sequence analysis were recovered as described by Ibekwe *et al.,* (2003).

Results

Microbial community diversity analysis

From the DGGE analysis, T3A was selected for further analysis. PCR products were prepared using universal archaeal and universal bacterial primers and clone libraries were produced to identify the bacterial and archaeal communities in T3A.

To examine whether temperature was driving the microbiology of the transects, a DGGE analysis was performed using bacterial and archaeal primers. The DGGE analysis showed that there was more diversity present within the bacteria, compared with the Archaea. There was no pattern present in the bacterial DGGE analysis. For the archaeal DGGE, little diversity was shown with only 1 or 2 bands present for each sample. There was no pattern present for Archaea.

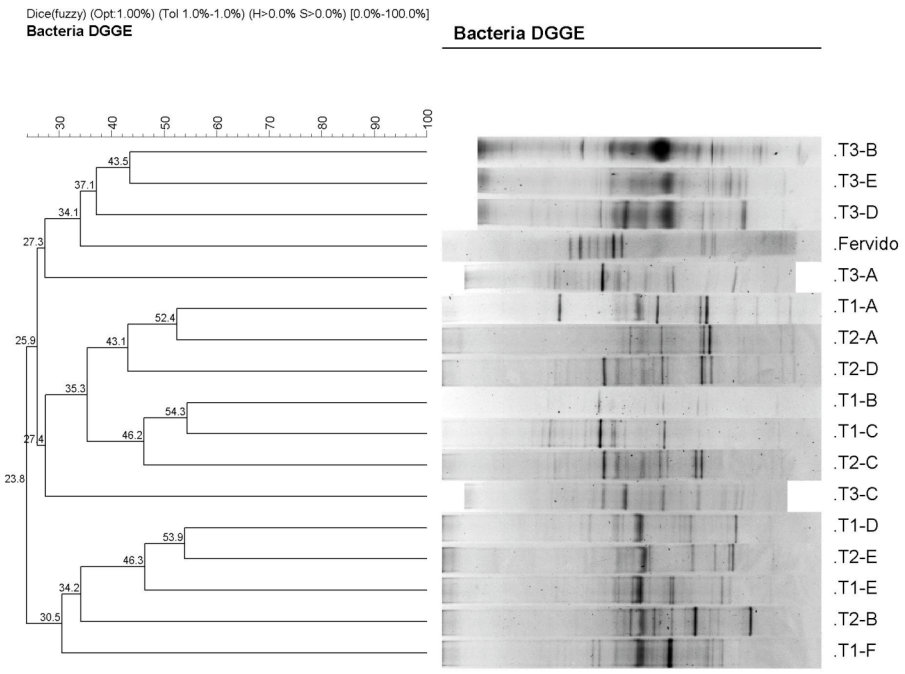

Fig. 2. Bacterial DGGE analysis of 16S rRNA gene segments for transect 1, 2 and 3. A dendrogram using the dice method with fuzzy logic and UPGMA shows the percentage similarity of the bands.

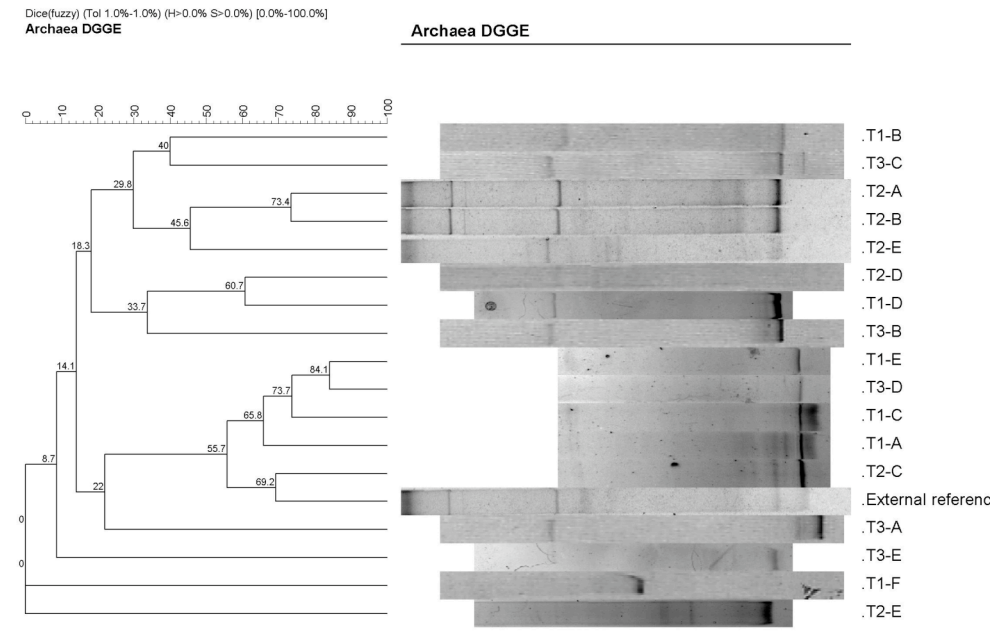

Fig. 3. Archaeal DGGE analysis of 16S rRNA gene segments for transects 1, 2 and 3. A dendrogram using the dice method with fuzzy logic and UPGMA shows the percentage similarity of the bands.

To see if the bands in the DGGE gel were dominant in the libraries. A comparison was made between the bands in the gel and the clone library. Prominent bands in the archaeal DGGE profiles were purified and sequenced to identify potential thermophiles. Sequences from the band stabs, T-1A, T-2A and T-2B, from the archaeal DGGE was determined and compared to previously reported sequences in the GenBank database by using the BLAST programme. The T-1A bandstab was 89% related to an uncultured archaeon clone from a hydrothermal system in souther Mariana Trough. The band stabs from T-2A and T-2B were 95% and 94% related to an uncultured archaeon clone SK289 from a hot spring at Yellowstone National Park, accession AY882838.

DGGE analysis was used to semiquantitatively assess the temperature gradient. It showed that there was a change in the microbial community diversity across a thermal continuum for bacteria but not for archaea. The archaeal DGGE showed that there was little diversity found in the Tramway Ridge site with only one or two bands present in the gel. It is thought that the lower temperature geothermal areas are mainly inhabited by thermophilic bacteria whereas volcanic geothermal fields (solfataras) seem to be exclusively dominated by hyperthermophilc Archaea (Nicolaus *et al.,* 1998). However, our results showed that bacterial diversity was much greater than those found for the Archaea. This may be due to the temperature at Tramway Ridge being on the boundary between bacteria and archaea dominance as seen in other thermal gradients (Nunoura *et al.,* 2005). The bacterial DGGE showed that bacterial communities were clustered closely based on temperature. Due to the area of sampling being larger than the probe, temperature gradients may not be completely accurate due to microgradients in the soil. Studies identifying soil microbial community structure across thermal gradients have shown that bacterial communities increase in diversity over a temperature gradient (Norris *et al.,* 2002). In the results found at Tramway Ridge, we did not observe any change in the number of bands in the DGGE, which ranged between 10 and 13 bands. This is considered a relatively low number compared with typical patterns from other soils, which can be in the hundreds, making it difficult to observe banding patterns (Nakatsu *et al.,* 2000). This could be a result of the extreme conditions and the isolation of the site making Tramway Ridge a perfect sampling area to look at diversity. This low level of richness has

allowed us to attain a depth of coverage in our survey. From the bacterial and archaeal DGGE, T-3A was chosen for further analysis.

Appendix D - Microbial community structure in soils of Northern Victoria Land, Antarctica

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Running Title: Antarctic dry valley soil microbial diversity

Summary

Biotic communities and ecosystem dynamics in terrestrial Antarctica are limited by an array of extreme conditions including low temperatures, moisture and organic matter availability,high salinity, and a paucity of biodiversity to facilitate key ecological processes. Recent studies have discovered that the prokaryotic communities in these extreme systems are highly diverse and patchy. Investigating the physical and biological controls over the distribution and activity of microbial biodiversity in Victoria Land is essential to understanding ecological functioning in this region. Currently, little information on the distribution, structure and activity of soil communities in the far southern regions of Victoria Land (south of 79º) are available, and their sensitivity to potential climate change remains largely unknown. We investigated the soil microbial communities from low and high productivity habitats in an isolated Antarctic location to determine how the soil environment impacts microbial community composition and structure. The microbial communities in Luther Vale, Northern Victoria Land were analysed using bacterial 16S rRNA gene clone libraries and were related to soil geochemical parameters and classical morphological analysis of the soil metazoan invertebrate communities. A total of 323 16S rRNA gene sequences analyzed from four soils spanning a productivity gradient indicated a high diversity (Shannon-Weaver values >3) of phylotypes within the clone libraries and distinct differences in community structure between the two soil productivity habitats linked to water and nutrient availability. In particular, members of the Deinococcus/Thermus lineage were found exclusively in the drier, low-productivity soils, while Gammaproteobacteria of the genus *Xanthomonas* were found exclusively in the high-productivity soils. However, rarefaction curves for all soils have not reached their diversity plateau indicating that these microbial habitats remain under-sampled. Our results add to the recent literature suggesting that there is a higher biodiversity within Antarctic soils than previously expected.

Introduction

Molecular investigations of microorganisms in diverse environments indicate that microbial community structure is subject to spatial variability (Cho and Tiedje, 2000, Whitaker *et al*., 2003; Papke and Ward, 2004; Oline, 2006; Hughes Martiny

et al., 2006) and that microbial species are not ubiquitously distributed as has been proposed by Fenchel and Finlay (2004). Spatial heterogeneity of microbial communities may be especially pronounced in extreme ecosystems where the "environment selects" for organisms with suitable physiology (Whitaker *et al*., 2003; Hughes Martiny *et al*., 2006; Oline, 2006). For example, desert environments have long been noted for their high degree of spatial structure and patchy distribution of above- and below-ground communities (Virginia *et al*., 1992; Schlesinger *et al*., 1990). Recent work shows that polar soils are spatially complex ecosystems (Barrett *et al*., 2004; Sjogersten *et al*., 2006), though few studies have examined spatial variation in polar microbial communities (e.g., Aislabie *et al*., 2006; Barrett *et al*., 2006a).

The Antarctic continent contains ice-free regions which encompass a wide range of terrestrial environments including dry mineral soils occuring on glacial till, intermittently saturated sediments on the margins of aquatic ecosystems, rich ornithogenic soils in coastal zones and exposed bedrock, and volcanic soils (Doran *et al*., 2002; Cowan and Tow 2004; Barrett *et al*., 2006b; Gooseff *et al*., accepted). The largest extant organisms are metazoan invertebrates; free-living soil nematodes are the most abundant and widely distributed and are related to gradients in soil chemical and physical factors such as availabilty of water, and carbon and salt concentrations (Freckman and Virginia, 1997; Virginia and Wall, 1999). Distribution of soil invertebrates varies markedly over these geochemical gradients (Treonis *et al*., 1999; Barrett *et al*., 2006a; Adams *et al*., 2006), including soils with abundance comparable to warm deserts and those where no metazoans are present (Freckman and Virginia, 1997; Poage *et al*., in press).

These terrestrial habitats experience severe environmental conditions such as extreme cold, broad temperature fluctuations, aridity, and steep chemical gradients including high salt concentrations and low carbon and nitrogen supply (Cowan and Tow, 2004; Aislabie, *et al*., 2006; Barrett *et al*., 2006b; Poage *et al*., in press). Coupled with high doses of damaging solar radiation (Hughes *et al*., 2003) and low water availability (Friedmann, 1993), conventional wisdom would suggest that extreme environments should generally support communities of low biomass and diversity. However, extremely dry Antarctic mineral gravels have

been shown to contain microbial biomass at levels four orders of magnitude higher than presumed; 10^6 to 10^8 prokaryotic cells g^{-1} (Cowan *et al*., 2002; Barrett *et al*., 2006a).

Culture-independent studies of Antarctic mineral soils are scarce with most culture-independent analyses being reported from Antarctic lakes or ponds (Bowman *et al*., 2000; Brambilla *et al*., 2001; Pearce *et al*., 2003; 2005; Sjöling and Cowan 2003; Taton *et al*., 2003; Cowan and Tow, 2004; Morgan-Kiss *et al*., 2006). However, recent studies by Smith *et al*. (2006), Saul *et al*. (2005) and Aislabie *et al*., (2006) have provided the first in-depth DNA-based studies into microbial population structure of Antarctic soils. Collectively these studies have revealed a greater soil microbial diversity than those based solely on cultural techniques.

This present study continues work described by Barrett *et al.,* (2006a) and is a component of the ongoing Latitudinal Gradient Project, an international collaboration of New Zealand, U.S. and Italian researchers examining the structure and function of Victoria Land ecosystems (Howard-Williams, 2006). The aim of this previous study was to elucidate the physical and chemical controls driving the distribution of Antarctic soil biotic communities. Barrett *et al*., (2006a) described the microbial and metazoan communities inhabiting soils of varied geochemical properties from Victoria Land, Antarctica. Denaturing gradient gel electrophoresis (DGGE) profile comparisons among soil microbial populations revealed a high diversity of bacteria. Microbial community structures were not strongly correlated to any single soil property; however, soils with similar availability of water and nutrients shared similar DGGE profiles. Here we report results using more in depth analyses of microbial communities from Barrett *et al*., (1996a). Soil geochemical data as well as information about each soil's invertebrate community were compared to microbial community data obtained through 16S rRNA gene clone library sequence analysis to elucidate and compare the microbial inhabitants between the soil ecosystems. Results from our study helps further the understanding of the environmental factors driving microbial population structure in Victoria Land, Antarctica soils and provides an insight in the spatial heterogeneity of microbial communities within this extreme ecosystem.

Results

DGGE analysis and chemical characteristics of soil samples

The collection of samples, site descriptions and DGGE profiling among microbial communities from Taylor Valley, Luther Vale and Cape Hallett soils in Victoria Land are described by Barrett *et al*., (2006a). This initial work indicated significant differences in microbial community structures and revealed that geography and presumably potential soil productivity levels had the greatest influence on DGGE clustering. Soil productivity was defined as either: high productivity, soils with a high abundance of water from melt-water or marine/coastal zones, and nutrients from bird-associated (orthinogenic) soils or well established moss/microbial mat communities, respectively; and low productivity, soils with lower abundance of visually conspicuous vegetation, lower concentrations of organic matter and low water availability. The DGGE profiles of the four soils included in this study showed the most significant differences in microbial populations between the two productivity soil biotopes (Barrett *et al*., 2006a). Based on these data, we chose soil sub-samples (Table 1) collected from a single geographic location (Luther Vale) for an analysis of microbial communities using 16S rRNA gene clone libraries. Briefly, soils with elevated concentrations of organic matter (C and N), chlorophyll *a* (an indication of photosynthetic biomass) and soil moisture relative to soils on the north Shore of Luther Lake were designated as "high productivity soils", while those collected from the north shore of Luther Lake were designated as "low productivity soils" (Table 1). While organic C was up to 5x higher, and soil moisture levels were 2- to 3- fold greater than low productivity environments, high productivity soils also had lower microbial biomass, and metazoan abundance spanned very low to moderate populations of nematodes in both soil types (rotifers of the genus *Rotatoria* were found in Luther Vale soils, but nematodes dominated metazoan communities) (Table 1).

Construction and diversity of clone libraries

Clones from the four soil samples (Table 1) were initially screened by restriction fragment length polymorphism (RFLP) patterns generated using the restriction endonucleases RsaI and HaeIII and grouping similar RFLPs into operational

taxonomic units (OTUs). However, the resulting RFLP profiles within each library contained few identical patterns and therefore all clones were sequenced. Following the removal of nine sequences containing possible chimeric properties, a total of 323 16S rRNA sequences from all four libraries were included in the analysis. The total number of sequences for each library and the number of unique OTUs based on an evolutionary distance of 3% are shown in Table 2. Each clone library is dominated by OTUs consisting of only a single sequence, thus reflecting a high diversity of microorganisms (Table 2). The commonly used Shannon-Weaver index was used as an indicator of 16S rRNA clone library biodiversity (Martin, 2002). Shannon-Weaver indices analysis reflected high biodiversity within each of the clone libraries (Table 2), with the LV High 1-3 soil sample having the highest observed index. The apparent richness of the clone libraries was substantiated by rarefaction analysis which demonstrated that analyses of all samples (curves) have not reached a diversity plateau (Fig. 1). The sample with the highest Shannon-Weaver index, LV High 1-3 soil, had the steepest trend line.

Community structure of clone libraries

The taxonomic composition of each clone library is presented in Fig 2. A collective comparison encompassing the distribution of each taxonomic group within the clone libraries indicates strong differences between the community structures of the low and high productivity soils (Fig. 3). 16S rRNA gene sequences unique to the high productivity soils included sequences related to the *Cyanobacteria* and *Betaproteobacteria,* although other taxonomic groupings (*Verrucomicrobia*, *Thermomicrobia* and *Nitrospira)* were also detected in the most diverse sample, LV High 1-3, which had the highest Shannon-Weaver index (see Table 2). Signatures exclusive to the low productivity soils included relatives of the *Deinococcus/Thermus* assemblage, however, members related to the *Firmicutes*, *Chloroflexi* and *Bacteriodetes* were also detected independently in each of the low productivity libraries. Both soil productivity types contained a large proportion of 16S rRNA sequences closely related to *Acidobacteria* and *Actinobacteria.* The libraries shared a similar percentage (between 13.0 and 16.4%) of *Actinobacteria*-related signatures. However, the low productivity soils contained a higher proportion of uncultured *Acidobacteria*-related signatures, 21.5% and 50.7% for LV Low 1-3 and 3-3 respectively and 16.5% and 6.5% for

LV High 1-3 and 1-4 respectively. Members of the *Gammaproteobacteria* clade were also present in all 4 libraries; with the high productivity samples containing a greater proportion, 16.5 and 22.8% for LV High 1-3 and 1-4, as compared to the low productivity soils, LV Low 1-3 and 3-3 with 7.6% and 1.5% respectively. Unclassified fractions of the four libraries consisted of 20% for LV High 1-3, 10% for LV High 1-4, 4% for LV Low 3-3 and 18% for LV Low 1-3.

Phylogenetic analysis of clone libraries

The majority of the sequences recovered from both productivity soil types were affiliated (NCBI BLASTn) with environmental sequences obtained from soil-related samples and were typically not closely related to cultured microorganisms. Also, a large proportion of sequences within each clone library showed less than 95% sequence identity with their corresponding closest BLAST relatives; LV High 1-3, 41.2%; LV High 1-4, 64%; LV Low 1-3 16%; LV Low 3-3, 40%. Taxonomic affiliations of the sequences are indicated by the inclusion of representative signatures (level 97%) in phylogenetic dendograms in Fig 4 a-c. Overall the phylogenetic trees support the observations drawn from the taxonomic analyses (Fig 2 and 3). For example all *Deinococcus*- and *Bacteroidetes*-related clones were from low productivity soils, whereas *Cyanobacterial*-, *Verrucomicrobia*- and *Betaproteobacterial*-related clones were only from the high productivity soils. The *Acidobacteria* and *Gemmatimonas* were dominated by low productivity clones, 68% and 55%, respectively; while the high productivity 16S rRNA clones dominated all the *Proteobacterial* clones (84%). It was also notable that throughout the phylogenetic trees there were obvious clusters of only low or high productivity clones, as seen in the *Actinobacteria*, *Gemmatimonas*, *Planctomycetes* and *Gamma* and *Alphaproteobacteria* clades (Fig 4).

Members of the Deinococcus/Thermus clade. The closest cultured representative for 90% (20 sequences total) of all the phylotypes within the *Deinococcus/Thermus* subdivision was *Truepera radiovictrix* (Albuquerque *et al*., 2005). Sequence homologies varied between approximately 89 and 98%.

Members of the Acidobacteria. The majority of the *Acidobacteria* signatures were most closely related to uncultured temperate-soil clones; however, some

sequences (3 of 14 for LV High 1-3; 1 of 6 for LV High 1-4; 8 of 17 for LV Low 1-3; 4 of 34 for LV Low 3-3) were closely associated to *Acidobacteria* clones from other Antarctic habitats. Collectively, all four productivity libraries contained *Acidobacteria* signatures most closely related to environmental clones 354D, 356G, 352G and 351B obtained from Antarctic soils by Saul *et al*., (2005). Likewise, both the low productivity soils contained *Acidobacteria*-related sequences most closely associated to environmental clone FBP241 (AY250867) obtained from an Antarctic dry valley cryptoendolithic community (de la Torre *et al*., 2003).

Members of the Actinobacteria. The *Actinobacteria* sequences recovered from the high productivity soils were dominated by a number of signatures that were distantly related to other soil-related environmental sequences. However, 50% of *Actinobacteria* signatures of the low productivity soil LV Low 1-3 were closely related to *Rubrobacter radiotolerans* which has also recently been detected in soils of Cape Evans by DNA-based methods (Shravage *et al*., 2007) and 42% were related to species of *Actinomycetales.* Whereas, 73% of the *Actinobacteria* sequences within the LV Low 3-3 library were most closely related to *Pseudonocardia* sp. clone 343G from the study undertaken on soils collected at Scott Base, Victoria Land, Antarctica by Saul *et al*., (2005).

Members of the Proteobacteria. Phylotypes within the α-subdivision for both low and high productivity soils contained signatures related to the *Rhodobacterales*, *Sphingomonadales* and *Rhizobiales*. The *Betaproteobacterial* sequences of both the high productivity soils were dominated (71%) by relatives of the *Burkholderiales*. All sequences in the δ-subdivision for both the high productivity soils were related to the *Myxococcales*. LV Low 3-3 was the only low productivity soil to contain *Deltaproteobacteria* signatures with a single sequence being *Myxococcales* in identity and the remaining three sequences being related to members of the *Desulfuromonadales*. The closest relatives of the *Gammaproteobacterial* sequences were members of the *Xanthomonadaceae* family, dominated by species of *Xanthomonas*.

Other Phylotypes. All *Bacteriodetes* signatures were distantly (<90%) related to species of the *Rhodothermus* genus. The single *Thermomicrobia*-related sequence obtained from LV High 1-3 was closely related to an uncultured bacterium clone FBP471 (AY250886) from a lichen-dominated Antarctic cryptoendolithic community of Southern Victoria Land (de la Torre *et al*., 2003). The LV High 1-4 clone library had the greatest amount of *Gemmatimonadetes* sequences, encompassing signatures related to clone JG34-KF-231 (AJ532724) from a uranium mining waste pile, two clones from Alaskan soils (AY988869 and AY988846), clone 353B from Antarctic soils (Saul *et al*., 2005) and signatures related to *Gemmatimonas aurantiaca* (Zhang *et al*., 2003). The LV Low 1-3 library also held a majority of sequences related to *Gemmatimonas aurantiaca,* whereas LV Low 3-3 contained a total of three sequences grouping within the *Gemmatimonadetes*, with a single sequence being very closely related (99%) to clone 353B (AY571848) from the study by Saul *et al*., (2005).

Discussion

Although, DNA-based analysis of microbial populations are prone to various biases, including differing DNA extraction efficiencies (LaMontagne *et al*., 2002) differential PCR bias (Reysenbach *et al*., 1992; Suzuki and Giovannoni, 1996) and 16S rRNA gene operon copy number (Klappenbach *et al*., 2001), it is widely accepted that these molecular methods provide a more accurate representation of the microbial populations when compared to traditional culture-based analyses. However, due to the polyphyletic nature of many metabolic traits, resolving the phenotypes of the dominant phyla within soil clone libraries is problematic.

Even though the LV High 1-3 soil showed slightly higher diversity and a steeper rarefaction curve, major differences in microbial diversity were not apparent by Shannon-Weaver analysis between all productivity soils. High Shannon-Weaver values (>3) have also been reported in other Antarctic soils including, Marble Point, Bull Pass, Vanda (Aislabie *et al*., 2006) and Scott Base (Saul *et al*., 2005), all in Southern Victoria Land. However, rarefaction curves for all the soils described here have not reached their diversity plateau suggesting that we have grossly under-sampled these microbial habitats. Although, this study provides an insight into the microbial populations of these soil biotopes, we may have

underestimated the true diversity of these habitats. A more comprehensive sampling of soils with multiple individuals is required to provide a complete examination into the microbial community composition of these soils.

Paired comparisons between all 4 clone libraries by the LIBSHUFF program (Singleton *et al*., 2001) resulted in low P-values (P = 0.001) indicating significant differences among the compositions of clone libraries. This is particularly striking as paired samples from each of the environments (low and high productivity) were collected from within 200 m of one another, indicating a striking shift in microbial communities over fine scales with moderate variation in soil environmental conditions. These results support the notion that there is both high biodiversity and high spatial variability of micro-organisms inhabiting Antarctic Dry Valley soils. Moreover, the significant differences (based on P-values) in microbial communities between similar soil productivity types adds to the evidence that micro-organisms in these habitats are not ubiquitously distributed. The question of whether large-scale regional and global patterns of soil biodiversity exist is hotly debated (Foissner, 1999; Finlay, 2002; Fierer and Jackson, 2006). One view is that many soil organisms, especially microfauna and microbes, are highly cosmopolitan: the "everything is everywhere" theory (Roberts and Cohan 1995; Finlay *et al*., 1999). The soils of Luther Vale (LV) at the eastern end of the Admiralty Range in Northern Victoria Land which are relatively undisturbed by humans, are remote areas of Antarctica and therefore the soil biodiversity patterns of these habitats are likely to be defined more by physical and chemical characteristics of the soil habitat, i.e. it is unlikely that human activity has introduced cosmopolitan microbes.

The two productivity soil types under investigation differed significantly in their geochemical characteristics, in particular water and organic matter levels. As described by Barrett *et al*., (2006b), these differences may be the major driving force for the observed patterns of distribution in soil communities, particularly for soil nematodes and other metazoan invertebrates. For example, the soil with the highest microbial diversity according to both the Shannon-Weaver index and rarefaction analysis (High 1-3) had the highest levels of soil organic matter and chlorophyll *a*, in contrast to the soil with the lowest diversity (Low 3-1) which

had the lowest concentrations of organic matter and biomass. These results are consistent with Barrett *et al.*, (2006a) who reported that microbial diversity (as indicated by DGGE) shared the greatest variance with soil organic C, chlorophyll *a* and moisture in a principle component analysis.

A large proportion of the signatures recovered from the soils were low in sequence homology to other environmental sequences which is in accord with other studies of cold soils (Lipson and Schmidt, 2004; Saul *et al*., 2005; Smith *et al*., 2006; Shravage *et al*., 2007). Likewise, a major contribution of signatures within all the clone libraries was from the *Acidobacteria* and *Actinobacteria* which is consistent with the molecular-based studies of Antarctic microbial habitats including, soil biotopes (Saul *et al*., 2005; Smith *et al*., 2006; Aislabie *et al*., 2006), cryoconite holes (Christner *et al*., 2003) and cryptoendolithic communities (de la Torre *et al*., 2003).

Members of the *Verrucomicrobia* or *Planctomycetes*, which are recognized as common inhabitants of soils (Buckley and Schmidt, 2001; Rondon *et al*., 1999), have previously been absent from clone libraries of Antarctic soil (Saul *et al*., 2005; Shravage *et al*., 2007; Aislabie *et al*., 2006), a small number of signatures of the *Verrucomicrobia* or *Planctomycetes* were recovered from the soil samples under investigation. These results corroborate the study by Smith *et al*., (2006) and indicate that these micro-organisms may exist in Antarctic soil environments at presumably low levels.

The high productivity soils had a greater quantity of all detected *Proteobacterial* (α, β, γ and δ) subdivisions as compared to both the low productivity soils. The high productivity *Proteobacterial* signatures were dominated by *Gammaproteobacteria,* and *Betaproteobacterial* signatures were exclusively recovered from the high productivity soils. Saul *et al*., (2005) also noted an increase of *Proteobacteria* in C-rich (hydrocarbon contaminated) Antarctic soils as compared to control soils. In addition, as was the case for this study, the *Gammaproteobacteria* signatures obtained by Saul *et al*., (2005) were exclusively species of *Xanthomonas*. Likewise, both Shravage *et al*., (2007) and Smith *et al*., (2006) noted the presence of *Proteobacterial* signatures from soils underneath

dead seals in the McMurdo Dry Valleys, with *Proteobacterial* signatures being absent from corresponding Dry Valley soils (Smith *et al*., 2006). Therefore, this and other studies point toward the likelihood that eutrophic Antarctic soil may enrich for members of the *Proteobacteria.*

It is difficult to address the relationships between metazoan invertebrates and microbial community structure due to the limited number of samples included in the study (and the range of nematode abundances occurring in each of the two sites, Table 1). However, it is worth noting that concentrations of microbial biomass C were lowest in the high productivity environments suggesting high microbial turnover given the otherwise favorable conditions. Spatial correlations in extreme environments which are prone to episodic changes in physicochemical conditions (e.g., melting, high-latitude diel cycles, etc.) may obfuscate relationships that develop temporally. For example, in the high productivity environment, a longer duration when liquid water is available -based on the physical (northern aspect) and geomorphic (located downhill from a snowpack) features of the site may facilitate greater activity of microbial and metazoan communities, which would contribute to invertebrate grazing and high rates of microbial turnover. To date, few studies have examined temporal variability in soil microbial communities in polar environments. Given the harsh physical conditions and brief periods of time when organisms can be metabolically active, temporal coupling between microbial and metazoan communities could be more significant to food web dynamics than spatial relationships.

The exclusive presence of *Cyanobacteria* and chlorophyll *a* in the high productivity samples was expected as *Cyanobacteria* distribution is typically restricted to wetter regions of polar environments (de los Ríos *et al*., 2004). On the other hand, the lack of water may be the major reason for the prominent difference in the distribution of signatures of the *Deinococcus/Thermus* group between the two sites. Members of this group have been shown to withstand both desiccation and high levels of ionizing radiation (Mattimore and Battista, 1996) and appear to be prevalent in a number of dry Antarctic microbial habitats (de la Torre *et al*., 2003; Saul *et al*., 2005; Shravage *et al*., 2007; Aislabie *et al*., 2006). Three species of *Deinococci* have recently been isolated from Antarctic soil and

rock samples and subsequently fully characterised (Hirsch *et al*., 2004). In this study, *Deinococcus/Thermus* signatures were exclusively recovered from the drier, low productivity soils with the closest cultured representative of the majority of these signatures being an extremely ionizing radiation resistant micro-organism, *Truepera radiovictrix* (Albuquerque *et al*., 2005). Likewise, a high proportion of the *Actinobacterial* clones of the low productivity soil, LV Low 1-3 were related to the radiation resistant genera of *Rubrobacter* (Ferreira *et al*., 1999). *Rubrobacter* related signatures are also present in clone libraries from other studies of Antarctic soils (Saul *et al*., 2005; Shravage *et al*., 2007; Smith *et al*., 2006; Aislabie *et al*., 2006) and are abundant in arid soils of Australia (Holmes *et al*., 2000) and thus may be a common component of desert soils in these two continents.

Surprisingly, a number of our phylotypes were related to thermophilic microorganisms including members of the genera of *Rhodothermus, Thermomicrobia* and *Truepera*. Thermophiles have been isolated from geothermal heated soils of Antarctica (Hudson *et al*., 1989; Bargagli *et al*., 2004) in Southern and Northern Victoria Land (Hudson and Daniel, 1998; Logan *et al*., 2000). Therefore, the presence of these assumed non-indigenous bacterial inhabitants found in our samples may be due to the high mobility of Antarctic soils during windy periods. The recovery of thermophilic-related signatures from cold Antarctic soils has previously been reported by Shravage *et al*., (2007). The presence of thermophilic micro-organisms in cool soils has been proven by culture-based approaches (Banat *et al*., 2004; Marchant *et al*., 2002; Rahman *et al*., 2004); however, physiological assumptions of the signatures obtained in this study cannot be made by 16S rRNA gene sequence homologies alone. Therefore, further cultured-based analysis is required to elucidate the metabolic properties of these microbes.

The observed differences between the microbial populations of low and high productivity soil types reflected their observed geochemical parameters, such as water, organic matter and nutrient availability. The microbial populations of these ecosystems contained a high number of original lineages indicating an abundance of novel taxa in Antarctic soils. Thus our data supports the accumulating evidence

to the high diversity of microbes inhabiting Antarctic soils (Barrett *et al*., 2006a) which is likely due to both geographical isolation and adaptation to environmental factors acting on the spatial heterogeneity of microbial populations.

Experimental Procedures

Field sites and soil sampling

The field site was located below Luther Peak in a dry cirque provisionally named Luther Vale (LV) (Barrett *et al*., 2006a) at the eastern end of the Admiralty Range in Northern Victoria Land. A large ice covered pond ("Luther Lake") was located in the centre of the Luther Vale cirque, along with several smaller ponds which are fed by snow-pack water runoff. The soil parent material was poorly weathered metamorphic rock and sandstone and macroscopic organisms were limited to springtails, mites, lichen, moss, and algal mats. Within the field site, two sampling locations were selected based on their presumed potential for primary productivity as determined by observations of available sunlight (i.e., aspect), moisture, and the presence of primary producers, herein referred to as LV Low and LV High (Luther Vale, low and high productivity, respectively). The LV High site consisted of 4 parallel 10 m transects across an elevation gradient extending up from the edge of Luther Lake under Luther Peak (S 72° 22.268, E 169° 53.062). The LV Low transects (S 72° 22.123, E 169° 169.53.128) was situated on the northern slope, directly across the lake from the high productivity transects. This transect appeared to have less sunlight and soil moisture, likely due to the lack of snow-pack water runoff and lacked conspicuous mats of algae at the lake edge. Also, in contrast to the high productivity transect springtails and mites were not observed at the lake edge. Separate soil samples for molecular and chemical analysis were collected aseptically at each site to a depth of approximately 15 cm, homogenized, and stored at -80°C for subsequent analysis.

Soil geochemical and nematode analysis

Soil geochemical parameters were determined as described by Barrett *et al*., (2006a). Extraction (sugar-centrifugation; Freckman and Virginia, 1997) identification and enumeration of metazoan invertebrates from soil followed that of Barrett *et al*., (2006a).

DNA extraction

DNA was extracted from bulk soil by the combination and modification of the methods by Coyne *et al*., (2001) and Miller *et al*., (1999) as described by Barrett *et al*., (2006a).

16S rRNA clone library construction

PCR of bacterial 16S rRNA genes from soil DNA extractions was performed using the universal bacterial primers 8F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1522R 5'-AAGGAGGTGATCCANCCRCA-3'. PCR reagents were used at the concentrations stated previously for DGGE PCR amplifications (Barrett *et al*., 2006a) with the exception of the use of 0.75 U *Taq* polymerase (Roche) and the corresponding PCR buffer as supplied by the manufacturer. Thermocycling conditions consisted of 94°C for 1 minute, followed by 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 minute (+2 seconds/cycle) with a final elongation step of 72°C for 7 minutes. PCR products were cloned for sequencing using a TOPO TA Cloning® Kit (Invitrogen Life Technologies). Clones were selected randomly and plasmids isolated by alkaline-lysis.

Sequencing of 16S rRNA clones

DNA inserts were PCR amplified from plasmids using the TA vector-specific M13F and M13R primers (Invitrogen). The quality of each amplification was evaluated through visualization on an ethidium bromide stained 1% agarose gel and PCR products subsequently purified using the QIAquick PCR Purification Kit (QIAGEN) and quantified spectrophotometrically (Nanodrop). Purified PCR products were sequenced using DYEnamic ET Dye Terminator Kit (Amersham Pharmacia Biotech) with either T7 or T3 primers (Invitrogen) and analysis undertaken by the University of Waikato Sequencing Facility using the MegaBACE system (Amersham Pharmacia Biotech).

Analysis of 16S rRNA clones

16S rRNA gene sequences were manually edited using SeqMan II Version 5.5.1 software and subjected to the CHIMERA_CHECK program of the Ribosomal Data Base Project (Cole *et al*., 2003) and suspected chimeras checked using the Pintail program (Ashelford *et al*., 2005). A total of 6 sequences from all 4 clone libraries

were not included in further analysis due to suspected chimeric properties. The sequences included in this study were deposited into the GenBank database as accession numbers EF464767 to EF465090. Sequences (approximately >500 bp) of each clone library were collectively aligned using ClustalX Version 1.83 (Thompson *et al*., 1997). A PHYLIP distance matrix output file was generated which was subsequently used for DOTUR-based OTU analysis (Schloss and Handelsman, 2005), encompassing OTU diversity, richness and rarefaction and collection analysis. Statistical comparison between clone libraries was undertaken using the LIBSHUFF program (Singleton *et al*., 2001). Phylogenetic affiliations of the 16S rRNA gene sequences were determined by submitting sequences to the classifier program of the Ribosomal Data Base Project (Cole *et al*., 2003). Bootstrap values of approximately greater than 80% were used to define well-supported phylogenetic groupings. Taxonomic groupings of all sequences were also confirmed by BLASTn comparison to the NCBI GenBank database.

Phylogenetic analyses

The ARB program (Ludwig *et al*., 2004) was used for sequence alignment, and phylogenetic analysis. A total of 306 clones were included in the phylogenetic trees with 17 clones excluded from the phylogenetic analyses due to erroneous or short alignments. Subsequently, 55% of clones included in the trees were high productivity-related and 45% being low productivity clones. Sequences that were closely related to the 16S rRNA gene clone sequences were identified in ARB and in BLAST (Altschul *et al*., 1990) searches of GenBank, and used in the analyses. A filter was generated that omitted alignment positions of sequence ambiguity (N), and where sequence data were not available for near full-length (>1400 bp) sequences. Tree topology was evaluated by reconstructing phylogenies using evolutionary distance (DNADIST, Jukes and Cantor model), maximum parsimony (DNAPARS) and maximum likelihood (FASTDNAML) analyses of the aligned near full length sequences (Ludwig *et al*., 1998). Aligned, partial 16S rRNA clone sequences were inserted into each tree using a parsimony tool available within ARB and the filter generated with complete sequence data. The phylogeny presented is based on the evolutionary distance analysis and neighbour-joining.

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Table 1. Geochemical parameters of the Luther Vale soil samples. N.D. = Not determined; MB C = microbial biomass carbon; MB N = microbial biomass nitrogen; SOC = soil organic carbon; TN = total soil nitrogen; Chla = Chlorophyll A.

Table 2. Operational taxonomic unit (OTU) comparison between bacterial clone libraries. OTUs are defined by an evolutionary distance level of 3%.

Sample	Date sampled	Soil moisture (% g/g)	pH	Conductivity (µS/cm)	N as NH_3 (mg/kg)	N as NO_3 (mg/kg)	MB C (mg/kg)	MB N (mg/kg)	MB CN	SOC (g/kg)	TN (g/kg)	C:N	Chl a (mg/kg)	Nematode Live (#/kg dry soil)
LV Low 3-1	18 Jan 2004	2.4	9.58	1351	0.18	0.02	56.61	33.46	1.69	0.48	0.17	2.91	0.01	0
LV Low 3-3	18 Jan 2004	3.8	9.50	71.8	0.17	0.03	64.41	49.62	1.30	0.60	0.19	3.25	0.06	645
LV High 1-3	15 Jan 2004	9.0	9.76	93.1	0.16	0.32	15.73	N.D.	-	2.29	0.48	4.79	0.52	121
LV High 1-4	15 Jan 2004	9.2	9.59	116.7	0.16	1.06	31.55	N.D.	-	2.00	0.42	4.71	0.23	1556

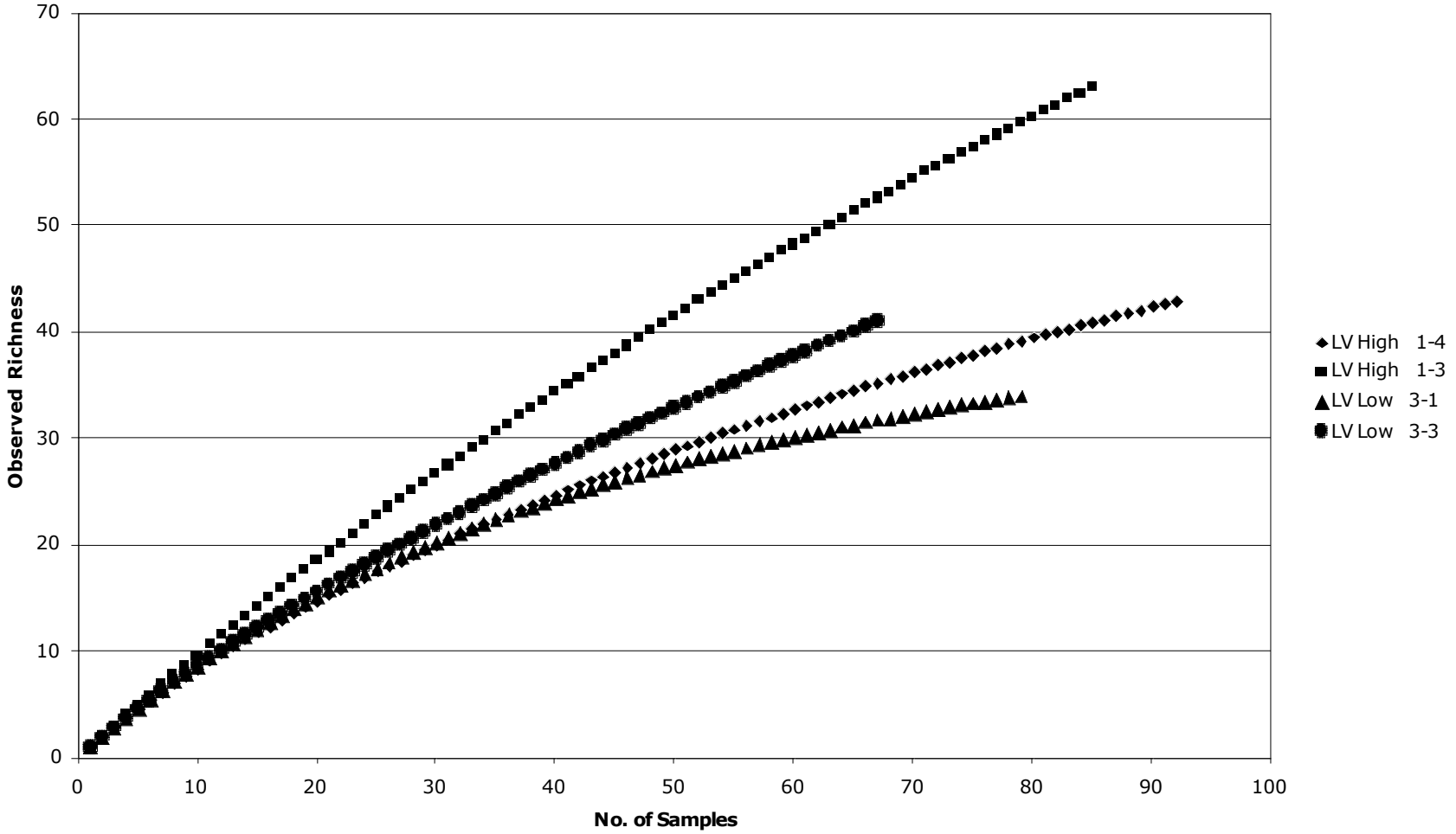
Soil sample	Total no. of sequences	No. of unique OTUs	No. of OTUs with n_x sequences											Shannon-Weaver index
			n_1	n_2	n_3	n_4	n_5	n_6	n_8	n_9	n_{10}	n_{11}	n_{13}	
LV Low 3-1	79	34	13	11	3	4	2	0	0	1	0	0	0	3.32
LV Low 3-3	67	41	30	8	1	0	0	0	1	0	1	0	0	3.40
LV High 1-3	85	63	47	11	4	1	0	0	0	0	0	0	0	4.04
LV High 1-4	92	43	26	9	2	3	0	1	0	0	0	1	1	3.37

Fig. 1. Rarefaction curve for accumulated 16S rRNA gene sequences from the clone libraries.

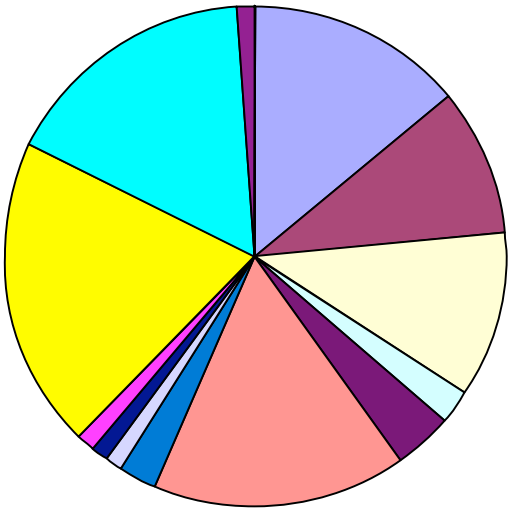
Fig. 2. Percentage abundance of each taxonomic grouping for each individual 16S rRNA gene clone library.

Fig. 3. The abundance of each taxonomic group within the Luther Vale soil 16S rRNA gene libraries.

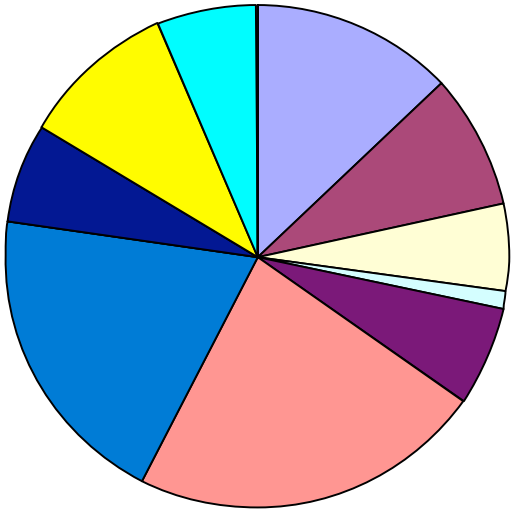
Fig 4. a-c Phylogenetic analysis of 16S rRNA gene sequences from clones from high and low productivity Luther Vale soils and representative bacterial isolates. Phylogenetic trees were constructed using near full length sequences from the representative bacterial isolates and the partial clone sequences were inserted using a parsimony tool available within ARB. The dendrograms show the result from analysis using DNADIST and neighbor-joining, and the bar represents 10% sequence divergence. Clone sequences are grouped where appropriate and the number of clones in each group indicated in parentheses. Groups containing only clones from low productivity soils are blue, those containing only clones from high productivity soils are red and those containing both are black. Individual clones are in bold and those prefixed with LVH are from high productivity soil, and those with LVL are from low productivity soil.


70
60
50
40
30
20
10
0
Observed Richness
0
10
20
30
40
50
60
70
80
90
100
No. of Samples
LV High 1-4
LV High 1-3
LV Low 3-1
LV Low 3-3

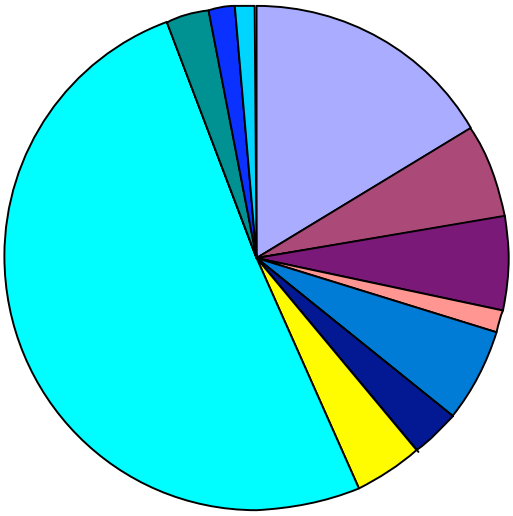
LV High 1-3



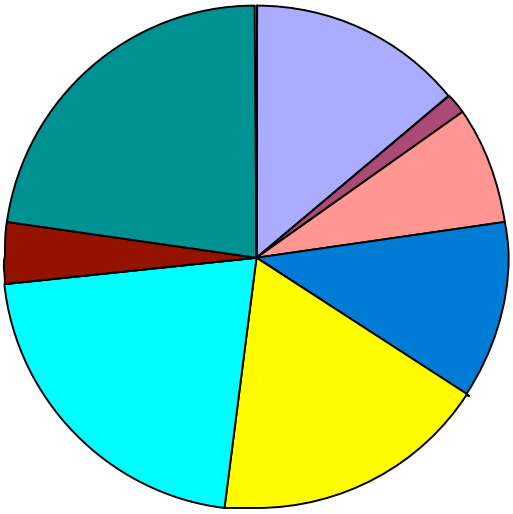
LV High 1-4

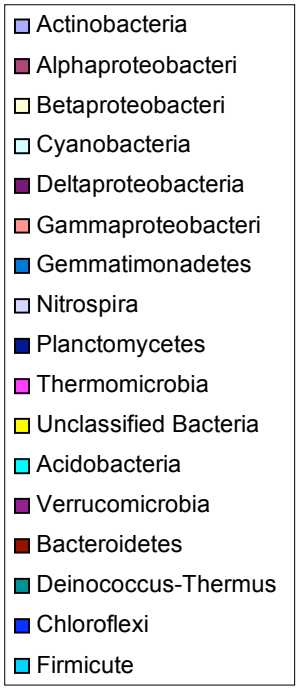


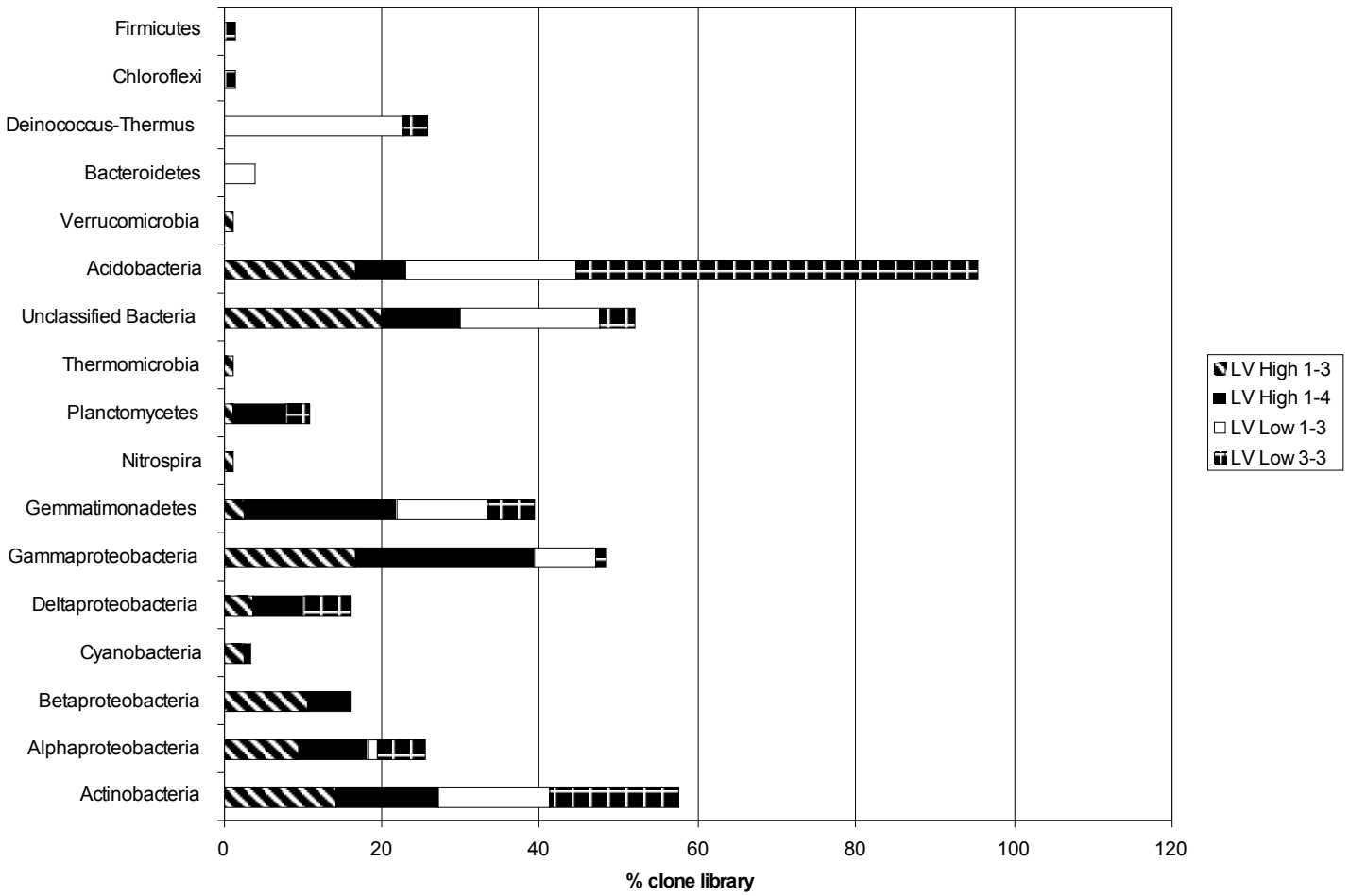
LV Low 3-3

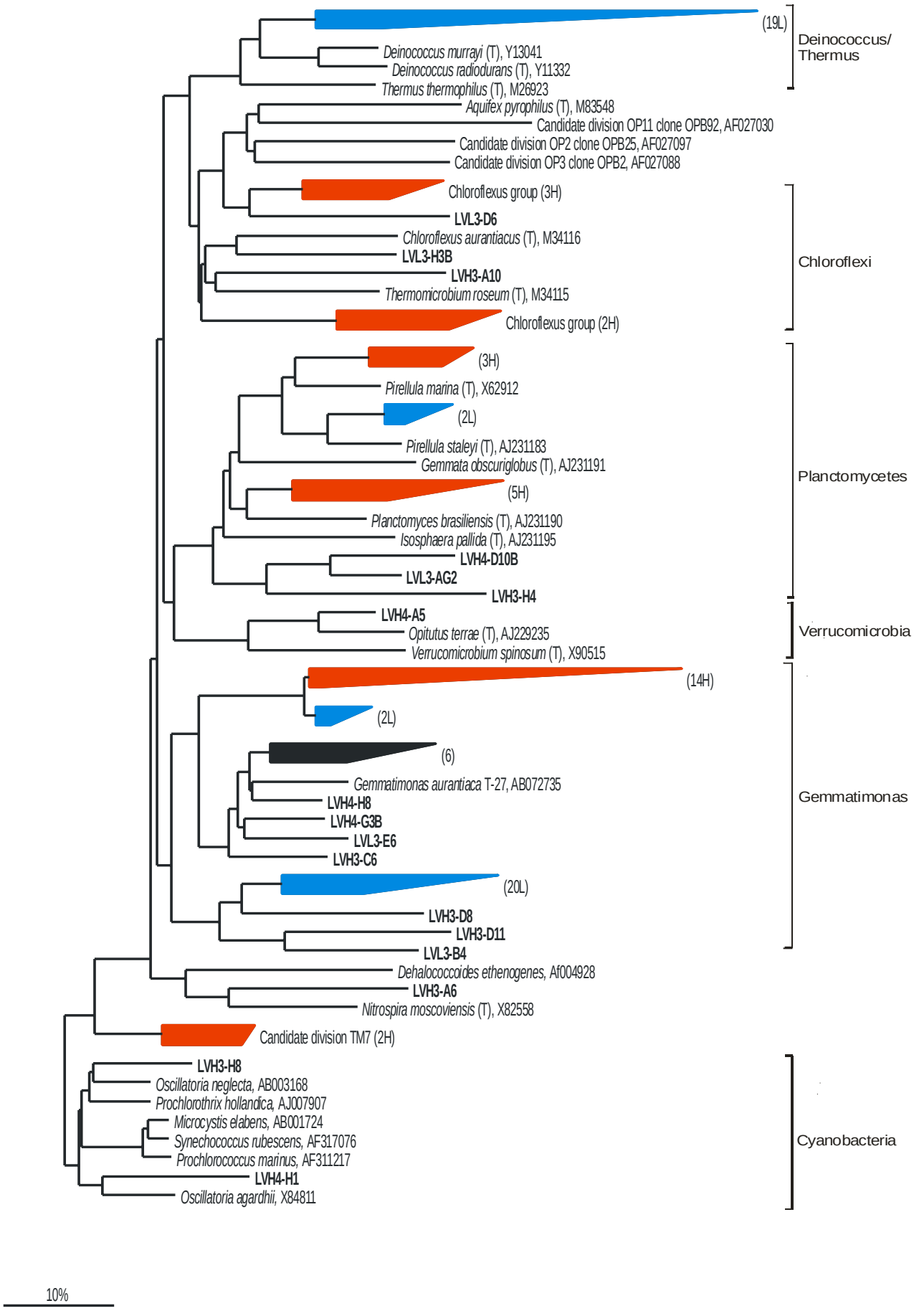


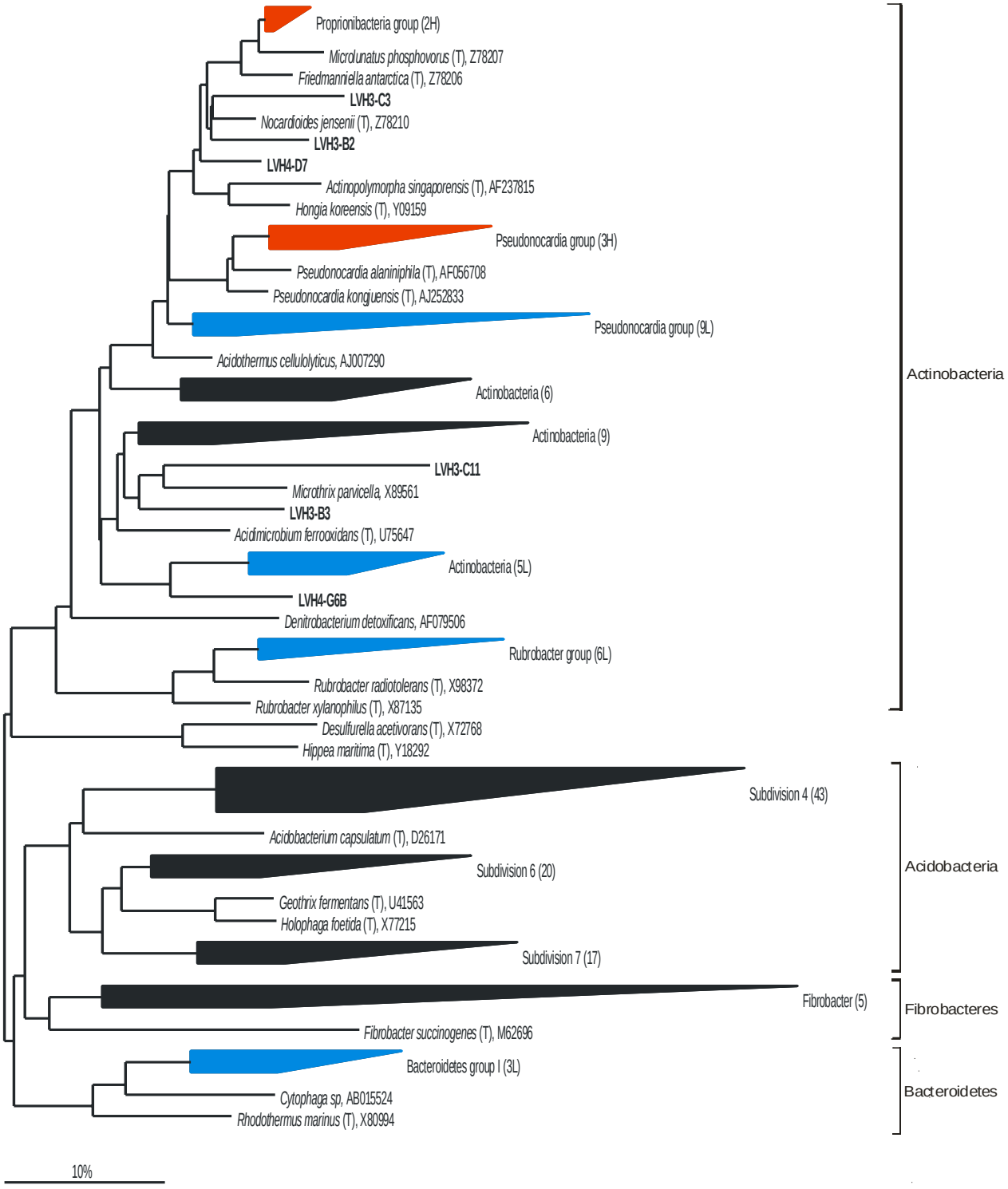
LV Low 1-3

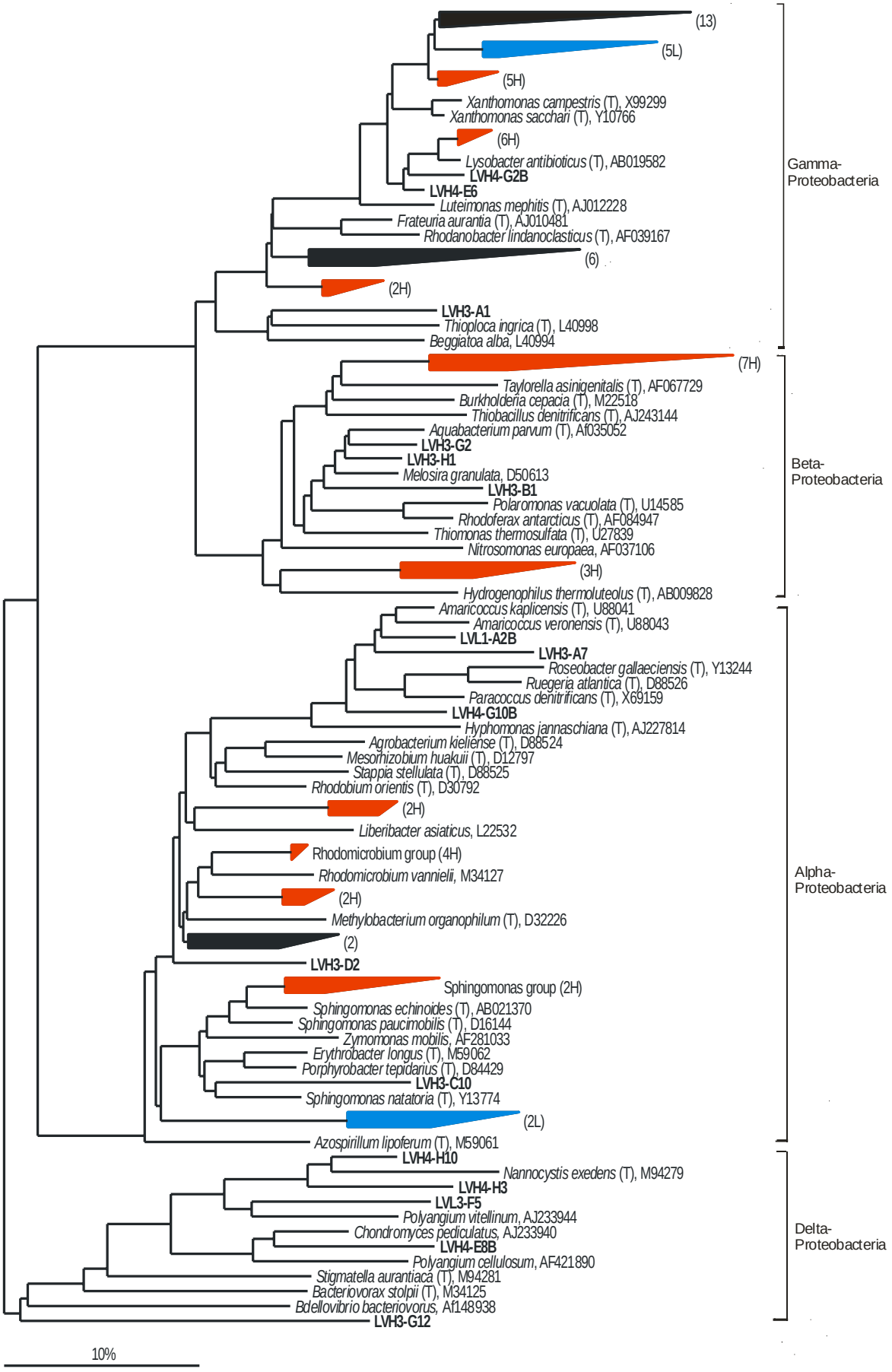



Actinobacteria
Alphaproteobacteri
Betaproteobacteri
Cyanobacteria
Deltaproteobacteria
Gammaproteobacteri
Gemmatimonadetes
Nitrospira
Planctomycetes
Thermomicrobia
Unclassified Bacteria
Acidobacteria
Verrucomicrobia
Bacteroidetes
Deinococcus-Thermus
Chloroflexi
Firmicute


Firmicutes
Chloroflexi
Deinococcus-Thermus
Bacteroidetes
Verrucomicrobia
Acidobacteria
Unclassified Bacteria
Thermomicrobia
Planctomycetes
Nitrospira
Gemmatimonadetes
Gammaproteobacteria
Deltaproteobacteria
Cyanobacteria
Betaproteobacteria
Alphaproteobacteria
Actinobacteria
0
20
40
60
80
100
120
% clone library
LV High 1-3
LV High 1-4
LV Low 1-3
LV Low 3-3


(19L)
Deinococcus murrayi (T), Y13041
Deinococcus radiodurans (T), Y11332
Thermus thermophilus (T), M26923
Deinococcus/ Thermus
Aquifex pyrophilus (T), M83548
Candidate division OP11 clone OPB92, AF027030
Candidate division OP2 clone OPB25, AF027097
Candidate division OP3 clone OPB2, AF027088
Chloroflexus group (3H)
LVL3-D6
Chloroflexus aurantiacus (T), M34116
LVL3-H3B
LVH3-A10
Thermomicrobium roseum (T), M34115
Chloroflexus group (2H)
Chloroflexi
(3H)
Pirellula marina (T), X62912
(2L)
Pirellula staleyi (T), AJ231183
Gemmata obscuriglobus (T), AJ231191
(5H)
Planctomyces brasiliensis (T), AJ231190
Isosphaera pallida (T), AJ231195
LVH4-D10B
LVL3-AG2
LVH3-H4
Planctomycetes
LVH4-A5
Opitutus terrae (T), AJ229235
Verrucomicrobium spinosum (T), X90515
Verrucomicrobia
(14H)
(2L)
(6)
Gemmatimonas aurantiaca T-27, AB072735
LVH4-H8
LVH4-G3B
LVL3-E6
LVH3-C6
(20L)
LVH3-D8
LVH3-D11
LVL3-B4
Gemmatimonas
Dehalococcoides ethenogenes, Af004928
LVH3-A6
Nitrospira moscoviensis (T), X82558
Candidate division TM7 (2H)
LVH3-H8
Oscillatoria neglecta, AB003168
Prochlorothrix hollandica, AJ007907
Microcystis elabens, AB001724
Synechococcus rubescens, AF317076
Prochlorococcus marinus, AF311217
LVH4-H1
Oscillatoria agardhii, X84811
Cyanobacteria
10%


Proprionibacteria group (2H)
Microlunatus phosphovorus (T), Z78207
Friedmanniella antarctica (T), Z78206
LVH3-C3
Nocardioides jensenii (T), Z78210
LVH3-B2
LVH4-D7
Actinopolymorpha singaporensis (T), AF237815
Hongia koreensis (T), Y09159
Pseudonocardia group (3H)
Pseudonocardia alaniniphila (T), AF056708
Pseudonocardia kongjuensis (T), AJ252833
Pseudonocardia group (9L)
Acidothermus cellulolyticus, AJ007290
Actinobacteria (6)
Actinobacteria (9)
LVH3-C11
Microthrix parvicella, X89561
LVH3-B3
Acidimicrobium ferrooxidans (T), U75647
Actinobacteria (5L)
LVH4-G6B
Denitrobacterium detoxificans, AF079506
Rubrobacter group (6L)
Rubrobacter radiotolerans (T), X98372
Rubrobacter xylanophilus (T), X87135
Desulfurella acetivorans (T), X72768
Hippea maritima (T), Y18292
Subdivision 4 (43)
Acidobacterium capsulatum (T), D26171
Subdivision 6 (20)
Geothrix fermentans (T), U41563
Holophaga foetida (T), X77215
Subdivision 7 (17)
Fibrobacter (5)
Fibrobacter succinogenes (T), M62696
Bacteroidetes group I (3L)
Cytophaga sp, AB015524
Rhodothermus marinus (T), X80994
Actinobacteria
Acidobacteria
Fibrobacteres
Bacteroidetes
10%


(13)
(5L)
(5H)
Xanthomonas campestris (T), X99299
Xanthomonas sacchari (T), Y10766
(6H)
Lysobacter antibioticus (T), AB019582
LVH4-G2B
LVH4-E6
Luteimonas mephitis (T), AJ012228
Frateuria aurantia (T), AJ010481
Rhodanobacter lindanoclasticus (T), AF039167
(6)
(2H)
LVH3-A1
Thioploca ingrica (T), L40998
Beggiatoa alba, L40994
Gamma-Proteobacteria
(7H)
Taylorella asinigenitalis (T), AF067729
Burkholderia cepacia (T), M22518
Thiobacillus denitrificans (T), AJ243144
Aquabacterium parvum (T), Af035052
LVH3-G2
LVH3-H1
Melosira granulata, D50613
LVH3-B1
Polaromonas vacuolata (T), U14585
Rhodoferax antarcticus (T), AF084947
Thiomonas thermosulfata (T), U27839
Nitrosomonas europaea, AF037106
(3H)
Hydrogenophilus thermoluteolus (T), AB009828
Beta-Proteobacteria
Amaricoccus kaplicensis (T), U88041
Amaricoccus veronensis (T), U88043
LVL1-A2B
LVH3-A7
Roseobacter gallaeciensis (T), Y13244
Ruegeria atlantica (T), D88526
Paracoccus denitrificans (T), X69159
LVH4-G10B
Hyphomonas jannaschiana (T), AJ227814
Agrobacterium kieliense (T), D88524
Mesorhizobium huakuii (T), D12797
Stappia stellulata (T), D88525
Rhodobium orientis (T), D30792
(2H)
Liberibacter asiaticus, L22532
Rhodomicrobium group (4H)
Rhodomicrobium vannielii, M34127
(2H)
Methylobacterium organophilum (T), D32226
(2)
LVH3-D2
Sphingomonas group (2H)
Sphingomonas echinoides (T), AB021370
Sphingomonas paucimobilis (T), D16144
Zymomonas mobilis, AF281033
Erythrobacter longus (T), M59062
Porphyrobacter tepidarius (T), D84429
LVH3-C10
Sphingomonas natatoria (T), Y13774
(2L)
Azospirillum lipoferum (T), M59061
Alpha-Proteobacteria
LVH4-H10
Nannocystis exedens (T), M94279
LVH4-H3
LVL3-F5
Polyangium vitellinum, AJ233944
Chondromyces pediculatus, AJ233940
LVH4-E8B
Polyangium cellulosum, AF421890
Stigmatella aurantiaca (T), M94281
Bacteriovorax stolpii (T), M34125
Bdellovibrio bacteriovorus, Af148938
LVH3-G12
Delta-Proteobacteria
10%