Comparison of actinobacterial diversity in Marion Island terrestrial habitats

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DECLARATION

I declare that "*Comparison of actinobacterial diversity in Marion Island terrestrial habitats"* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Walter Tendai Sanyika November 2008

Abstract

Marion Island is a sub-Antarctica Island that consists of well-characterised terrestrial habitats. A number of previous studies have been conducted, which involved the use of culture-dependent and culture-independent identification of bacterial communities from Antarctic and sub-Antarctic soils. Previous studies have shown that actinobacteria formed the majority of microorganisms frequently identified, suggesting that they were adapted to cold environments. In this study, metagenomic DNA was directly isolated from the soil and actinomycete, actinobacterial and bacterial 16S rRNA genes were amplified using specific primers.

Hierarchical clustering and multidimensional scaling were used to relate the microbiological diversity to the habitat plant and soil physiochemical properties. The habitats clusters obtained were quite similar based on the analysis of soil and plant characteristics. However, the clusters obtained from the analysis of environmental factors were not similar to those obtained using microbiological diversity. The culture-independent studies were based on the 16S rRNA genes. Soil salinity was the major factor determining the distribution of microorganisms in habitats based on DGGE and PCA. Nutrient availability was also an important factor. The bacterial and actinobacterial diversities were mainly influenced by different combinations of soil and plant characteristics. The distribution of actinobacteria was mainly influenced by pH. In addition, total carbon, exchangeable magnesium and total potassium. The plants included *Poa annua,* mire bryophytes and the *Bryum/ Breutelia* plant species. The distribution of bacteria was influenced by all the forms of calcium and potassium, total sodium and exchangeable sodium. The plants included *P. annua,* lichens, and epiphytic graminoids and brachythecium mosses.

The analysis of metagenomic clone libraries from three selected habitats suggested that oxygen availability was also an important determinant of microbiological diversity. Less than 1% of the phylotypes identified from the metagenomic libraries were identified as cultured. The rest showed 16S rRNA gene sequence identities of between 89 and 99% to other phylotypes. The actinobacteria identified belonged to the suborders,

Acidimicrobineae, Propionibacterineae, Streptomycineae, Frankineae, Corynebacterineae, Streptosporangineae and *Micrococcineae.* Habitat MI 1.2 (Coastal Fellfield Habitat) was dominated by the M*icrococcineae* (44%) and *Propionibacterineae* (18%). This habitat had higher species richness, diversity indices and evenness compared to habitats MI 5.1 (Cotula Herbfield Habitat) and MI 6.3 (Wet Mire Habitat). Habitat MI 51 was dominated by the *Frankineae* (38%) and *Streptosporangineae* (32%) whilst MI 6.3 was dominated by the *Acidimicrobineae* (54%) and *Frankineae* (24%). These microorganisms dominating Habitats MI 5.1 and MI 6.3 were associated with mineralization activities and generation of energy from inorganic compounds under anaerobic conditions. This was attributed the presence of compacted peat soils in habitat MI 5.1 due to peats compacted by trampling from animals and water-logging in habitat MI 6.3.

A total of 42 different actinomycetes were subsequently isolated using standard techniques and media. Only 1.3% of the actinomycete phylotypes identified from metagenomic clone libraries were subsequently isolated using culture-dependent techniques. The sequences from the rest of the isolates were not identified from metagenomic clone libraries, although were all related. The 13 isolates that showed less than 99% sequence identities to other known actinomycetes may be new species. Amongst these, 4 isolates showed sequence identities between 93 and 97% and may belong to new genera.

This study has therefore, used both culture-dependent and culture-independent techniques to describe the community structures of three Marion Island terrestrial habitats. This resulted in the identification the major factors that determine the distribution of microorganisms, phylogenetic analysis of actinobacteria in three of the habitats and isolation of novel actinomycetes. The study showed that environmental characteristics are strong determinants that influence the spatial distribution of microorganisms on Marion Island and that some actinobacterial species that are endemic to the island.

Dedication

This thesis is dedicated to the man and woman who taught me how to read and write, and selflessly committed their lives to my education: My father, Mr Webster Evans Gwanzura, and my mother Mrs Joanita Zhakata (Gwanzura).

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Glossary and Abbreviations

Abbreviation	Definition
APS	Ammonium persulphate
ARDRA	Amplified Ribosomal DNA Restriction Analysis
Bisacrylamide	*N, N*-Methylene bisacrylamide
BLAST	Basic Local Alignment Search Tool
CTAB	Cetyl trimethyl ammonium bromide
DGGE	Denaturing Gradient gel electrophoresis
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
H	Hour
IPTG	Isopropyl-beta-D-thiogalactopyranoside
Min	Minutes
mM	Millimolar
Ng	Nanogram
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
Pg	Picogram
PIPES	Piperazine-1,2-bis[2-ethanesulfonic acid]
PVPP	Polyvinylpolypyrrolidone

Table continued

Abbreviation	Definition
Rcf	Relative Centrifugal Force (xg)
R_f	Relative Retention Factor
Rpm	Revolutions per minute
S	Seconds
SDS	Sodium dodecyl Sulphate
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
TE	Tris EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
*M*g	Microgram
Ml	Microlitre

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CHAPTER 1

1.0 Literature review

1.1 Background and summary

Microbiological nutrient recycling activities are important in Antarctic ecosystems, which are usually limited in trophic structure (Smith, 1988). Marion Island (46º54′S, 37º57′S) is a small volcanic island (290 square km) located in sub-Antarctica, about 2300 km South East of Cape Town that formed the larger part of the two islands making up the Prince Edward Islands (Chown and Froneman, 2008) (Fig. 1.1). The island comprises of distinct, well-characterised terrestrial habitats (Smith & Steenkamp, 2001; Smith, *et al.,* 2001), which forms the basis for comparative studies. This study was intended at comparing the occurance of bacteria and actinobacteria in selected Marion Island terrestrial habitats using culture-dependent and culture-independent techniques. Multivariate statistical techniques were used to identify the important factors that influenced the distribution of bacteria and actinobacteria in habitats based on their community patterns on DGGE. Phylogenetic analysis was conducted based on the culture-independent studies for those habitats that potentially contained unique microbiological diversity. Identification of microorganisms was based on comparing their identities based on the 16S rRNA gene sequences. The

importance of such microbiological diversity studies is their contribution to facilitating commercial bio-prospecting ventures (Bull *et al.,* 1992).

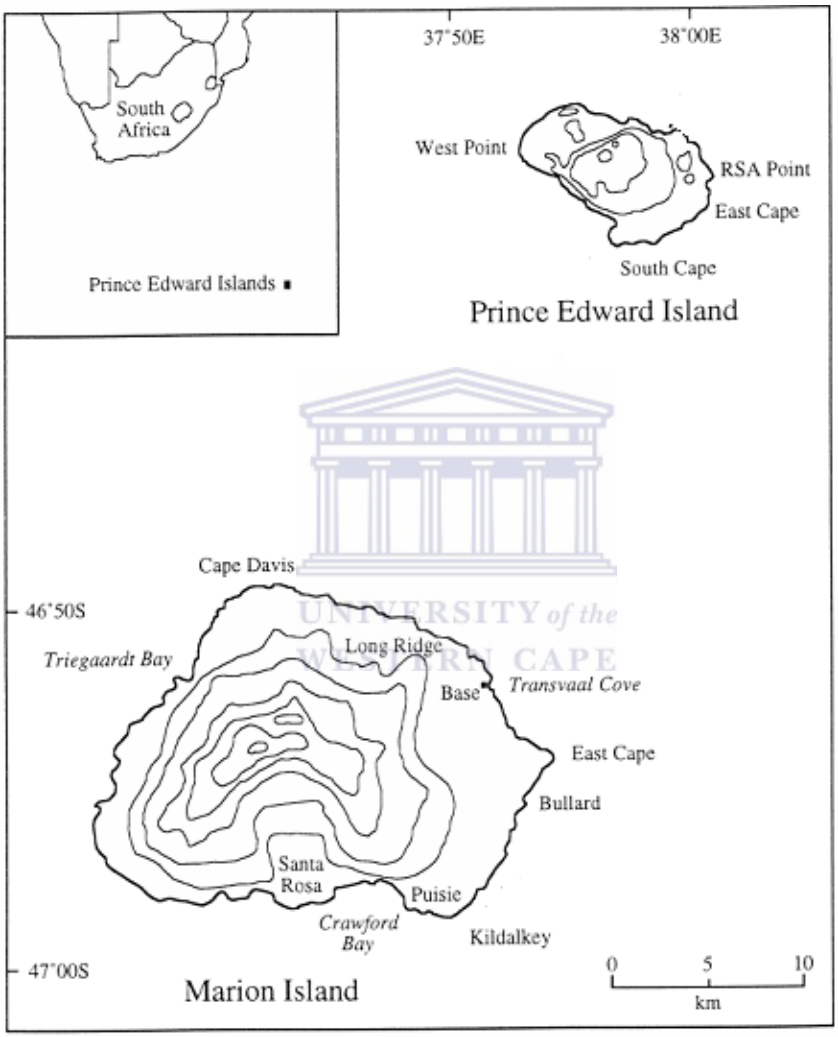


Fig. 1.1: The Prince Edward Islands and the location of Marion Island relative to South Africa. Contour intervals are 200m (Huyser *et al.,* 2000).

1.1.1 Biodiversity at Marion Island

A variety of marine birds and mammals occur and breed on Marion Island in vast numbers. The marine birds and mammals include estimated populations of Southern Elephant seals (1700) and sub-Antarctic (77 000) and Antarctic Fur Seals (3 600). Penguin populations include King Penguins (440 000), Gentoo Penguins (1 800), Macaroni Penguins (720 000) and Southern Rockhopper penguins (134 000). Albatrosses include the wandering Albatrosses (3 600), Grey-Headed Albatrosses (15 800), Light-Mantled Albatrosses (400), Dark-Mantled Albatrosses (2 400). These breed on the islands, providing localized nutrients around the nests. At least sixteen species of petrels occur on the islands, which all breed in burrows except for two species. Most of the species occur in breeding pairs of tens of thousands. These include the Blue Petrels (100 000), Greatwinged Petrels (20 000), Southern Giant Petrels (3 500), Northern Giant Petrels (700), Soft-plumaged Petrels (10 000), Kerguelen Petrels (20 000), and Whitechinned Petrels (20 000). Other seabirds include Crozet Shags (540), Sub-Antarctic Skuas (1 200), Kelp Gulls (200), Antarctic Terns (50) and Kerguelen Terns (100). Besides the seabirds, only the Lesser Sheathbills or Blackfaced Sheathbills breed on the island (Chown and Froneman, 2008).

Other organisms include the House Mice and invertebrates such as Diamondbacked Moths, German Cockroaches, nematodes, mites, earthworms, woodlice, caterpillars, flightless moths and springtails. The vegetation on Marion

Island is generally described as tundra (Chown and Froneman, 2008). A variety of vascular plants, bryophytes (mosses and liverworts) and lichens occur within different terrestrial habitats (section 1.2) (Chown and Froneman, 2008; Smith & Steenkamp, 2001).

Even though the diversity of microorganisms on Marion Island is well characterised, only a few studies few studies have been previously conducted on the microbiology of Marion Island. Most of the studies were focused on nitrogen fixation by microorganisms, particularly cyanobacteria (Smith and Ashton, 1981; Smith, 1988). Some of the studies were focused on the classical isolation and enumeration of bacterial populations using culture-dependent techniques (French and Smith, 1986), but were not extensive or primarily aimed at diversity studies. A number of studies have shown that Antarctic environments support a variety of microorganisms (section 1.8).

1.1.2 Climate and climate change

The island climate is typically sub-Antarctic, characterised by wet, cloudy and windy conditions (Chown and Froneman, 2008; Smith 1987). The island air temperature is generally low with an annual mean ranging between 5.0 and 6.5 ℃. The mean daily temperature is approximately 2 ℃ in winter and 3° C in summer. The average mean diurnal temperature is 3.6℃. Precipitation is very high with total monthly precipitation ranging between 1900 to 2800mm between

1960 and 2000. Rainfall is the major form of precipitation on the island (Smith *et al.,* 1993). The relative humidity is very high, averaging 80%. Sunshine is rare due to high cloud cover, which averages at least 75% of the sky per day (Chown and Froneman, 2008). North-westerly and west-north-westerly gale force winds blow up to an average of 107 days per year (Smith, 1987). However, the precipitation has declined over the years to an annual average of 1900mm and the island climate is gradually becoming warmer and drier (Chown and Froneman, 2008; Smith, 2002).

1.1.3 Energy production and nutrient recycling

Nutrient recycling is the major limiting factor in the mineralization activities of plant litter and consequently to the production of primary energy on the island. The primary production is very high as well as the plant requirements for nutrient inputs. However, there are no macroherbivores, which results in most of the energy and nutrients being incorporated into the detritus cycle (Smith, 1988). The activities of soil bacteria and microinvertabrates (nematodes, earthworms, springtails, mites and insect larvae) that feed on plant litter result in the bulk of the energy flow and nutrient recycling (decomposition) (Chown and Froneman, 2008; Smith and Steenkamp, 1992). These processes, and consequently primary production, are expected to increase with increasing temperature (Chown *et al.,* 1997). The high precipitation is associated with the waterlogging, which is the most important factor retards the soil microbiological processes (Smith, *et al.,*

1993). The warmer climate is most likely expected to influence the rate at which the island is invaded by alien plant and animal species (Bergstrom and Chown, 1999). The marine mammals cause significant erosion and trampling of coastal habitats and provide nutrients for the vegetation through excretion, skin moulting and dead corpses. Most of the nutrients are ammonium nitrogen nitrates, uric acid, proteins, phosphates and calcium (Hall and Williams, 1981). Amongst the bacteria, actinobacteria are the most important decomposers of complex materials. They are involved in nutrient recycling through the degradation of plant material (Ventura *et al.,* 2007; Vorob'ev, *et al.,* 2007; Pankratov *et al.,* 2006). A study of actinobacterial diversity, distribution and their relationships with soil and plant characteristics on Marion Island is therefore, very important.

1.2 The Marion Island terrestrial habitats

Marion Island consists of 23 different terrestrial habitats classified into seven Habitat Complexes (Smith & Steenkamp, 2001; Smith, *et al.,* 2001). The differences in physical, chemical, and biotic factors contribute to the variations in the properties of each habitat. The habitats are characterised by harsh environments and impoverished biota (Smith, 2002). Each habitat is assigned an identification (classification) code. In this study, the habitats were named by including the prefix MI, which refers to Marion Island. A summary of the habitat

names and locations is provided in Table 1.1 and details of soil physiochemical properties provided in Appendix A1.

Table 1.1: The terrestrial ecological habitats of Marion Island.

Habitat type	Code	Location	Elevation (m)	S	E
Coastal Salt-Spray Complex	**1**				
[a]Coastal Herbfield	MI 1.1	Archway	22	46º 53.892′	37º 53.557′
[a]Coastal Fellfield	MI 1.2	Archway	20	46º 53.895′	37º 53.560′
Fellfield Complex	**2**				
Xeric Fellfield	MI 2.1	Tafelberg	362	46º 53.750′	37º 40.12′
Mesic Fellfield	MI 2.2	Scoria Cone near Swartzkop	100	46º 55.481′	37º 35.912′
Slope Complex	**3**				
Open Fernbrake	MI 3.1				
Closed Fernbrake	MI 3.2		23	46º 52.877′	37º 51.927′
[a]Mesic Fernbrake	MI 3.3	Archway	22	46º 53.892′	37º 53.557′
Dwarf-Shrub Fernbrake	MI 3.4				
Slope Drainage Lines	MI 3.5				
Spring and Flush Habitat	MI 3.6		32	46º 55.644′	37º 35.66′
Biotic Grassland Complex	**4**				
Coastal Tussock Grassland	MI 4.1	Archway	32	46º 33.866′	37º 53.485′
Inland Tussock	MI 4.2				
[a]Pedestalled Tussock Grassland	MI 4.3	Archway	30	46º 53.868′	37º 53.485′

[a]Samples used in this study

Table continued

Habitat type	Code	Location	Elevation (m)	S	E
Biotic Herbfield Complex	5				
[a]Cotula Herbfield	MI 5.1		11	46º 53.018´	37º 52.151´
[a]Biotic Mud	MI 5.2	Near Trypot beach	2	46º 53.131´	37º 52.092´
[a]Biotic Lawn	MI 5.3		22	46º 53.055´	37º 52.044´
Mire Complex	**6**				
Dry Mire	MI 6.1	Ship's Cove	34	46º 52.661´	37º 51.567´
[a]Mesic Mire	MI 6.2	Ship's Cove	64	46º 51.532´	37º 50.664´
[a]Wet Mire	MI 6.3	Ship's Cove	91m	46º 51.616´	37º 50.818´
[a]Mire Drainage	MI 6.4	Ship's Cove	46	46º 51.975´	37º 51.037´
Biotic Mire	MI 6.5	Lake Edge near Swartzkop	148	46º 55.665´	37º 35.665´
Polar Desert	**MI 7**				
Polar Desert	MI 7.0		-	-	-

[a]Samples used in this study

1.2.1 The Coastal Salt-Spray Complex

Salt-spray communities are restricted to shore-zone areas that are heavily affected by wind-blown sea spray. The Coastal Salt-Spray Habitat complex comprises two habitats, the Coastal Herbfield and the Coastal Fellfield Habitats. The two habitats occur on fibrous brown-black peats dominated by the dicotyledonous *Crassula moschata* and *Cotula plumosa* and are frequently

influenced by manuring from marine birds and fur seals (Smith & Steenkamp, 2001; Smith, *et al.,* 2001).

1.2.2 The Fellfield Complex

The Fellfield Complex consists of two habitat types, the Xeric Fellfield and the Mesic Fellfield Habitats. The habitats occur on the exposed plateaus and ridges, dominated by cushion dicotyledonous plants and lichens. Bare rock or scoria makes up a larger part of the surface. Neither animals nor salt-spray influences the habitats in the Fellfield Complex. The soils contain higher bulk density and lower concentrations of salts. The Xeric Fellfield Habitat comprises of sparse vegetation dominated by lichens and cushion bryophytes (Smith & Steenkamp, 2001; Smith, *et al.,* 2001).

1.2.3 The Slope Complex

The Slope complex consists of the Open Fernbrake, Closed Fernbrake, Mesic Fernbrake, Dwarf Shrub Fernbrake, Slope Drainage Line and Streambank, and the Spring-and-Flush Habitats. The first four habitats consist of fernbrake communities dominated by *Blechnum penna-marina* (Smith & Steenkamp, 2001; Smith, *et al.,* 2001).

The Open Fernbrake Habitat occurs on slopes up to 300m above sea level (a.s.l), co-dominated by vegetation also found in the Mesic Fellfield and Closed Fernbrake habitats. The Open Fernbrake and Closed Fernbrake Habitats consist of soils with higher organic and moisture contents, and lower pH, total Calcium, and total Magnesium than the Fellfield habitats due to increased organic input by plants. The Closed Fernbrake Habitat occurs on slopes below 300m down to sea level, on deep, well-drained soils with vegetation dominated by *B. penna-marina* (Smith & Steenkamp, 2001; Smith, *et al.,* 2001).

The Mesic Fernbrake Habitat mainly occurs on slopes that are less steep and have more peaty soils than those in the Open and Closed Fernbrake Habitats. Vegetation is co-dominated by mire graminoids and mire bryophytes. The Dwarf Shrub Fernbrake Habitat is less common, occurring on slopes with impeded drainage and depressions. The deciduous shrubs *B. penna-marina,* the *Acaena magellanica* and the *Brachythecium* mosses dominate the vegetation. Mosses and other bryophytes also occur in this habitat.

The Slope Drainage Line and Streambank Habitat occur in slope drainage lines, and on banks and streams. Vegetation is dominated by *B. penna-marina* in combination with either *A. magellanica* or *Brachythecium* mosses. The Spring-and-Flush Habitat is the wettest of the slope habitats, which occurs not only on slopes, but also in water tracks, springs, and level areas. The Spring-and-Flush

Habitat is the wettest amongst the Slope complex Habitats, consisting of vegetation similar to that found in the Slope Drainage Line and Streambank Habitat. The soils of the Spring-and-Flush Habitat are at higher pH and contain higher mineral content and less organic matter than those found in other Slope complex habitats. In addition, the Spring-and-Flush Habitat consists of soils similar to those found in the Mire Drainage Line Habitat (Smith & Steenkamp, 2001; Smith, *et al.,* 2001).

1.2.4 The Biotic Grassland Complex

The Biotic Grassland complex consists of three habitats, the Coastal Tussock Grassland, Inland Tussock Grassland, and the Pedestalled Tussock Grassland Habitats. Tussock grasslands are composed of "nitrogen-loving" plants. These occur mainly around seal and penguin colonies in well-drained slopes where burrowing petrels nest. The Coastal Tussock Grassland Habitat occurs in coastal areas exposed to heavy trampling and manuring by burrowing petrels, penguins, and seals. Most of the vegetation consists of the mat dicotyledonous plants, dominated by the tussock-forming grass *Poa Cookii*. The Inland Tussock Grassland Habitat is common on inland slopes and near the coast. The inland areas also support colonies of burrowing petrels. Marine birds and animals affect the coastal parts of the habitat to some extent. Mosses form an important part of the vegetation, which is dominated by *P. cookii*, although not pedestalled.

The Coastal Tussock Grassland soils are the most and acidic, with an average pH of 4.0. The soils in the Biotic Grassland Complex habitats contain high levels of organic and inorganic forms of nitrogen and phosphorous due to the manuring influence of animals and the highest vegetation crop on the island. The Pedestalled Tussock Grassland Habitat occurs around the King penguin rookeries, with vegetation that includes *C. antarctica* and *Montia fontana*, dominated by the *P. cookii* tussocks on peat pedestals (Smith & Steenkamp, 2001; Smith, *et al.,* 2001).

1.2.5 The Biotic Herbfield Complex

The Biotic Herbfield complex consists of three distinct habitats, the Cotula Herbfield, Biotic Mud, and the Biotic Lawn Habitats. These are all influenced by seabirds and seals. The Cotula Herbfield Habitat occurs near the coast, dominated by *C. plumosa.* The habitats are characterised by high soil fertility and high salinity indices.

The Biotic Mud Habitat occurs near the coast and immediately adjacent to the seal wallows and penguin rookeries and is influenced by the impact of heavy trampling and manuring by seals and seabirds. The mat forming dicotyledonous plants dominate the vegetation and soils are wet organic mud, usually anaerobic, with very high levels of nitrogen and phosphorous. The Biotic Lawn Habitat occurs in similar areas to the Biotic Mud Habitat, but the surface derives from

volcanic material and is composed of thin peat underlain by a scoria of fine ash or pebbles. The vegetation includes *P. cookii, P. annua,* and *C. plumosa* in addition to the mat forming dicotyledonous plants found in the Biotic Mud Habitat (Smith & Steenkamp, 2001; Smith, *et al.,* 2001).

1.2.6 The Mire Complex

The Mire complex consists of some of the wettest parts of Marion Island and is made-up of six habitat types with similar soil and vegetation characteristics. The habitats from this complex cover a total area of approximately 50% of the island below 300m a.s.l. Mire graminoids, mire bryophytes, and mosses dominate the vegetation. The soils are organic with high moisture content and low bulk density.

Of the all the Mire habitats, the Dry Mire Habitat contains least organic matter and vegetation is dominated by *B. penna marina*. The Mesic Mire Habitat occurs in wetter and deeper regions and contains more organic peat than the Dry Mire Habitat. The Wet Mire Habitat is composed of a range of plant communities. These include graminoids and thick mats of bryophytes around ponds, tarns and lakes. Bryophytes dominate in wet depressions where the water table is at the surface. The Mire Drainage Habitat occurs around the drainage lines and water tracks of the mire areas. These surfaces are composed of slightly compacted peat containing an appreciable amount of volcanic ash.

The Biotic Mire Habitat occurs where manured underground water from the surrounding areas seeps and results in mire vegetation. The enrichment of the water found in the Biotic Mire Habitat occurs elsewhere by manuring from seabirds or seals. A single bryophyte species, *Clasmatocolea vermicularis* and the mat dicot *M. fontana* strongly dominate the vegetation. In addition, other mat dicotyledonous plants such as *Callitriche antarctica* and *Ranunculus biternatus* and the graminoids *Agrostis magellanica* and *P. cookii* occur in the Biotic Mire Habitat.

The Saline Mire Habitat is uncommon and occurs on compacted peat with impeded drainage due to heavy deposition of salt-spray, characterised by very high salt salinity values (Smith & Steenkamp, 2001; Smith *et al.,* 2001).

1.2.7 The Polar Desert complex

The Polar Desert Complex has no sub-habitats. It is dominated by physical rather than biological processes. The habitat covers approximately 41% of the total area of the island and occurs at altitudes above 500m on the western and southern parts of the island. The surface of the Polar Desert Habitat consists of a thin grit of volcanic ash that occurs under and between deposits of pebble-sized scoria, with occasional rocks or boulders on the surface. However, this habitat is not composed of soils. The vegetation consists of cushion or ball-forming mosses and crustose forms of lichens. These generally cover less than 1% of the surface

of the complex, but may occasionally reach up to 50% in localized areas. These include areas such as where snowmelt accumulates or protected areas. Large expanses of bare rock with the occasional cushion-forming moss or crustose lichens normally surround the spots with high vegetation cover (Smith & Steenkamp, 2001; Smith, *et al.,* 2001).

1.3 Actinomycetes

In this study, more emphasis was on the actinomycetes because they constitute the majority of the class *Actinobacteria* (Stackebrandt *et al.,* 1997). In addition, they are producers of most secondary metabolites of commercial and biomedical importance (Bull & Stach, 2007; Bull *et al.,* 2000; Lange, 1996; Lazzarini *et al.,* 2000). Actinomycetes are a broadly defined heterogeneous group of bacteria, mainly found in soil habitats, where they play important ecological roles, breaking down complex organic matter and recycling nutrients (Alexander, 1977; Embley & Stackebrandt, 1994; Goodfellow & Williams, 1983; Stackebrandt, *et al.,* 1994; Waksman, 1957). Actinomycetes are Gram-positive, mostly true-branching filamentous bacteria that are free-living saprophytes and are phylogenetically related based on the 16S ribosomal RNA gene (Embley & Stackebrandt, 1994; Goodfellow & Williams, 1983). Most actinomycetes are generally aerobic to microaerophilic, spore-forming bacteria (Embley & Stackebrandt, 1994) that share a number of similar characteristics with fungi in terms of morphology,

pathology, and habitat adaptation (Alexander, 1964; Lechevalier & Lechevalier, 1967; Waksman, 1957).

1.3.1 The general life cycle of actinomycetes

The morphological diversity within the actinomycete genera include the coccus and rod life cycles, fragmenting hyphal forms, and highly differentiated, branched mycelium. Some genera form spores, which include motile zoospores and specialized propagules that resist desiccation and mild heat (Waksman, 1957). However, the actinomycete spores are different from the bacterial endospores in their organization and resistance properties (Goodfellow & Williams, 1983).

Filamentous actinomycetes usually occur as spores or small fragments of mycelium, mostly in soils and a variety of other habitats (Waksman, 1957). Under favourable conditions, germination occurs by formation of branching threads or rods that develop into unicellular mycelia. The hypha formed is generally non-septate, except in some few actinomycetes. Vegetative mycelium grows within the substrate of solid media whilst aerial mycelium grows above the vegetative growth. Many actinomycetes reproduce through either special sporulating bodies, or the vegetative tips of the mycelium (Alexander, 1977; Waksman, 1940).

1.3.2 Properties of actinomycetes similar to fungi

Actinomycetes were initially mistaken for fungi because most of them exhibit filamentous hyphae, but of generally smaller diameter compared to eukaryotes. Similarly, actinomycetes reproduce by spores, conidia and sporangia just like fungi (Alexander, 1977; Lechevalier & Lechevalier, 1967; Waksman, 1957).

1.3.3 Actinomycetes as true bacteria

Even though similar to fungi, actinomycetes are true prokaryotes. The flagella consist of typical bacterial morphology and organization. Unlike the actinomycetes and other bacteria, fungi consist of eukaryotic flagella consisting of 11 fibrils, each resembling a single bacterial flagellum. There are no strictly anaerobic or chemoautotrophic fungi, but some actinomycetes, like other bacteria, are strictly anaerobic or strictly chemoautotrophic (Lechevalier & Lechevalier, 1967).

Unlike the fungi, actinomycetes are generally sensitive to the antibiotics that affect other Gram-positive bacteria, resistant to the strictly antifungal antibiotics, sensitive to acids, and subject to attack by phages that infect other bacteria (Lechevalier & Lechevalier, 1967; Waksman, 1957). Some actinomycetes synthesize lysine using α, ε-diaminopimelic acid pathway commonly used by other bacteria instead of the α-aminoadipic acid pathway used by fungi. Likewise, the actinomycete cell walls do not contain chitin or cellulose as in fungi, and

consists of the chemistry found in other Gram-positive bacteria (Lechevalier & Lechevalier, 1967).

1.4 Methods used for classifying actinomycetes

Different classification systems assign actinomycetes to various generic groups largely based on morphology, chemotaxonomy and DNA-DNA reassociation (Lechevalier & Lechevalier, 1967; Palleroni, 1983; Stackebrandt *et al.,* 1997; Waksman, 1940). Classification based on the 16S rRNA gene (Stackebrandt *et al.,* 1997) is the most reliable marker for the classification of actinomycetes. Morphological and chemotaxonomic traits are additionally used to delineate the phenotypic differences that exist between the phylogenetically homologous groups which 16S rRNA gene may not completely resolve (Embley & Stackebrandt, 1994). Since the outcomes of the different classification systems do not vary much, the polyphasic classification strategy is used, which comprehensively combines all the classification approaches (Stackebrandt, *et al.,* 1994; Stackebrandt *et al.,* 1997). Some of the classification methods are described below:

1.4.1 Chemotaxonomy

Chemosystematics involves the classification of organisms based on the chemical variations amongst them (Priest & Austin, 1995). Chemotaxonomy

mostly involves the analysis of chemical composition of the cell constituents and whole cell lysates, such as the cell wall and peptidoglycan, lipids, sugars, fatty acids, isoprenoid quinones, cytochromes and DNA base composition (Romano & Sohler, 1956; Stackebrandt *et al.,* 1997; Waksman, 1957). The distribution of chemotaxonomic traits is also reflected in the phylogenetic trees based on the 16S rRNA genes (Embley & Stackebrandt, 1994).

1.4.1.2 Lipid composition

The lipids found in actinomycetes are characteristic for a genus and differ in fatty acid composition, solubility, and pyrolysis properties (Minnikin *et al.*, 1984; Minnikin *et al.*, 1977a; Minnikin *et al.*, 1977b). Mycolic acids are lipids that occur in mycobacteria, containing approximately 80 carbons in the lipid skeleton. Nocardic acids contain about 50 carbons and occur in *Nocardia*, whilst corynomycolic acids occur in corynebacteria containing about 32 carbons (Embley & Stackebrandt, 1994; Lechevalier & Lechevalier, 1967).

1.4.1.3 Cell wall composition

The differences in cell wall chemistry are central in the classification of actinomycetes (Embley & Stackebrandt, 1994; Stackebrandt *et al.,* 1997; Waksman, 1957). Most bacterial cell walls consist of a peptidoglycan layer of repeating (β1→4)-linked *N*-acetylglucosamyl and *N*-acetylmuramic acids cross-

linked via short peptides chains. The differences in the amino acids substituted in the interpeptide bridges and the different linkage units result in immense diversity of cell wall chemotypes amongst the Gram-positive bacteria (Mascaretti, 2003).

The amino acids found in peptidoglycan tetrapeptide unit are unique only to the cell walls. Diaminopimelic acid (A_2pm, DAP) is unique to bacterial cell walls, including actinomycetes, and is therefore, a suitable marker for bacterial taxonomic classification. Due to chirality, A_2pm exists as DD-, LL-, DL-, and LD-A_2pm, but the DL- and LD-A_2pm are similar and cannot be separated. They are therefore, referred to as the *meso*-A_2pm (Kawasaki *et al.,* 2007; Sasaki *et al.,* 1998).

All the different A_2pm isoforms occur in actinomycetes (Šuput *et al.,* 1967). The variations in menaquinone, fatty acid, and mycolic acid composition of the cell wall provide additional information for classifying actinomycetes (Busse *et al*., 1996; Dover *et al*., 2004). All actinomycetes containing the group B peptidoglycan also contain long unsaturated menaquinones, made up of more than nine isoprene units (Embley & Stackebrandt, 1994).

Cell wall chemotyping reveals the presence of major amounts of glucosamine, muramic acid, alanine, and glutamic acid in all actinomycete cell wall preparations. However, the differences that exist due to the presence or absence

of other sugars and amino acids generally result in different cell wall chemotypes (Cummins, 1965; Lechevalier & Lechevalier, 1967; Šuput *et al.,* 1967) (Table 1.2). The anaerobic–microaerophilic actinomycetes containing lysine in cell walls form a distinct major group amongst the aerobic forms containing diaminopimelic acid (DAP). The actinomycetes containing lysine form two subgroups, one with major amounts of lysine and ornithine, and the other containing major amount of lysine and aspartic acid (Embley & Stackebrandt, 1994; Lechevalier & Lechevalier, 1967).

Table 1.2: The major constituents present in cell wall preparations of representative actinomycetes. From Lechevalier & Lechevalier, (1967).

Cell wall type	Lysine	Ornithine	Aspartic acid	Glycine	L- A_2pm	*Meso*- A_2pm	Arabinose	Galactose	Oxygen requirement
I. *Streptomyces*				+	+				Mainly aerobic
II. *Micromonospora*				+		+			Mainly aerobic
Actinoplanes				+		+			Mainly aerobic
III *Streptosporangium*						+			Mainly aerobic
Dermatophilus						+			Mainly aerobic
Thermoactinomyces						+			Mainly aerobic
Microbispora						+			Mainly aerobic
Nocardia (actinomadura type)						+			Mainly aerobic
IV *Nocardia (asteroids type)*						+	+	+	Mainly aerobic
V. *Actinomyces (Israeli type)*	+	+							Mainly anaerobic, microaerophilic, to Facultative Aerobic
VI *Actinomyces (bovis type)*	+	+	+						Mainly anaerobic, microaerophilic, to facultative Aerobic

+ Denotes the presence of a sugar.

1.4.2 DNA nucleotide composition

The base composition of the deoxyribonucleic acid (DNA) characteristically varies amongst organisms in guanine and cytosine content (mol% G+C content), forming a basis for classifying microorganisms (Lechevalier & Lechevalier, 1967). Actinomycetes are generally made up of DNA containing more than 55 mol% G+C content, which is considered high (Embley & Stackebrandt, 1994; Goodfellow & Williams, 1983; Paul & Clark, 1996; Stackebrandt *et al.,* 1997).

1.4.3 The ribosomal RNA gene

The phylogenetic relatedness of actinomycetes is based on the 16S ribosomal RNA (rRNA) gene sequences (Embley & Stackebrandt, 1994; Goodfellow & Williams, 1983; Paul & Clark, 1996; Stackebrandt *et al.,* 1997). The small-subunit ribosomal ribonucleic acid (SSU rRNA) genes are important molecular markers for identifying microorganisms and phylogenetic studies. The rRNA genes are suitable biomarkers because rRNAs are essential for protein synthesis in all organisms. In addition, the rRNA genes are conserved, with highly variable sequences in both primary and secondary structure. The rRNAs are easy to isolate and identify, they mutate slowly and do not exhibit horizontal gene transfer. The 16S rRNA gene consists of about 1500 nucleotides (Woese, 1987, Embley & Stackebrandt, 1994; Paul & Clark, 1996). Using bioinformatics analysis, known sequences deposited in the database serve as references for the identification and classification of unknown microorganisms (Woese, 1987, Embley & Stackebrandt, 1994).

1.4.4 Restriction analysis

Low-frequency restriction fragment analysis (LFRFA) involves digesting the total chromosomal DNA with rare-cutting restriction enzymes and identifying the resulting fingerprint patterns (Anderson & Wellington, 2001). Randomly amplified polymorphic DNA (RAPD) analysis is based on single primers and is alternatively used to determine the fingerprint restriction pattern for identifying a particular group of microorganisms (Anderson & Wellington, 2001; El-Fiky *et al.,* 2003). Cook and Meyers (2003), identified actinomycetes to genus level, based on the patterns produced from the amplified ribosomal DNA restriction analysis (ARDRA) of the 16S rRNA gene (Cook & Meyers, 2003).

1.4.5 Numerical taxonomy

Phenetic classification refers to the relatedness of organisms based on their overall similarity or affinity with respect to molecular structure, physiology, and habitat. Numerical taxonomy involves in the use of as many traits (characteristics) as possible to generate a large set of data for computational grouping of organisms (Priest & Austin, 1995).

Some of the traits can be characterised by using morphological and biochemical analysis (Embley & Stackebrandt, 1994; Gordon & Smith, 1955; Lechevalier & Lechevalier, 1967; Waksman, 1940, 1957). Morphological characterization includes the description of the growth and appearance on various selected

media, including mycelia, spores and life cycle. The biochemical properties include formation of pigments, utilization of carbon and nitrogen sources, production of specific chemical compounds, and sensitivity to the effect of various types of phages (Anderson & Wellington, 2001; Waksman, 1957).

1.4.6 DNA-DNA reassociation

The phylogenetic relatedness of actinomycetes is based on DNA pairing studies (Embley & Stackebrandt, 1994; Paul & Clark, 1996; Stackebrandt *et al.,* 1997). DNA-DNA reassociation, also referred to as DNA-DNA relatedness, involves thermal or alkaline denaturation of double-stranded DNA (dsDNA) from two organisms, and the subsequent annealing of the respective single-stranded DNA (ssDNA) strands to form homologous duplexes by complementary base pairing (Wayne *et al.,* 1987).

The melting temperature (T_m) corresponds to the midpoint of the transition from double-stranded to single-stranded DNA and determines the stability of the nucleic acid duplex. The hybridization conditions, at temperatures as low as 25°C below the $T_{m,}$ allow for heteroduplex formation by mismatches. An increase in 1% mismatching normally reduces the T_m by approximately 1°C. The T_m can be monitored by measuring the absorbance at 260nm using a spectrophotometer. The use of high temperature and low salt concentration increases stringency that minimizes mismatches (Wayne *et al.,* 1987, Priest & Austin, 1995).

Microorganisms of the same species show at least 70% DNA homology, with less than 5% melting temperature (T_m) divergence when reassociation is measured under stringent conditions (Anderson & Wellington, 2001; Priest & Austin, 1995; Wayne *et al.,* 1987).

1.5 The class *Actinobacteria*

The modern classification system, as according to Stackebrandt *et al.* (1997), is entirely based on the comparison of actinobacterial 16S rRNA gene sequences and recognition of the signature nucleotides. However, the rRNA classification does not necessarily change the descriptions previously defined using the other methods, especially at lower taxonomic levels. Under the current system, the actinomycetes are members of the domain *Bacteria* and form one complex phylum (division), *Actinobacteria*, together with other high G+C Gram positive bacteria. The phylum *Actinobacteria* consists of one class, *Actinobacteria* and 5 subclasses (*Acidimicrobidae, Rubrobacteridae, Coriobacteridae, Sphaerobacteridae, and Actinobacteridae*), 6 orders (*Acidimicrobiales, Rubrobacterales, Coriobacteriales, Sphaerobacteriales, and Actinomycetales)*, 10 sub-orders (*Actinomycineae, Micrococcineae, Corynebacterineae, Micromonosporineae, Propionibacterineae, Pseudonocardineae, streptomycineae, Streptosporangineae, Frankineae, and Glycomycineae*), and 40 families (Fig. 1.2) (Stackebrandt *et al.,* 1997).

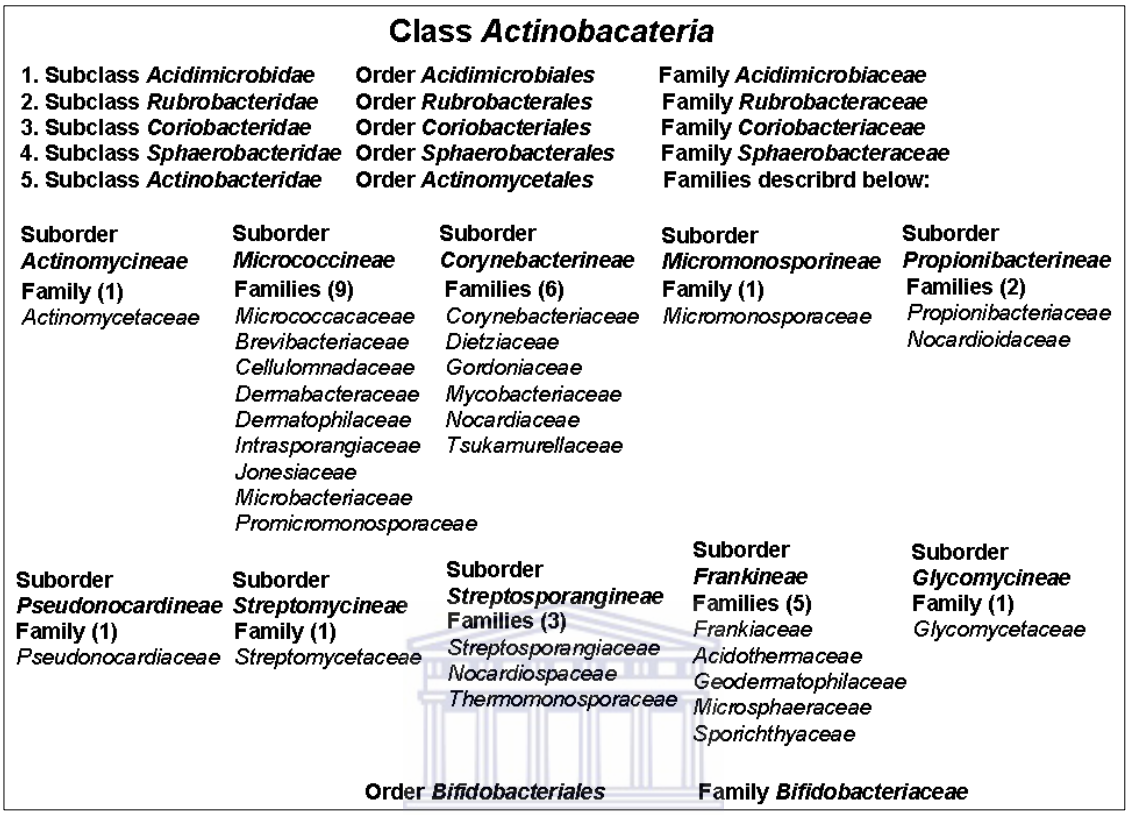


Fig. 1.2: The phylogenetic groups of class *Actinobacteria* based on numerical taxonomy and 16S rRNA gene sequences. Adapted from Stackebrandt *et al.* (1997).

1.5.1 Phylogeny of actinomycetes

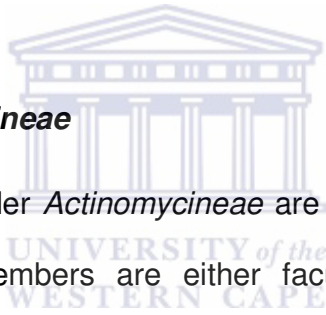
Early classification systems generally assigned actinomycetes to 7 to 8 aggregate groups: Actinobacteria, Actinoplaetes, Maduromycetes, Multilocular Sporangia, Nocardioforms, Streptomycetes, and Thermomonosporas (Goodfellow & Williams, 1983; Paul & Clark, 1996). Some authors also include the Micropolysporas (Goodfellow & Williams, 1983) and Thermoactinomycetes

as additional groups in the classification (Paul & Clark, 1996). The genus *Thermoactinomyces* has since been proposed based on phenotypic, chemotaxonomic and 16S rRNA gene phylogenetic analysis (Yoon *et al*., 2005). These earlier classification systems, based upon morphological, chemotaxonomic, and physiological characteristics as well as DNA-DNA reassociation experiments conducted over three decades, provide a working basis for defining the phylogenetic taxonomic units (Stackebrandt *et al.,* 1997).

Embley & Stackebrandt (1994) and Stackebrandt *et al.* (1997) comprehensively reviewed and related the old classification to the systems based on the 16S rRNA genes. The phylogenetic clades identified based on morphological properties, chemotaxonomy, and other conserved macromolecules such as peptidoglycan, menaquinones, phospholipids, sugars, and mycolic acids are also reflected in the 16s rRNA trees. However, most of these macromolecules used as molecular markers are polyphyletic and cannot therefore resolve at higher taxonomic levels (species or genus levels). They are therefore, unsuitable for phylogenetic studies, except for the mycolic acids (Embley & Stackebrandt, 1994). The actinomycetes evolved relatively recently (less than 1 billion years ago), over a short period on the bacterial evolution scale. Such rapid evolution has resulted in diversity that consists of homogenous clades that are in some instances, difficult to delineate at species level, due to close 16S rRNA sequence relatedness (Embley & Stackebrandt, 1994; Stackebrandt *et al.,* 1997).

1.6 Description of the *Actinomycetales* suborders

The characteristics of the various actinomycete suborders are summarized below as according to Stackebrandt *et al.,* 1997 (Fig. 1.2), and described using additional physiological, chemotaxonomic, morphological, and other information from the previous classification systems. Even though this review highlights the 16S rDNA signature nucleotides for the suborders, the families also exhibit distinct characteristic signature nucleotides (Stackebrandt *et al.,* 1997), some of which are not provided here.



1.6.1 Suborder *Actinomycineae*

The members of the suborder *Actinomycineae* are generally, referred to as the *Actinomycetaceae.* The members are either facultative or strict anaerobes requiring CO_2 for growth (Alexander, 1977). Members of the suborder *Actinomycineae* contain the 16S rRNA signature nucleotides at positions 598-640 (U-G), 1059-1198 (U-A), and 1061-1195 (G-U) (Stackebrandt *et al.,* 1997).

1.6.2 Suborder *Micrococcineae*

The suborder consists of nine families of different genera that include *Micrococccaceae, Brevibacteriaceae, Cellulomonadaceae, Dermabacteraceae, Dermatophilaceae, Intrasporangiaceae, Jonesiaceae, Microbacteriaceae,* and *Promicromonosporaceae*. Different forms characterize the members, including

mycelial (*Promicronospora*), coccus (*Micrococcus*), thallus (*Dermatophilus*), pleomorphic (*Microbacterium*), and rod-coccus morphologies (*Arthrobacter*) (Embley & Stackebrandt, 1994). The 16S rRNA is characterised by the signature nucleotides at positions 66-103 (A-U), 70-98 (U-A), 82-87 (G-C), 127-234 (A-U), 449 (A), 598-640 (U-G), 722-733 (A-A), 952-1229 (C-G), 986-1219 (A-U), 987-1218 (A-U), and 1059-1198 (U-A) (Stackebrandt *et al.,* 1997).

1.6.3 Suborder *Corynebacterineae*

The suborder *Corynebacetrineae* consists of six families, *Corynebactericeae, Dietziaceae, Gordoniaceae, Mycobacteriaceae, Nocardiaceae, and Tsukumurellaceae.* Some members contain mycolic acids with a cell-wall chemotype IV consisting of *meso*-A_2pm, arabinose, and galactose. Mycolic acids are a unique and coherent phylogenetic molecular marker for the suborder *Corynebacterineae* (Stackebrandt *et al.,* 1997). The suborder consist of 16S rRNA signature nucleotides at positions 127-234 (G-C), 131-231 (U-Purine), 502-543 (A-U), 658-748 (A-A), 564 (C), 600-638 (G-C), 601-638 (G-C), 601-637 (U-G), 660-745 (U-A), 986-1219 (U-A), 1116-1184 (U-G), and 1414-1486 (U-G) (Stackebrandt *et al.,* 1997).

1.6.4 Suborder *Micromonosporineae*

The suborder consists of one family, *Micromonosporaceae* include the genera *Micromonospora, Actinoplanes, Catellatospora, Couchioplanes, Catenuloplanes, Dactylosporangium,* and *Pilimelia* (Stackebrandt *et al.,* 1997). The members are generally belong to the group Actinoplanetes or Micromonospora (Embley & Stackebrandt, 1994; Lechevalier & Lechevalier, 1967). All members of the suborder *Micromonosporineae* are chemically homogeneous and contain hydrogenated menaquinones and phosphatidylethanolamine (Embley & Stackebrandt, 1994). The family *Micromonosporaceae* is characterised by the 16S rRNA signature nucleotides at positions 66-103 (G-C), 127-234 (A-U), 153-168 (C-G), 502-543 (G-C), 589-650 (C-G), 747 (A), 811 (U), 840-846 (C-G), 952-1229 (C-G), 1116-1184 (C-G), and 1133-1141 (G-C) in 16S rDNA (Stackebrandt *et al.,* 1997).

1.6.5 Suborder *Streptomycineae*

The suborder *Streptomycineae* consists of one family *streptomycetaceae*, characterised by the 16S rRNA signature nucleotides at positions 80-89 (G-C), 81-88 (C-G), 82-87 (U-G), 127-234 (G-C), 209 (C), 210 (C), 211 (G), 610 (G), 671-735 (U-A), 819 (G), 837-849 (C-G), 950-1231 (U-G), 955-1255 (C-G), 965 (C), 1254-1283 (A-U), and 1409-1491 (C-G), (Stackebrandt *et al.,* 1997). All members are filamentous and contain partially saturated menaquinones with nine

isoprene units. They usually contain LL-A_2pm in peptidoglycan, and a number of them are important antibiotics producers (Embley & Stackebrandt, 1994).

1.6.6 Suborder *Streptosporangineae*

The members of *Streptosporangineae* are morphologically diverse, chemically homogenous, and related based on sequence analysis and nucleic acid pairing. The members also contain partially saturated menaquinones with nine isoprene units as in suborder *Streptomycineae*. They also contain glucosamine and madurose, and a similar type of peptidoglycan based on *meso*-A_2pm (Embley & Stackebrandt, 1994). The suborder contains three families *Streptosporangiaceae, Nocardiopsaceae, and Thermomonosporaceae* with 16S rRNA signature nucleotides at positions 127-234 (A-U), 657-749 (G-Pyrimidine), and 955-1225 (C-G) (Stackebrandt *et al.,* 1997).

1.6.7 Suborder *Frankineae*

The suborder *Frankineae* consists of five families, *Frankiaceae, Acidothermaceae, Geodermatophilaceae, Microsphaeraceae,* and *Sporichthyaceae*. They are characterised by 16S rRNA signature nucleotides at positions 82-87 (C-G), 127-234 (G-C), 141-222 (G-C), 371-390 (G-C), 502-543 (A-U) and 1003-1037 (G-G). The family *Frankiaceae* contains the genus *Frankia*, *Acidothermaceae* the genus *Acidothermus* and *Geodermatophilaceae* genera

Geodermatophilus and *Blastococcus.* The family *Microsphaaeraceae* contains genus *Microsphaera* and *Sporichthyaceae,* the genus *Sporychthya* (Stackebrandt *et al.,* 1997). *Frankia* and *Geodermatophilus* form the multilocular sporangia consisting clusters of spores with many compartments when the hyphae divide. *Geodermatophilus* is an aerobic soil microorganism that forms motile spores. The family *Frankiaceae* forms non-motile sporangiospores (Paul & Clark, 1996).

1.6.8 Suborder *Pseudonocardineae*

The suborder *Pseudonocardineae* consists of only one family *Pseudonocardiaceae*. This includes the genera *Pseudoniocardia, Actinopolyspora, Actinosynnema, Amycolaptosis, Kibdelosporangium, Kutzneria, Lentzea, Saccharomonospora, Saccharopolyspora, Saccharothrix, Streptoalloteichus* and *Thermocrispum*. The 16S rRNA signature nucleotides occur at positions 127-234 (G-C), 183-194 (G-U), 502-543 (A-U), 603-635 (C-G), 610 (A), 747 (A), 952-1229 (U-A), 986-1219 (U-A), 987-1218 (G-C), 101-1039 (Pyrimidine-G), and 1308-1329 (C-G) (Stackebrandt *et al.,* 1997).

The members of *Pseudonocardiceae* vary in chemotaxonomic and morphological properties, but contain the cell wall chemotype IV cell wall, consisting of meso-A_2pm, arabinose, and galactose. The only exceptions are the genera *Kutzneria, Actinosynnema* and *Saccharothrix,* which contain cell wall chemotype III. In

addition, most members with type IV cell wall chemotype contain either phosphatidylethanolamine, or phosphatidylcholine, or both phospholipids in cell membrane (Embley & Stackebrandt, 1994). The genus *Actinopolyspora* consists of moderate to extreme halophiles, and *Kutzneria* and *Kibdelosporangium* form large sporangia-like structures. Some members of *Pseudonocardineae* produce commercially important antibiotics including rifamicins, vancomycin and erythromycin amongst others (Embley & Stackebrandt, 1994).

1.6.9 Suborder *Propionibacterineae*

The suborder *Propionibacterineae* consist of two families, *Propionibacteriaceae*, and *Nocardioidaceae*, characterised by 16S rRNA signature nucleotides at positions 127-234 (A-U), 603-635 (A-U), 657-749 (G-C), 671-735 (A-U), 986-1219 (U-A), 987-1218 (G-C), 990-1215 (U-G), and 1059-1198 (C-G) (Stackebrandt *et al.,* 1997).

Members of both *Propionibacteriaceae* and *Nocaradioidaceae* contain LL-A_2pm, but the two families arise from two separate lineages. The *Nocaradioidaceae* include the genera *Nocardioides* and *Aeromicrobium*. *Propionibacteriaceae* include the genera *Propionibacterium, Lutecoccus* and *Propioniferax* (Stackebrandt *et al.,* 1997). The propionibacteria consist of anaerobic and microaerophilic genera. *Aeromicrobium erythreum* is important for producing the macrolide antibiotic erythromycin A (Embley & Stackebrandt, 1994).

1.6.10 Suborder *Glycomycineae*

The Suborder *Glycomycinae* consists of one family, *Glycomycetaceae* represented by the genus *Glycomyces* and consisting of the 16S rRNA signature sequences at positions 70-98 (A-U), 127-234 (G-Pyrimidine), 140-223 (A-U), 229 (G), 366 (U), 415 (C), 449 (C), 534 (G), 681-709 (A-U), 825-875 (G-C), 999-1041 (C-G), 1059-1198 (C-G), 1064-1192 (G-G0, 1117-1183 (A-U), and 1309-1328 (C-G) (Stackebrandt *et al.,* 1997).

Glycomyces does not closely relate with any of the known cultured actinomycetes. However, members contain hexa-hydrogenated menaquinones with hydrogens on isoprenyl units II, III, and IX, similar to those found in a number of other bacteria and a few actinomycetes (Embley & Stackebrandt, 1994). The cell wall contains *meso*-A_2pm and glycine. Phosphatidylinositol, phosphatidylinositol mannosides, phosphatidylglycerol, and a number of phosphoglycolipids occur in cell membrane. Members also contain techoic acids and predominantly 15-17-carbon fatty acids of both the *iso* and *anteiso* series. The menaquinones predominantly consist of 10-12 isoprene units (Labeda & Kroppenstedt, 2004; Potekhina *et al.,* 1993). The members of *Glycomyces* have variable morphology, including mycelial and non mycelial, spores spore-forming and non-sporulating form of different colours (Labeda & Kroppenstedt, 2004).

1.7 Ecology and roles of actinomycetes

The actinomycetes widely occur in soils and a variety of other habitats, including manure and composts, water bodies, dust and food (Alexander, 1977; Embley & Stackebrandt, 1994; Goodfellow & Williams, 1983; Waksman, 1957). Actinomycetes play a number of important ecological functions in different habitats as follows:

1.7.1 Ecological roles in soil and rhizosphere

The rhizosphere is the soil zone immediately surrounding the plant root system. Most actinomycetes are free-living saprophytes that adapt to a wide variety of nutrients (Goodfellow & Williams, 1983). Members of the genus *Streptomyces*, play a role in soil mineralization and degradation of complex materials and plant litter, especially cellulose, hemicellulose, chitin, and lignin. The members of the genera *Nocardia* and *Rhodococcus* also degrade lignin and related compounds. Members of the genera *Rhodococcus* and *Arthrobacter* degrade hydrocarbon materials in soil (Goodfellow & Williams, 1983; Paul & Clark, 1996).

Most members of the genus *Frankia* consist of nodule-forming, non-infectious, nitrogen-fixing endophytes and non nitrogen-fixing members that form symbiotic associations with non-leguminous plants (Goodfellow & Williams, 1983; Paul & Clark, 1996).

1.7.2 Ecological roles in composts and related materials

Thermophilic actinomycetes are common in most compost, aquatic habitats, sewage and faeces of various animals. The members of the genus *Saccharomonospora* are obligate thermophiles whilst members of *Thermomonospora* (*Thermomonospora vulgaris*)*, Micropolyspora, Streptomyces* (*Streptomyces diastaticus*), and *Pseudonocardia* (*P. thermophila*) are either obligate or facultative thermophiles that dominate in self-heated substrates. In addition, *Rhodococcus coprophilus* is a truly coprophilus actinomycete that grows on a variety of decomposing material (Goodfellow & Williams, 1983).

Mesophilic actinomycetes, such as members of the genera *Corynebacterium, Microbacterium, Arthrobacter* and *Rhodococcus* occur in dairy activated and sewage sludge. The activated sludge from municipal sewage contains *Nocardia amarae*, which acts as a strong demulsifier and *Rhodococcus erythropolis,* which removes phthalate esters preventing deflocculating (Goodfellow & Williams, 1983). Mesophilic actinomycetes also play a role in the deterioration of poorly stored grain and food, especially under moist conditions. Examples include members of the genera *Saccharomonospora* and *Saccharopolyspora, Micropolyspora faeni, Thermoactinomyces vulgaris*, and a *Thermomonospora* species that results in allergies caused by airborne spores such as “farmer’s lung” or “bagasosis”, and “mushroom worker’s lung” caused by

Thermoactinomyces sacchari (Embley & Stackebrandt, 1994; Goodfellow & Williams, 1983).

1.7.3 Ecological roles in freshwater habitats

A number of actinomycetes, including the genera *Micromonospora, Streptomyces, Rhodococcus, Thermoactinomyces*, and the actinoplanetes (some members of the suborders *Streptosporangineae* and *Micromonosporineae*) are readily isolated from freshwater, rivers and lakes, where they are associated with decomposing chitin, lignin, and cellulose in twigs, leaf litter, and woody material. The genus *Micromonospora* occurs in abundance in streams, rivers, and lakes, and their sediments. *Actinomadura madurae, Mycobacterium kansaii,* and species of *Arthrobacter, Corynebacterium*, and *Nocardia* also frequently occur in freshwater habitats. Some actinomycetes are specifically adapted to decomposing particular substrates, for example, the *Actinoplanes* grow on river plant litter, *Micromonospora* on timber, *Nocardia asteroids* on rubber material in freshwater drainage pipes, whilst *Streptomyces* grows on chitinous materials in woodland streams. In addition, the actinomycetes produce volatile compounds and odours such as geosmin and methyl *iso*-borneol during decomposition, which affect the taste of drinking water. Most of the freshwater actinomycetes seem to arise from endospores washed from terrestrial habitats into the water bodies, where they germinate in the presence of suitable substrates (Goodfellow & Williams, 1983).

1.7.4 Ecological roles in marine habitats

Actinomycetes were initially believed not to occur in marine habitats (Waksman, 1957), but subsequent studies revealed that the genera *Rhodococcus, Streptomyces* and *Micromonospora* were frequently isolated from marine environments. Most of the actinomycetes isolated from marine habitats mainly spore-forming types, isolated from shallow sea mud, leading to the assumption that they were of terrestrial origin. The consistent isolation of these three genera led to the belief that the marine temperatures, salinity, nutrient availability, and hydro-pressure exerted a selection pressure on the survival of the spores (Okami & Okazaki, 1974). Okami & Okazaki (1974) concluded that many actinomycetes could survive in the salty and low temperature marine environments over considerable periods if enough nutrients and oxygen are available. Their studies of the physiological responses to simulated marine environmental conditions showed that some actinomycetes were adapted to marine environments (Colquhoun *et al.,* 1998a; Colquhoun *et al.,* 1998b; Okami & Okazaki, 1974) suggesting marine environments haboured endemic groups. For example, some members of the genera *Dietzia* and *Rhodococcccus* inhabiting deep seas in Polar Regions are psychrotolerant with specific salt requirements (Yu *et al.,* 2005).

The marine actinomycetes occur in different habitats, including the deep sea floor, coral reefs, sediments, marine plants, reef sponges, algal samples, and marine invertebrates at all depths (Lam, 2006). The limited understanding of

marine actinomycete microbiology was mainly due to the lack of effort in studying marine environments (Bull *et al.,* 2000; Lam, 2006). Recent findings have shown the existence of actinomycetes in deep-sea habitats (Colquhoun *et al.,* 1998a; Colquhoun *et al.,* 2000; Lam, 2006; Magarvey *et al.,* 2004; Maldonado *et al.,* 2005; Maldonado *et al.,* 2005a; Stach *et al.,* 2003a).

Improved culture techniques and the use of 16S rRNA technology resulted in the isolation and identification of a broad diversity of deep-sea actinobacterial genera. These include *Actinomadura, Dietzia, Gordonia, Microbacterium, Nocardiopsis, Pseudonocardia, Saccharopolyspora, Streptosporangium, Nonomuraea, Verrucosispora*, *Williamsia,* and a number of novel species (Bull *et al.,* 2000; Colquhoun *et al.,* 1998b; Maldonado *et al.,* 2005).

Some actinobacteria, for example members of the genera *Dietzia, Rhodococcus, Streptomyces, Salinispora, Marinophilus, Solwaspora, Verrucosispora, Salinibacterium, Aeromicrobium marinum* and *Williamsia,* are indigenous to the marine environments (Lam, 2006). Some of the actinomycetes were found to be endemic to the hypersaline marine environments. These include members of the genera *Salinispora* (Maldonado *et al.,* 2005) and *Salinibacterium* (Han *et al.,* 2003). The genus *Salinispora* consists of members that are only metabolically

active in the natural marine environment (Maldonado *et al.,* 2005). In addition, marine actinomycetes are a rich source of most recently discovered antibacterial, antifungal, anticancer, anti-inflammatory, and anti-malarial compounds (Bull *et al.,* 2000; Lam, 2006).

1.7.5 Ecological roles as plant pathogens

Many actinomycetes cause plant pathogenesis, for example members of the genera *Curtobacterium, Clavibacter*, and *Rathayibacter* that belong to the suborder *Micrococcineae* (Embley & Stackebrandt, 1994). Members of the genus *Corynebacterium* cause plant wilt (*Corynebacterium flaccumfaciens, Corynebacterium insidiosum, Corynebacterium nebraskense,* and *Corynebacterium sepedonicum*), leaf spots (*Corynebacterium betae, Corynebacterium oortii,* and *Corynebacterium poinsettiae*), and crankling of leaves and stems (*Corynebacterium michiganense* and *Corynebacterium poinsettiae*). *Arthrobacter ilicis* causes blight of holly, *Nocardia vaccinii* and *Rhodococcus fascians* cause the proliferation of galls in plants. *Streptomyces aureofaciens, Streptomyces flaveolus* and *Streptomyces griseus* cause common potato scab. *Streptomyces ipomoeae* causes sweet potato scab, and *Streptomyces scabies* causes russet scab of potatoes. All these infections result in reduced plant yields (Goodfellow & Williams, 1983).

1.7.6 Ecological roles as human and animal pathogens

The actinomycetes, mostly members of the families *Corynebacteriaceae, Mycobacteriaceae,* and *Nocardiaceae,* cause many human and animal diseases. *Corynebacterium diphtheria* causes diphtheria in humans, *Mycobacterium tuberculosis* causes tuberculosis in humans and cattle, and *Mycobacterium leprae* causes leprosy in humans. Actinomycosis is caused by some members of the suborder *Actinomycineae (Actinomyces bovis, Actinomyces israelii* and *Arachnia propionica*). Some members of the genus *Nocardia* cause Norcadiosis and nocarditis. Actinomycetoma, the infection of the feet, legs, and extremities is caused by *Actinomadura madurae* (suborder *Streptosporangineae*), some members of the genus *Nocardia* including *Nocardia asteroides,* and *Streptomyces somaliensis*. Different forms of pneumonia result from infections by actinomycetes such as *Rhodococcus, Micropolyspora faeni, Saccharomonospora viridis* and *Thermoactinomyces vulgaris* (Goodfellow & Williams, 1983). The suborder *Micrococcineae* contains animal pathogens and bacteria that cause skin infections in humans (Goodfellow & Williams, 1983).

1.7.7 The survival and growth of actinomycetes in soil

Streptomyces exists as resting spores in soil that may germinate in the presence of exogenous nutrients to form mycelia, rapidly colonizing the substrate to produce aerial spores (Lechevalier & Lechevalier, 1967; Waksman, 1940). Non-sporulating genera such as *Arthrobacter* can exist for longer periods as resting

cocci (Lechevalier & Lechevalier, 1967). *Streptomyces* spores disperse above the soil by water and air currents, and by water and arthropods within the soil. The soils structure and chemical composition influences the distribution of actinomycetes in soil. These factors include the competition from other microorganisms, nutrient availability, soil temperature changes, soil pH, moisture tension, carbon dioxide, soil structure, humic acids and other exogenous soil pollutants (Goodfellow & Williams, 1983; Paul & Clark, 1996).

Most actinomycetes are mesophiles, with a temperature growth optimum at around 28°C. The optimum pH for the majority of soil actinomycetes is between 5.0 and 9.0 with an optimum around neutrality, but acidophilic and acidoduric actinomycetes grow in soils of pH as low as 4.5. Soil actinomycetes are sensitive to anaerobic conditions, which normally occur in water-filled pores such as in peat and clay soils. The low soil moisture content can result in the desiccation of microorganisms (Goodfellow & Williams, 1983; Paul & Clark, 1996).

1.8 The microbiology of Antarctic and cold habitats

Microbiological communities are highly diverse as different types are favored in different ecological niches (Kassen and Rainey, 2004). The complex heterogenous environments, such as those of Marion Island provide more niches that are most likely to maintain diversity compared to the simpler niches (Kassen

and Rainey, 2004). The numbers and diversity of soil microorganisms decreases under low temperature conditions as they usually fail to produce spores and other dormant structures (Paul & Clark, 1996; Vorobyova *et al.,* 1997). Lower temperatures slow biological processes in microorganisms, allowing for longer periods of cell viability (Kochkina *et al.,* 2001). Some of the cold-adapted microorganisms resist freezing because they are structurally equipped with intracellular antifreeze compounds, proteins and flexible membranes, which enable them to carry out growth and metabolic activities at lower temperatures (Callaghan *et al.,* 2004).

The bacterial populations associated with the sea-ice diatom assemblages from Antarctic coastal habitats were found to be rich in diversity (Bowman *et al.,* 1997). The bacteria isolated belonged to the alpha and gamma proteobacteria, gram-positive bacteria and the *Flexibacter- Bacteroides-Cytophaga* phylum. Uncultured groups detected using culture-independent techniques belonged to the genera *Colwellia, Shewanella, Marinobacter, Planococcus,* and the *Flexibacter-Bacteroides-Cytophaga* phylum. Psychrotrophic strains included members of the genera *Pseudoalteromonas, Psychrobacter, Halomonas, Pseudomonas, Hyphomonas, Sphingomonas, Arthrobacter, Planococcus,* and *Halobacillus* (Bowman *et al.,* 1997).

Even though the microbiology of Marion Island is not well characterised, a number of studies indicate that cold Antarctic environments support a wealth of microorganisms. Using the culture-independent approach based on the 16S rRNA gene, *Methylobacterium, Sphingobacteriales,* acidobacteria, actinobacteria, cyanobacteria, alphaproteobacteria, gammaproteobacteria and verrucomicrobia were detected from Falkland, South Georgia, Signy and Anchorage Islands (Yergeau *et al.,* 2007).

Kochkina *et al.* (2001) isolated microorganisms in abundance from ancient Arctic and Antarctic permafrost sediments samples aged between five thousand and three million years. The actinobacteria, particularly the actinomycetes dominated the bacterial diversity, contributing 50-90% of the viable counts. In a related study, actinobacteria constituted the largest group of bacteria identified in Antarctic cold desert mineral soils, and were mostly of uncultured groups (Smith *et al.,* 2006). Sjöling & Cowan (2003) also reported the presence of actinobacteria from Bratina Island (Antarctica) glacial meltwater lake sediments using culture-independent techniques. However, other groups of bacteria including Proteobacteria and the *Cytophaga-Flavobacterium-Bacteroides* phylum dominated the diversity in the sediments (Sjöling & Cowan, 2003). Bacteria that belong to the genera *Pseudomonas, Sphingobacterium, Micrococcus, Planococcus* and *Arthrobacter* were isolated from Schirmacher Oasis in Antarctica (Shivaj *et al.,* 1994).

Cowan & Ah Tow (2004) reviewed the microbiological diversity and community structures of different Antarctica habitats and concluded that they varied according to habitats. They recommended more studies to be conducted because the knowledge on the microbiology of cold habitats was not complete (Cowan & Ah Tow, 2004). Our study was therefore, intended at gathering more knowledge on the microbiological diversity in Marion Island terrestrial habitats. In this study, the microbiological diversity was expected to vary according to the characteristics of different habitats.

1.9 Industrial importance of actinomycetes

The Actinomycetes are the richest source of naturally occurring, biologically active metabolites, and contributing 65-70% of such compounds (Lange, 1996; Lazzarini *et al.,* 2000). By the 1999-2000, about 50-55% of the known biologically active compounds were produced by actinomycetes of the genus *Streptomyces,* 21-22% from fungi, 12-16.9% from other bacteria and 11-16% from strains that belong to other genera of actinomycetes (Demain, 1999; Lazzarini *et al.,* 2000). These biologically active compounds are of clinically important applications in human medicine as antibacterial, antifungal, therapeutic and antitumour agents. They are also important as protection agents and growth promoters in plants (Demain, 1999). A number of actinomycete genera are also important producers of vitamins and enzymes (Hopwood, 2007; Waksman, 1957). In addition, the

genus *Corynebacterium* consists of a number of members that produce food additives and amino acid in the fermentation industry (Hopwood, 2007).

The rate of discovering new antibiotics from actinomycetes significantly declined over the past decades. In fact, the rate of rediscovery of the known biologically active compounds from microorganisms approached 99.9%, but mathematical modeling postulates that a large number of antibiotics are still undiscovered (Watve *et al.,* 2001).

1.9.1. Biosynthesis of biologically active compounds

The mechanism of secondary metabolism and production of biologically active compounds by actinomycetes occurs during the phase of physiological differentiation. This involves environmental control in response to the supply of nutrients and chemicals that stimulate cell signaling and cascade reactions (Horinouchi, 2007).

1.9.1.1 Actinomycete biosynthetic genes

Many actinomycete secondary metabolite genes are highly conserved, and organized into synthetic clusters of separate functions such as precursor synthesis, backbone synthesis, tailoring, and other functions. The non-ribosomal polypeptide synthetase (NRPS) and polyketide synthase (PKS) genes encode for

the most important enzymes involved in the biosynthesis of such important substances in actinomycetes (Amoutzias *et al.,* 2008; Donadio *et al.,* 2007; Hutchinson, 1999, 2003; Weber *et al.,* 2003).

1.10 Scope of this study

A number of culture-dependent and culture-independent strategies are used to characterize the diversity and community structures of microorganisms. In addition, the exploration of new and extreme environments provides better prospects for identifying new microorganisms compared to commonly known habitats.

The details for techniques and strategies used in this study are provided in respective chapters of this thesis as follows: Chapter 2 describes the materials and methodologies used in this study. Chapter 3 describes the extraction of metagenomic DNA from Marion Island soils, PCR amplification of the 16S rRNA genes, DGGE and statistical analysis. Chapter 4 describes the phylogenetic analysis of the diversity and identification of community structures based on the sequencing of the 16S rRNA genes. Chapter 5 describes the isolation of actinomycetes from Marion Island terrestrial habitats soils using the classical isolation techniques and conditions adjusted to suit the habitats. Chapter 6 is an overall discussion with conclusions and recommendations for the study.

1.11 Justification of the study

The lack of detailed microbiological studies, the unique and extreme sub-Antarctic climate, and a combination of other habitat factors make Marion Island a relevant target for exploration of microbiological diversity and novelty. Actinomycetes are particularly important because of their diverse ecological roles, their economic importance, and their uses in medicine. In addition, the well-characterised habitats of Marion Island provide an excellent model for studying the relationships between the microbial diversity and physiochemical factors. The sub-Antarctic ecosystems are simple and sensitive to disturbances, which make them ideal for studying interactions of organisms with their environment (Smith, 2002). The findings from this study will contribute to our knowledge of microbiological diversity and understanding the factors that determine the distribution of microorganisms in Marion Island ecosystems.

1.12 Aims of the study

The major aim of this study is to determine and compare the distribution of bacteria and actinobacteria in Marion Island terrestrial habitats.

The specific objectives are as follows:

1. To compare the distribution of actinobacterial in Marion Island terrestrial habitats using the 16S rRNA gene as a phylogenetic marker.

2. To identify the major factors that may contribute to the differences in microbiological diversity in Marion Island soils.
3. To identify how the bacterial and actinobacterial diversity relate to the habitat factors in Marion Island terrestrial habitats.

4. To isolate and identify the culturable actinomycetes from Marion Island terrestrial soils.

1.12.1 Research questions

This study intended to answer the following research questions:

1. What are the community structures of actinomycetes within and across the distinct habitats of Marion Island?

2. Is it possible to relate the habitats according to the patterns of microbiological diversity observed? Thus, do the physiochemical and biotic factors that define the distinctions between the habitats influence the distribution of microorganisms amongst the habitats? If yes, then what are the major factors that determine the diversity? Is there an association that relates the occurrance of specific actinomycetes with changes in those factors?

3. What is the composition of actinobacterial community structures in Marion Island terrestrial habitats?

4. How do the actinomycetes found in Marion Island terrestrial habitats relate to those identified elsewhere? What ecological roles do they play in these habitats, and are there any reasons to justify the existence of adapted, endemic, or novel actinomycetes on Marion Island?

5. Are there any novel actinomycetes that can be isolated from these habitats, and what strategies can be used?

CHAPTER 2

2.0 Materials & Methods

This chapter describes the methods and materials used for conducting the experiments during the study. The full names for abbreviations are provided in the glossary.

2.1 Reagent sources

All the reagents used in this study were of the highest grade and purchased from Merck (Darmstardt, Germany), Bioline (England), Fermentas (Vilnius, Lithuania), BIO Rad (Munich, Germany), BDH, England, Kimix (South Africa), Saarchem (South Africa), Promega (Madison, Wis. U.S.A), Fluka (Germany) and Sigma (Deissenhofen, Germany). Taq DNA polymerase was prepared in-house at IMBM. InqabaBiotech, South Africa (www.Inqabqbiotech.co.za) and Integrated DNA Technologies (IDT) (http://eu.idtdna.com) synthesized the oligonucleotide primers. All biological media and components were purchased from Oxoid Ltd., (Hampshire England), Merck Biolab (Gauteng, South Africa) and Difco (Becton Dickinson, Sparks, MD, U.S.A). Antibiotics were obtained from Fermentas, Vilnius, Lithuania (ampicillin), Sigma, Deissenhofen, Germany (cycloheximide), and Merck Calbiochem, Darmstadt, Germany (tetracycline).

2.2 Autoclaving conditions

Unless otherwise stated, all reagents, buffers, culture media, and durable lab-ware were sterilized by autoclaving at 121 ºC for 30 minutes.

2.3 Preparation of culture media

The culture media and protocols used in this study are described below. All the components were mixed, dissolved, and the pH adjusted before making up to the final volume and autoclaving.

Humic acid agar (HA) (Hayakawa & Nonomura, 1987: values in g/L)

Humic acid (sodium salt) [a]	1.00
Na_2HPO_4	0.50
KCl	1.71
$MgSO_4.7H_2O$	0.05
$FeSO_4.7H_2O$	0.01
$CaCO_3$	0.02
Yeast extract[b]	1.00
Agar	15.0

[a]Dissolved in 2M NaOH (10ml).

[b]Modification of the original protocol. Yeast extract used instead of B-vitamins solution.

The medium was adjusted to pH 7.3 using 5N NaOH before autoclaving, and supplemented with Cycloheximide (50 µg/ml) after cooling to less than 55 ºC.

Middlebrook (7H9) agar (Becton, Dickinson & Co. Sparks, MD, U.S.A: values in g/L)

Difco™ Middlebrook broth	4.70
Agar	15.0
100 mM glucose solution [c]	100ml

[c]Autoclaved separately before adding to the other components of the medium.

The medium was adjusted to pH 7.3 using 5N NaOH before autoclaving, and supplemented with Cycloheximide (50 µg/ml) after cooling to less than 55 ºC.

Czapek (CZ) agar (Waksman, 1957: values in g/L)

Sucrose	30.00
$NaNO_3$	2.00
K_2HPO_4	1.00
KCl	0.50
$MgSO_4.7H_20$	0.50
$FeSO_4.7H_2O$	0.01
Agar	15.0

The medium was adjusted to pH 7.3 using 5N NaOH before autoclaving, and supplemented with Cycloheximide (50 µg/ml) after cooling to less than 55 ºC.

MC agar (Nonomura & Ohara, 1971: values in g/L)

Glucose	2.00
$NaNO_3$	0.50
K_2HPO_4	0.30
KCl	0.30
$MgSO_4.7H_20$	0.3
$FeSO_4.7H_20$	0.01
$CuSO_4.5H_20$	0.001
$ZnSO_4.7H_20$	0.001
$MnSO_4.7H_20$	0.001

The medium was adjusted to pH 7.4 using 5N NaOH before autoclaving, and supplemented with Cycloheximide (50 µg/ml) after cooling to less than 55 ºC.

Yeast extract-Malt extract (YM) agar (values in g/L)

Yeast extract	2.00
Malt extract	3.00
Bacteriological peptone	3.00
Glucose	10.00
Agar	15.0

Medium is a modification of Yeast peptone agar (Waksman, 1957) and ISP medium 2.

The medium was supplemented with Cycloheximide (50 µg/ml) after cooling to less than 55 ºC.

Luria-Bertai agar (LB) (Sambrook *et al.,* 1989: values in g/L)

Yeast extract	10.0
Tryptone	5.00
NaCl	10.0
Agar	15.0

The pH was adjusted to 7.0 and before autoclaving and the medium was supplemented with 100mg/ml of ampicillin and 100 mM $MgCl_2$ after cooling to less than 55 ºC.

SOB agar (LB) (Sambrook *et al.,* 1989: values in g/L)

Yeast extract	0.50
Tryptone	20.0
NaCl	0.50
250 mM KCl	1.87
Agar	15.0

The pH was adjusted to 7.0 before autoclaving and the medium was supplemented with 100mg/ml of ampicillin and 100 mM $MgCl_2$ after cooling to less than 55 ºC.

2.3.1 Supplementation of culture media with antibiotics

Where necessary, media was supplemented with appropriate antibiotics or $MgCl_2$ after cooling to less than 55 ºC. All additives were dissolved in appropriate

solvents and filter-sterilized through 0.2 µm cellulose acetate filters (Lasec SA (Pty) Ltd., Cape Town, South Africa).

2.4 Buffers

Table 2.1: Buffers used in this study

Buffer	Components	pH
6x agarose loading buffer	30% (v/v) Glycerol	
	0.25 % (w/v) Bromophenol blue	-
	15% (w/v) ficoll type 400	
10X Orange G loading buffer	60% (v/v) Glycerol	-
	0.25 % (w/v) Orange G dye	
Inoue transformation buffer	55 mM $MnCl_2$.4H2O (w/v)	
	15mM $CaCl_2.2H_2O$ (w/v)	
	250mM KCl (w/v)	6.7
	10mM PIPES (pH 6.7)	
50X TAE	2M TRIS base (w/v)	
	10mM Glacial acetic acid (w/v)	8.0
	0.5 M EDTA (w/v)	
0.5x TBE	45mM Tris-borate (v/v)	8.3
	1mM EDTA (w/v)	
TE	1mM EDTA (w/v)	
	10mM Tris-HCl (pH 8.0)	8.0
1XPCR buffer	100mM Tris-HCl pH 8.8, 10mM KCl, 10mM $(NH_4)SO_4$, 0.1% (w/v), 0.1% (w/v) Triton X-100, 15mM $MgCl_2$	

Cycloheximide was prepared in absolute ethanol and added to the media at a final concentration of 50µg/ml (Porter *et al.,* 1960). Ampicillin was prepared in sterile, double distilled water and added to media at a final concentration of 100µg/ml (Sambrook *et al.,* 1989). Antibiotics were stored at -20 ºC until required for use.

2.5 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 2.2. Stock cultures were maintained at -80 ºC as cell suspensions in 25% (v/v) glycerol (Sambrook *et al.,* 1989).

Table 2.2: *E. coli* strains and plasmid vectors used in this study

Strain or plasmid	Genotype or relevant description	Source or reference
***E. coli* strains**		
XL1-Blue	*recA*1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F' *pro*AB *lacI*^{q} Z∆M15 Tn10 (Tet^{r})]	Stratagene Ltd. (La Jolla, CA, U.S.A)
Plasmids		
pTZ57R/T	Size 2888 bp, *lac* operator, *Lac*Z start codon, phage f1 region, pUC M13 priming sites, 3' - T overhangs, rep (pMB1), Ampr	Fermentas

2.6 Primers

Table 2.3: Primers used in this study for PCR amplification of genes.

Primer name and Sequence (5'-3')	Position on gene	Annealing Temp. (ºC)	Specificity	Reference
S-C-Act-235-a-S-20 CGCGGCCTATCAGCTTGTTG	-	68	Actinomycete 16S rRNA	Stach *et al.* (2003b)
S-C-Act-878-a-S-19 CCGTACTCCCCAGGCGGGG	-	68	Actinomycete 16S rRNA	Stach *et al.* (2003b)
F243 (Act 226-243) GGATGAGCCCGCGGCCTA	226-243	63	Actinomycete and other bacterial 16S rRNA	Heuer, *et al.* (1997)
E9F GAGTTTGATCCTGGCTCAG	9-27	50	Bacteria, universal, 16S rRNA	Farrelly *et al.* (1995)
U1510R GGTTACCTTGTTACGCATT	1510-1492	50	Bacteria, universal, 16S rRNA	Reysenbach & Pace (1995b)
16S-F1 AGAGTTTGATCITGGCTCAG	17F	-	Bacterial, universal, 16S rRNA	Weisberg *et al.* 1991
16S-R5 ACGGITACCTTGTTACGACTT	33R	-	Bacterial, universal, 16S rRNA	Weisberg *et al.* 1991
M13F GTAAAACGACGGCCAGT	598-615	50	-	Yanisch-Perron *et al.* (1985)

Table continued

Primer name and Sequence (5'-3')	Position on gene	Annealing Temp. (ºC)	Specificity	Reference
M13R ATTACCGCGGCTGCTGG	734-751	50	-	Yanisch-Perron *et al.*, (1985)
534R ATTACCGCGGCTGCTGG	534-518	55	Bacteria, universal, 16S rRNA	Muyzer *et al.* (1993)
341F-GC[a] CCTACGGGAGGCAGCAG	341-357	55	Bacteria, universal, 16S rRNA	Muyzer *et al.* (1993)

[a]GC clamp added to primer 5' end: CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG

2.7 Enzymes and kits

Table 2.4: Kits and enzymes used in this study

Kit/ Enzyme	Source
Kits	
FastDNA® SPIN kit for soil (Catalog number 6560-200)	QBIOgene, BIO 101® Systems, Carlsbad, California
Invisorb® Spin Plasmid Mini *Two*	Invitek, Roche
GFX PCR DNA and Gel Band purification Kit (Product code 27-9602-01)	Amersham Biosciences
InsTAclone™ PCR Cloning Kit	Fermentas, Vilnius Lithuania
Enzymes	
RNAse A	Bio Basic, Markham, Ontario, Canada
Proteinase K	Fermentas, Vilnius, Lithuania

All kits supplied with respective buffers.

2.8 Soil sampling from Marion Island terrestrial habitats

Joanne Rapley conducted soil sampling in June 2005 from Marion Island terrestrial habitats, described by Smith *et al.* (2001). The GPS positions and information about the sampling sites were provided and the habitats described (Table 1.1, Section 1.2, Chapter 1). The samples used in this study were from the following 11 terrestrial habitats: MI 1.1 (Coastal Herbfield habitat), MI 1.2 (Coastal Fellfield Habitat), MI 3.3 (Mesic Fernbrake Habitat), MI 4.3 (Pedestalled Tussock Grassland Habitat), MI 5.1 (Cotula Herbfield Habitat), MI 5.2 (Biotic Mud Habitat), MI 5.3 (Biotic Lawn Habitat), MI 6.2 (Mesic Mire Habitat), MI 6.3 (Wet Mire Habitat), MI 6.4 (Mire Drainage Line Habitat) and MI 6.5 (Biotic Mire Habitat).

For each habitat, six 50g topsoil (1-10cm deep) samples were collected using a sterile spatula. These were homogenously mixed at the laboratory in sterile plastic bags. The samples were redistributed into sterile containers and stored at -80ºC until required and at -20 ºC during the period of use.

2.9 Extraction and purification of soil metagenomic DNA

The FastDNA® SPIN kit, based on the bead-beating method was used for soil metagenomic DNA extraction according to the manufacturer's instructions, but eluted in 1XTE buffer (Tris-HCl and EDTA pH 8). The DNA extracts were purified

on PVPP in columns constructed from pipette tips (Berthelet *et al.,* 1996): Caps were removed from 1.5ml and 0.6ml microcentrifuge tubes. The lower parts of the 0.6ml tubes were excised. Filtered 20*µ*l pipette tips were cut approximately 3mm below the filter, placed into 0.6ml tubes, and into the 1.5ml tubes. Suspensions of 200*µ*l autoclaved 50% (w/v) PVPP were placed onto the filters and centrifuged at 2x*g* for 2 min. The step was repeated and an additional centrifugation step included. The columns were washed twice using 150*µ*l of 1XTE buffer and centrifuging at 2x*g* for 2 min. The columns were dried by centrifuging at 34x*g* for 10 min. 50*µ*l DNA extracts were applied onto each column, incubated at room temperature for 2 min and eluted by centrifuging at 600x*g* for 5 min, and further centrifugation at 271x*g* for 10 min. The quality of the DNA was verified using spectrophotometry and on 1% (w/v) agarose gels.

2.9.1 RNAse treatment of genomic DNA

All the genomic DNA samples were treated with 10ng/µl of RNAse A (10mM Tris-EDTA, pH 8.0) and incubated at 37 ºC for 1 h.

2.10 Extraction of genomic DNA from bacterial cultures

Genomic DNA extraction from bacterial cultures was performed according to Sambrook *et al.* (1989). Bacterial were harvested from liquid media by centrifugation at 10 000x*g* for 3 min in 2.0ml microcentrifuge tubes. The pellets

were resuspended in 567μl of 1XTE buffer (Tris-HCl and EDTA pH 8) with 30μl of 20% (w/v) SDS and 6μl of 20mg/ml proteinase K. The final concentrations were 1% (w/v) SDS and 200μg/ml proteinase K in approximately 600μl. The contents were thoroughly mixed and incubated at 37 ºC for 1 hr in a shaking incubator. Thereafter, 100μl of 5M NaCl was added to avoid the formation of a CTAB-nucleic acid precipitate. The contents were thoroughly mixed and 80μl CTAB/NaCl solution (10% (w/v) CTAB in 0.7M NaCl) added. After mixing, the contents were incubated at a 65 ºC for 10 min in a shaking water bath. The cell lysates were extracted using equal volumes of chloroform/isoamyl alcohol (24/1 (v/v)) and centrifuged at 16000x*g* for 5 min. The aqueous phase obtained was extracted using an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1 (v/v)) and phases separated by centrifugation at 16000x*g* for 5 min. Nucleic acids were precipitated by adding 0.6 times volume isopropanol to the aqueous phase in a clean tube. The precipitate was centrifuged at 16000x*g* for 5 min, and resulting pellet washed with 70% (v/v) ethanol, dried at room temperature, and resuspended in 20μl of autoclaved distilled water (sterile distilled water).

2.11 PCR amplification of genes

The PCR amplifications were performed in 0.2ml thin walled tubes using Techne (Techne, Cambridge) and Hybaid (Thermo Hybaid, Ashford, GB) thermocyclers, equipped with heated lids. Different primer sets were required for specific PCR

reactions (Table2.3). The standard 50*µ*l PCR reaction solution contained approximately 100*n*g metagenomic DNA template, 0.5*µ*M of each primer, 200*µ*M of each dNTP (dATP, dTTP, dCTP, dGTP), 1XPCR buffer (100mM Tris-HCl pH 8.8, 10mM KCl, 10mM $(NH_4)SO_4$, 0.1% (w/v) Triton X-100, 2mM $MgCl_2$) and 1U/*µ*l DNA Taq polymerase. The PCR products for all reactions were visualized by electrophoresis on 1% (w/v) agarose gels.

2.11.1.1 Actinobacterial 16S rRNA gene

Actinobacteria were amplified using the primers S-C-Act-878-a-S-19 and S-C-Act-235-a-S-20. The PCR involved an initial denaturation at 94 ºC for 4 min followed by 10 three-temperature cycles of denaturation at 94 ºC for 45s, annealing at 68 ºC for 45s and touching down at -0.5ºC per cycle. Primer extension was at 72 ºC for 1.5 min. Additional 15 three-temperature cycles followed, involving denaturation at 95 ºC for 45s, annealing at 68 ºC for 45s and primer extension at 72 ºC for 1.5 min. The reaction was held at 15 ºC after the final extension step at 72 ºC for 5 min. (Stach *et al.,* 2003b).

2.11.1.2 Bacterial 16S rRNA genes (E9F and U1510R)

The almost complete bacterial 16S rRNA genes were amplified using the primers E9F and U1510R. The PCR involved an initial denaturation at 94 ºC for 4 min followed by 10 three-temperature cycles of denaturation at 94 ºC for 30s,

annealing at 70 ºC for 30s and touching down at -1.8ºC per cycle, and primer extension at 72 ºC for 2 min. An additional 25 three-temperature cycles followed, involving denaturation at 92 ºC for 30s, annealing at 52 ºC for 30s, and extension at 72 ºC for 1.5 min. The reaction was held at 15 ºC after the final extension at 72 ºC for 5 min.

2.11.1.3 Actinomycete 16S rRNA genes

The actinomycete 16S rRNA genes were amplified using the primers F243 and U1510R. The PCR components were: 50*n*g metagenomic DNA template, 0.25*μ*M of each primer, and 2% (v/v) acetamide added to the standard PCR buffer (containing 2mM $MgCl_2$ final concentration). Thermo-cycling conditions were the same as those used for the E9F and U1510R amplification of bacterial 16S rRNA genes.

2.11.1.4 Bacterial 16S rRNA genes (16S-F1 and 16S-R5)

The almost full length 16S rRNA genes were amplified using the primers 16S-F1 and 16S-R5. Thermo-cycling PCR reaction involved initial denaturation at 96 ºC 4 min. Thereafter, 35 three-temperature cycles followed involving denaturation at 95 ºC for 45 sec, primer annealing at 56 ºC for 30 sec and extension at 72 ºC for 2 min. The reaction was held at 15 ºC after the final extension at 72 ºC for 7 min.

2.11.1.5 Bacterial 16S rRNA genes (341F-GC and 534R)

The bacterial 16S rRNA genes were also amplified using the primers 341F-GC and 534R. Thermo-cycling conditions were the same as those used for the E9F and U1510R amplification of bacterial 16S rRNA genes.

2.11.2 Nested PCR-DGGE (341F-GC and 534R)

All the primary PCR products were diluted 100 times and 80*p*g of template DNA amplified using primers 341F-GC and 534R for the nested PCR-DGGE. Thermo-cycling conditions were the same as those used for the E9F and U1510R amplification of bacterial 16S rRNA genes.

2.11.3 Colony PCR (M13F and M13R)

Metagenomic library clones with correct insert were identified by screening using colony PCR with the M13 primers in (Yanisch-Perron *et al.,* 1985). The PCR components were adjusted to 0.4*µ*M of each primer and 160*µ*M of each dNTP. Template DNA was added by pipetting minute clone material into the PCR solutions and lysing cells by extending the initial denaturation at 94 ºC to 10 min during the thermo-cycling reaction. Thereafter, 20 three-temperature cycles followed involving denaturation at 94 ºC for 30 sec, primer annealing at 52 ºC for 30 sec and extension at 72 ºC for 1 min. The reaction was held at 15 ºC following final extension was at 72 ºC for 7 min.

2.12 Analytical procedures

Spectrophotometry and agarose gel electrophoresis were used to verify the quality and quantity of the DNA in this study. The reference DNA fragments were developed from PCR amplification of known 16S rRNA genes or from the digestion of λ DNA with *Pst*I restriction enzyme.

2.12 .1 Spectrophotometry

The DNA concentration (calculated as OD_{260nm} x 50*ng*/µl) and purity (ratio OD_{260nm}/ OD_{280nm}) were measured using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, USA). The solvent used for DNA elution was the reference sample for the determination of the DNA spectrophotometric parameters.

2.12.2 Agarose gel electrophoresis

DNA fragments were separated on TAE agarose gels by electrophoresis in 1% (w/v) TAE (40mM TRIS base (w/v), 0.2mM Glacial acetic acid (w/v), 10mM EDTA (w/v), pH 8.0) buffer at 5 volts per cm of electrode separation. Alternatively, TBE agarose gels in 0.5% (w/v) TBE buffer (45 mM Tris-borate (v/v), 1mM EDTA (w/v), pH 8.3) were used for electrophoresis. All gels were containing 0.05µg/ml EtBr for staining DNA (Sambrook *et al.,* 1989). The DNA fragments were visualised and gel images captured under UV illumination using the Alphaimager

3400 Imaging System (AlphaInnotech Corporation™ San Leandro, CA). DNA fragments of known concentrations and sizes were used as reference markers.

2.13 Gel extraction and purification of DNA fragments

DNA fragments were briefly visualized under UV illumination, using the long wavelength, and excised from agarose gels using sterile scalpels. The GFX PCR DNA and Gel Band purification Kit was used to purify the DNA according to the manufacturer's instructions. The DNA was eluted in 10mM Tris-buffered, double distilled, sterile water at pH 8.0.

2.13.1 Desalting of gel-purified products

Excess salts were removed from DNA solutions by adding 1/10 volume of 3M sodium acetate (pH 5.2), mixing and adding 2.5 volume ethanol. The solutions were mixed by inverting the microcentrifuge tube several times, incubated at -80 ºC for 10 min and centrifuged at 16000x*g* for 10 min. The pellets were washed using 500*µ*l absolute ethanol and centrifuged at 16000x*g* for 2 min. After removing ethanol, the tubes were dried at room temperature and pellets resuspended in 10mM Tris-buffered, double distilled, sterile water at pH 8.0.

2.14 DGGE

20µl of nested PCR-DGGE products (approximately 500ng DNA) were separated by DGGE as described by Muyzer *et al. (*1993). The DGGE plates were thoroughly cleaned using methanol, ethanol and double-distilled water (Millipore), and dried before each use. Urea-formamide (Fluka) gel denaturing gradients were developed using the Bio-Rad Gradient-former (Bio-Rad, Hercules, USA). 0.5% (w/v) APS and 0.02% (v/v) TEMED were added to the acrylamide:bisacrylamide (37.5:1(w/w)) solutions, as catalysts for gel polymerization prior to gradient development. Actinobacterial DNA was separated on 45-80% and bacterial DNA on 30-80% urea-formamide gradients respectively (A 100% urea-formamide solution contains 7M urea and 40% (v/v) formamide). DGGE was performed using the Scie-Plas (V20-HCDC) Vertical Electrophoresis apparatus (www.scie-plas.com) on 16.5mm x 16.5mm x 1mm thick 9% (w/v) polyacrylamide gels, at 100V for and 60 ºC for 16 h in 1XTAE buffer (40mM TRIS base (w/v), 0.2mM Glacial acetic acid (w/v), 10M EDTA (w/v)). After electrophoresis, the gels were stained using 0.5µg/ml EtBr in 1XTAE for 15 min and destained in 1XTAE for 30 min before visualizing and capturing the image using the Alphaimager 3400 Imaging System UV transilluminator (AlphaInnotech Corporation™ San Leandro, CA).

2.14.1 Statistical analysis of DGGE patterns

The AlphaEase FC image processing and analysis software (AlphaInnotech Corporation™ San Leandro, CA) was used to analyze the community profiles of DNA fragments generated using DGGE. The resulting nominal data was suitable for statistical analysis and coded for the presence (coded 1) or absence (coded 0) of a DNA fragment. PCA was conducted using S and S plus programming languages (Spector, 1994; Venables & Ripley, 1996) with R software, version 2.6.1 (2007) (The R Foundation for Statistical Computing, http://www.r-project.org/). All the environmental data for the habitats analyzed was first transformed using log(x+1) (where x is an absolute value) (Appendix A2). In order to follow a normal distribution function, the data for each variable was normalized. This was achieved by dividing the difference between each value and variable mean (x-mean) with the variable standard deviation to achieve a variance of 1.0. The transformed data and eigenvalues for PCA analysis are shown in Appendix A2. The first three principal components, which explained up to 75% of the variability, were selected for the biplots using the eigenvalues obtained.

The software PRIMER5 (Clarke and Warwick, 2001) was used for hierarchical clustering, multidimensional scaling (MDS) analysis and correlation analysis. The correlation analysis was based on the inbuilt function, BIOENV (Biota-Environment matching). For BIOENV analysis, the biological data was first

transformed using the Bray-Curtis similarity matrix and compared to an environmental data matrix. The SIMPER (Similarity percentages-Species contributions) function of the software was used to identify the similarities between environmental variables and biological characteristics and the contributions of species. A cut-off value of 90% was considered for the SIMPER analysis. Hierarchical clustering was conducted using the Unpaired Group Mean Weight Average (UPGMA, average) pairing based on the Euclidean distance methods. MDS analysis was based on the Bray-Curtis similarity of the original data. The proportion of each genospecies identified in the metagenomic clone library (a separate genotype on DGGE) was used for the calculation of diversity indices. The Shannon-Wiener diversity indices (H) were calculated using the formula, $H = -\sum p_i \ln p_i$ (from $i = 1$ to $i = S$), where i = species, S = the total number of genospecies in the community (richness); p_i = proportion of S made up of the ith genospecies (the number of clones in each genospecies group divided by the total number of clones in each library) and $\ln p_i$ = natural logarithm of p_i. The Shannon-Wiener equitability (E_H) (evenness) was calculated as $E_H = H/H_{max} = H/\ln S$ (Begon *et al.,* 1996). Multidimensional scaling ordination was based on the Bray-Curtis similarity matrix of the original data and 10 iterations.

2.15 Cloning of DNA fragments-preparation of metagenomic libraries

Ligation reactions were performed in 0.2ml tubes containing 10*µ*l volumes using the pTZ57R/T. The reaction mixtures consisted of approximately 75*n*g DNA in a 3:1 molar ratio to plasmid vector, 1X ligation buffer and 1U T4-DNA ligase, supplied by manufacturer. The contents were mixed and centrifuged for 2 s before incubating at 16 ºC overnight.

2.15.1 Preparation of competent cells

Competent *E. coli* XL1-Blue cells were prepared and transformed according to the Inoue Transformation method Sambrook *et al.* (1989). An *E. coli* XL1-Blue culture was streaked onto SOB agar and incubated at 37℃ for 20 h. A pre-culture was prepared by inoculating the resulting pure, single colonies into 25ml of SOB broth, supplemented with 10mM of $MgCl_2$, in a 250ml conical flask. The cells were grown by incubating at 37 ℃ and 250 rpm for 6-8 hours. Different volumes of the pre-culture were inoculated into 250ml of SOB broth supplemented with 10mM $MgCl_2$ in 1000ml conical flasks, and incubated overnight at 18-22 ℃ and 120 rpm. The cells were chilled on an ice bath for 10 min on reaching log phase growth ($O.D_{600nm}$ between 0.4 and 0.6), and harvested by centrifugation at 2 500x*g* for 10 min at 4℃. The medium was poured off and traces removed by adsorbing onto a stack of paper towel for 2 min. The cells were gently resuspended in 80ml ice-cold Inoue transformation buffer (55 mM

$MnCl_2$.4H2O (w/v), 15mM $CaCl_2$.2H_2O (w/v), 250mM KCl (w/v), 10mM PIPES (pH 6.7) whilst swirling on ice, and centrifuged at 2 500x*g* for 10 min at 4°C. The buffer was poured off and traces removed by adsorbing onto a stack of paper towel for 2 min. The cells were gently resuspended in 5ml ice-cold Inoue transformation buffer on ice, and 375*µ*l DMSO added. Working quickly, 50*µ*l cell suspensions were aliquoted into 1.5ml microcentrifuge tubes on an ethanol bath cooled at -80 °C. The competent cells were kept at -80 °C until required for use.

2.15.2 Transformation of competent *E. coli* cells

The competent *E. coli,* XL1-Blue cells were transferred into chilled 1.5ml Eppendorf microcentrifuge tubes containing approximately 25*n*g ligated DNA. The mixtures were incubated on ice for 25 min, transferred into a water bath at 80 °C for 90 sec and incubated on ice for 2 min before adding 950*µ*l of LB broth to allow for cell recovery. The transformed cells were incubated at 37 °C and 120 rpm for 1 h and 100*µ*l aliquots plated onto LB agar plates supplemented with ampicillin (100µg/ml), and IPTG (20*µ*g/ml) and X-gal (30*µ*g/ml). The plates were incubated overnight at 37 °C until colonies were visible for screening recombinants (insertional inactivation of β-galactosidase).

2.15.3 Screening of metagenomic libraries

2.15.3.1 DGGE screening

The insertional inactivation of the *lac Z* genes of the plasmid cloning vectors enabled the identification of recombinant clones, which were white colonies. The colonies were subjected to colony-PCR to identify if they contained the correct size of recombinant DNA fragment. This was followed by nested PCR-DGGE and separation using a urea-formamide denaturing gradient. Fragments migrating with different R_f values were considered genetically different. The clones containing these different genotypes were re-amplified using PCR-DGGE and mixed to form a composite reference DNA standard. This was used as an aid to further screen (probe) and de-replicate each library. Each different DGGE fragment was assumed to represent a bacterial genospecies.

2.16 Sequencing

DNA sequencing of plasmid DNA was carried out at the University of Stellenbosch and University of Cape Town (UCT) using the M13F and M13R oligonucleotide primers. Sequencing was conducted using the Hitachi 3730xl DNA Analyzer (Applied Biosystems) using the Big Dye Terminator v3.1 system that is based on the Sanger method. The sequencing of gel-purified DNA fragments of the bacterial 16S rRNA gene PCR amplification was carried out with primers 16S-F1 and 16S-R5 and at the University of Cape Town. Sequencing

was conducted using the 3130 Genetic Analyser and Big Dye terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and BIOLINE Half Dye Mix. The sequencing was based on the Sanger method.

2.17 Phylogenetic analyses

The program CHECK-CHIMERA was used to inspect the Sequences for inverted tandem repeats. Sequence alignments and editing were done using the program Bio-Edit and phylogenetic trees constructed using *MEGA 4* (Tamura *et al.,* 2007). The trees were based on the Maximum Composite Likelihood method and substitution model using Neighbor-Joining. A 1000 bootstraps of replicates were used with pair-wise deletion of gaps. Substitutions included transitions and transversions, and the pattern among lineages was assumed to be homogeneous (Felsenstein, 1985; Saitou & Nei, 1987; Tamura *et al.,* 2004). The amplified DNA sequences were identified through homology searches using BLAST against the NCBI non-redundant database.

2.18 Isolation of culturable actinomycetes from soil samples

1.0 g soil samples were asetically added to test tubes containing 10ml of sterile distilled water. The tubes were mixed by vigorous vortexing for 60 s to dislodge bacteria from soil particles. The soil particles were allowed to settle for 3 min and

1.0ml of suspension used for serial dilutions up to 10^{-5} times. All diluted soil suspensions were plated onto HA (Hayakawa *et al.,* 2000), MC (Nonomura & Ohara, 1971), CZ (Waksman, 1957) and 7H9 (Difco) agar plates supplemented with 50µg/ml cycloheximide, at pH 5.0, 5.1 and 7.3-7.4. The plates were incubated for up to three months at 16 ºC in sealed plastic bags and inspected weekly.

2.18.1 Purification and maintenance of cultures

Colonies of different morphology were periodically isolated and purified by streaking onto new agar plates of the same medium they were isolated. Pure isolates were kept on agar plates at 4 ºC. The cultures were sub-cultured from plates into YM broth (pH 7.0) and incubated at 16 ºC and 100-120 rpm for up to 4 weeks until growth was observed. The cells or mycelia were harvested by centrifuging at 1 150x*g* for 10 min and resuspending in fresh YM medium containing 25% (v/v) glycerol. The cultures were maintained at -80 ºC until required for further use.

CHAPTER 3

3.0 Relating habitats to microbiological diversity using statistical analysis

3.1 Aims

The aim of this chapter was to assess the diversity and distribution of bacteria and actinobacteria in selected Marion Island terrestrial habitats. This preliminary assessment was entirely based on the analysis of the microbiological community patterns generated using DGGE without use of sequence data. The DGGE analysis followed soil metagenomic DNA extraction and PCR amplification of the 16S rRNA genes. The DGGE patterns were statistically analyzed using multivariate techniques. The resulting conclusions led to the selection of habitats that were showing microbiological novelty. These were used for culture-independent studies. The environmental factors associated with the microbiological diversity were also identified.

The following 11 Marion Island terrestrial habitats were analyzed in this study: MI 1.1 (Coastal Herbfield habitat), MI 1.2 (Coastal Fellfield Habitat), MI 3.3 (Mesic Fernbrake Habitat), MI 4.3 (Pedestalled Tussock Grassland Habitat), MI 5.1 (Cotula Herbfield Habitat), MI 5.2 (Biotic Mud Habitat), MI 5.3 (Biotic Lawn

Habitat), MI 6.2 (Mesic Mire Habitat), MI 6.3 (Wet Mire Habitat), MI 6.4 (Mire Drainage Line Habitat) and MI 6.5 (Biotic Mire Habitat).

3.2 Background & literature review

The terrestrial habitats of Marion Island are largely undisturbed by human activities. The climate is relatively constant (Smith *et al.,* 2001) and this provides a controlled environment to serve as a model for ecological studies. The habitats are distinct and have been adequately described in terms of geology, soil characteristics, and climate (Smith & Steenkamp, 2001; Smith, *et al.,* 2001) (Appendix A1). This enables comparative ecological studies involving environmental variables and community microbiological diversity. A number of external factors influence the habitats, allowing for the investigation of additional dimensions. The niche space for microorganisms is determined by both biotic and abiotc factors. These continually create opportunities for the survival of well-adapted microorganisms through natural selection. This leads to ecological specialization of microorganisms (Kassen and Rainey, 2004).

The total DNA directly isolated from soil can be used to provide representative information on the diversity of microorganisms within a soil community (Duarte *et al.,* 1998; Marsh, 1999; Picard *et al.,* 1992; Szekely *et al.,* 2008; Tsai & Rochelle, 2001). A variety of soil nucleic acid extraction protocols and kits are optimised to

suit different soil samples (Bürgmann *et al.,* 2001; Holben *et al.,* 1988; Jacobsen & Rasmussen, 1992; More *et al.,* 1994; Roose-Amsaleg *et al.,* 2001; Stach *et al.,* 2001; Steffan & Atlas, 1988; Yeates *et al.,* 1997). However, all protocols involve common steps including cell lysis, separation of nucleic acids from proteins and polysaccharides and the extraction of nucleic acids. The initial step requires chemical and/or physical lysis of cells. DNA purification relies on differential solubility in aqueous and organic phases of solvents and/or adsorption to silica column matrices (Roose-Amsaleg *et al.,* 2001; Tsai & Rochelle, 2001; Yeates *et al.,* 1998). The variations in the methods affects the quality of DNA obtained (Bürgmann *et al.,* 2001; Carrigg *et al.,* 2007; Zhou *et al.,* 1996). The purity of DNA is mostly affected by humic substances and salts, which interfere with applications such as PCR amplification and restriction digestion of DNA (Tsai & Rochelle, 2001).

Genetic fingerprinting techniques include Denaturing Gradient Gel Electrophoresis (DGGE), Thermal Gradient Gel Electrophoresis (TGGE) and Terminal Restriction Fragment Length Polymorphism (T-RFLP). While DGGE uses chemical denaturants, TGGE uses a temperature gradient for denaturing DNA (Heuer *et al.,* 2001; Muyzer & Smalla, 1998) for analyzing microbial communities. T-RFLP is based on the automated analysis of the patterns generated from the digestion of the fluorescently labeled 5′-terminal of a gene sequence using a single restriction endonuclease (Marsh, 1999). Multiplex T-

RFLP involves the use of a number of primer sets to target different genetic markers, followed by restriction endonuclease digestion (MacDonald *et al.,* 2008a).

Muyzer *et al.* (1993) conducted some of the earliest published microbiological community studies using DGGE (Muyzer *et al.,* 1993). The technique has since been applied to microbiological communities from a number of environmental samples. These include marine environments (Diez *et al.,* 2001), polluted waters (Ringbauer *et al.,* 2006) and lakes. DGGE has been used to study soil (Brons & van Elsas, 2008) and soil rhizosphere microbiological communities (Maarit Niemi *et al.,* 2001; Nunan *et al.,* 2005) and may provide information that is useful to agriculturists.

In some cases, the bioremediation of contaminated sites and polluted soils is performed using information gathered from DGGE analysis (Kozdrój & van Elsas, 2001; MacNaughton & Stephen, 2001; Roling *et al.,* 2004). The DGGE analysis of gut (Bibiloni *et al.,* 2006; Kurokawa *et al.,* 2007; Wang *et al.,* 2007) and human breast-milk communities (Delgado *et al.,* 2008) shows its importance in clinical microbiology. Sulphate-reduction (Dar *et al.,* 2005; Dar *et al.,* 2007), ammonia oxidation and nitrogen cycling communities have also been studied using DGGE (Demba Diallo *et al.,* 2004; Kowalchuk *et al.,* 1997; McCaig *et al.,* 1999; Zhang *et al.,* 2008). This contributes to our understanding of the roles of microorganisms in

biogeochemical cycles and nutrient recycling. Other communities studies were performed on peat soils associated with disease (Morgan & Whipps, 2006), corroded water pipes (Vincke *et al.,* 2001), biofilms (Gillan *et al.,* 1998) and microbial mats (Desnues *et al.,* 2007).

Statistical analysis aids in the interpretation of metagenomic community data. A number of multivariate statistical techniques are frequently used in ecological studies of microorganisms. These relate the diversity of microbiological community data to environmental factors (Fromin *et al.,* 2002; Muyzer & Smalla, 1998). A number of ecological studies described microbiological diversity in relation to environmental factors using various multivariate statistical approaches (Table 3.1).

Some of these methods include Principal Component Analysis (PCA), Principal Coordinate Analysis (PCoA), Non-metric Multidimensional Scaling (NMDS) ordination, Significance testing, variance analysis, Redundancy Analysis (RDA), Canonical Correspondence Analysis (CCA), Linear Discriminant Analysis (LDA), Multiple regression and correlation analysis, and Diversity indices (Ramette *et al.,* 2001). The problems and limitations associated with DGGE also affect statistical analysis (Heuer *et al.,* 2001; Ramette *et al.,* 2001).

Table 3.1: Studies involving multivariate statistical analysis of community data generated from microbiological community analysis using DGGE or other genetic fingerprints.

Reference	Description of study
Allgaier *et al.* (2007)	Applied MDS ordination analysis, and Pearson's product moment correlation analysis to DGGE profiles. This was used to compare lake actinobacterial communities in relation to environmental variables (Allgaier *et al.,* 2007).
Mauriello *et al.* (2003)	Used MDS, distance cluster analysis, and PCA to relate the biochemical properties of cheese to the geographic origins of starter cultures identified using DGGE analysis (Mauriello *et al.,* 2003).
Bennett *et al.* (2008)	Used significance testing to assess the sensitivity of T-RFLP and to establish the reliable threshold levels required for identifying key genospecies in soil communities. The study was also used to verify the outcomes using non-metric MDS ordinations of the detected fingerprints (Bennett *et al.,* 2008).
Boon *et al.* (2002)	Investigated DGGE patterns of activated sludge communities using distance cluster analysis, MDS, and PCA (Boon *et al.,* 2002).
Desnues *et al. (*2007)	Compared the spatial differences in denitrifying communities of a microbial mat population generated by DGGE using significance testing (Desnues *et al.,* 2007).
Gafan *et al. (*2005)	Used the Shannon-Wiener diversity index, logistic regression, chi-square test, distance cluster analysis, and significance testing to associate the development of gingivitis (a disease) with the plaque microbiota based on DGGE patterns (Gafan *et al.,* 2005).

Table continued

Reference	Description of study
Dilly *et al. (*2004)	Used the Shannon-Wiener diversity index, significance testing, and PCA to account for the succession during the litter decomposition is soils based on DGGE (Dilly *et al.,* 2004).
Jones & Thies, (2007)	Used PCA and distance cluster analysis to compare soils with different Zn concentrations using a two dimensional DGGE (Jones & Thies, 2007).
Ranjard *et al. (*2003)	Associated the diversity of microorganisms based on the fingerprints generated from the automated ribosomal intergenic spacer analysis (A-RISA). The diversity was associated to soil particle size using PCA. The thresholds of soil quantities required to provide reproducible community fingerprints for bacteria and fungi were statistically determined (Ranjard *et al.,* 2003).
Mathur *et al. (*2007)	Used correlation analysis and PCA to analyze analyzed the 16S rRNA gene sequences of bacteria from acidic thermal springs. Temperature and mineral chemistry were identified as major factors determining bacterial distribution (Mathur *et al.,* 2007).
Zhou *et al. (*2004)	Related the occurrence of specific bacterial groups from different sandy subsurface soils to ecological regions (Zhou *et al.,* 2004).
Park *et al.,* 2006)	Compared the T-RFLP profiles of rhizosphere bacterial communities using PCA and discriminant analysis (Park *et al.,* 2006).
Staddon *et al. (*1997)	Calculated the evenness and diversity of bacteria isolated from the same forest soils using the diversity indices (Shannon, McIntosh, and Simpson), and in addition related the diversity to carbon utilization patterns using PCA (Staddon *et al.,* 1997).

3.2.1 A review of methods used in this study

Cluster analysis methods group objects according their similarities and dissimilarities, based on measures of association. Hierarchical clustering is based on linkage rules such as the nearest or furthest neighbour and Unweighted Paired Mean Group Average methods (UPMGA). However, the resulting clusters are subject to interpretation based on the similarity or dissimilarity distance, which varies. PCA calculates linear combinations of variables that account for the most variability (variance) in the original data set. The data should follow a normal distribution function and thus, be normalized. PCA is based either on a variance-covariance matrix or on a correlation matrix. The correlation matrices rely on standardized data in which variables are independent of the original scales and units. In this case, all variables have equal contribution to variance. Arrows on the orthogonal projection indicate the direction of most variability and angles between the arrows, correlations between the variables. If the data is not standardized, the length of the arrows shows the strength of contribution of each species to variability (Clarke and Warwick, 2001). Unlike PCA, MDS does not require data to be normally distributed. MDS non-linearly ranks the distances between the objects. The distances are presented on a two-dimensional ordination space with similar objects appearing in close proximity. The MDS algorithm involves an iteration of steps to achieve the best goodness of fit characterised by the lowest stress value. MDS is more efficient at identifying the relationships and gradients compared to hierarchical clustering

(Ramette, 2007). The main interpretation of the relationships amongst the Marion Island terrestrial habitats will therefore, be mainly based on MDS analysis. The analysis will be supported by hierarchical clustering and PCA analysis. The clusters identified from hierarchical clustering can be superimposed onto MDS plots (Clarke and Warwick, 2001).

The BIOENV (Biota-Environment matching) function selects environmental variables that best explain the community pattern, by maximizing a rank correlation between their respective similarity matrices. The SIMPER (Similarity Percentages-Species Contributions) function measures the contribution of each species to the average Bray-Curtis dissimilarity between groups of samples and determines the contribution to similarity within a group (Clarke and Warwick, 2001).

In a related study, Smith & Steenkamp (2001) used CCA, cluster analysis and correlation analysis to classify vegetation on Marion Island based on the soil physiochemical properties. In their study, CCA was used to identify the main determinants of soil variability. Hierarchical clustering was used to group the habitats, which were related to vegetation type. The choice, quality, and depth of statistical analysis depend on the objectives of the research (Ramette, 2007). In this study, the genospecies of microbiological communities were analyzed using DGGE and related using hierarchical clustering. None of these previous studies

attempted to identify the genospecies that were similarly distributed in different ecological habitats. The Marion Island soil variables were analyzed using PCA, MDS, correlation-based techniques and hierarchical clustering methods. The habitats were classified according to microbiological diversity.

3.3 Extraction of metagenomic DNA from soil

High quality, high molecular weight metagenomic DNA was extracted using the Bio 101® Fast Prep kit (Fig. 3.1). The protocol involved a combination of bead-beating and chemical methods for cell lysis and DNA extraction. These methods were found to be more effective for more representative community DNA extraction, especially for bacteria that are difficult to lyse such as actinomycetes (Frostegård *et al.,* 1999; Tsai & Rochelle, 2001).

The DNA obtained in this study had absorbance maxima at 260nm, and the $A_{260/280nm}$ ratios varied from 1.6 to 1.8 showing an acceptable purity. The extractions were conducted independently and in triplicate for each soil sample. The resulting DNA yields ranged from 78 to 210ng/µl, equivalent to approximately between 7.8 and 21.0µg of DNA per gram of soil after the Polyvinylpolypyrolidone (PVPP) purification step (Table 3.2).

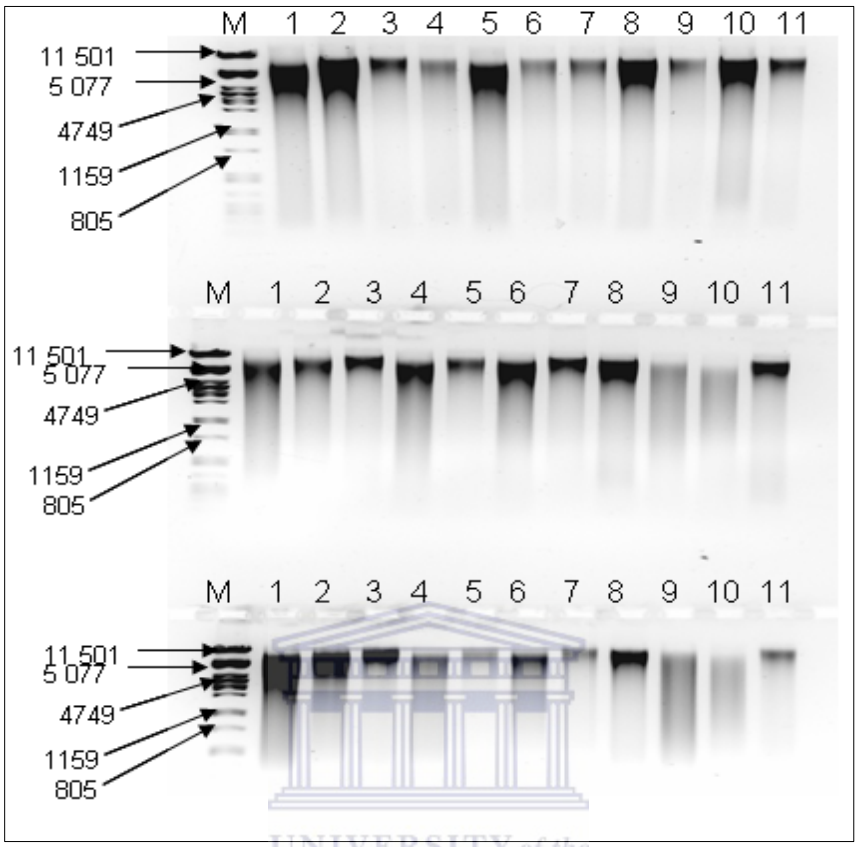


Fig. 3.1: Metagenomic DNA isolated (in triplicate) from soils using the Bio 101® Fast Prep kit. Lanes: M; molecular marker (bp); 1-11, samples from Marion Island terrestrial habitats MI 5.3, MI 5.1, MI 1.2; MI 6.5; MI 6.2; MI 5.2; MI 3.3; MI 1.1; MI 6.4; MI 6.3 and MI 4.3 respectively.

PVPP complexes with phenolic compounds, especially humic substances that are commonly associated with soil DNA. This inhibits PCR reactions (Tsai & Rochelle, 2001; Zhou *et al.,* 1996). The soil humic substances include humic acids, fulvic acids and humins (Tsai & Rochelle, 2001).

The metagenomic DNA yields varied depending on soil sample (Table 3.2), and thus, according to the nature of soil. On average, the lowest DNA yields were obtained from soils of habitat MI 3.3. This habitat occurs on well drained slopes and consists of very peaty soils with low nutrients. The highest yields were obtained from habitats MI 1.2 and MI 5.1. These habitats consist of soils rich in nutrients due to manuring from birds and marine mammals (Smith, *et al.,* 2001).

Table 3.2: Metagenomic DNA yields from Marion Island terrestrial habitat soils.

Sample/ habitat	Average DNA yield and standard deviation (µg of DNA/g soil)
MI 1.1	136 ± 35.9
MI 1.2	190 ± 35.5
MI 3.3	78 ± 29.0
MI 4.3	135 ± 6.4
MI 5.1	210 ± 48.0
MI 5.2	140 ± 36.5
MI 5.3	171 ± 51.2
MI 6.2	118 ± 25.4
MI 6.3	170 ± 13.9
MI 6.4	136 ± 46.1
MI 6.5	122 ± 25.3

3.4 Amplification of the 16S rRNA genes

The Polymerase Chain Reaction (PCR) amplification targeting the 16S rRNA genes as a molecular marker relies on the specificity of the primers. The primers

are designed from the alignment and comparative analysis of representative 16S rRNA gene sequences (Heuer *et al.,* 2001; Hugenholtz & Goebel, 2001).

3.4.1 Amplification of the actinobacterial 16S rRNA gene

The actinobacterial 16S rRNA genes were amplified using the primer set S-C-Act-235-a-S-20 and S-C-Act-878-a-S-19 (Stach *et al.,* 2003b) and yielded 642bp DNA fragments in all samples (Fig. 3.2).

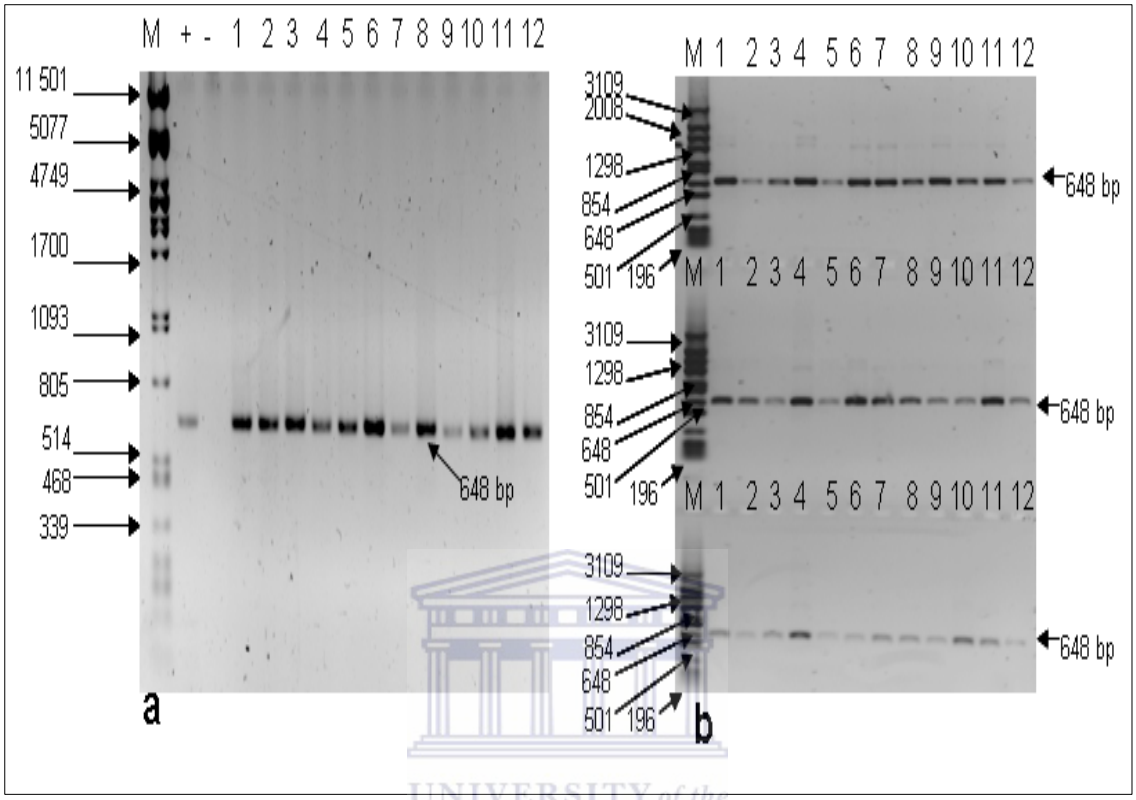


Fig. 3.2: Agarose gel (1%) of PCR products of the 16S rRNA genes amplified using the actinobacterial-specific primers S-C-Act-235-a-S-20 and S-C-Act-878-a-S-19 (a) Unpurified (b) Gel-purified PCR product for each sample in triplicate. Lanes: M, molecular weight marker (bp); +, or 12, positive control for actinobacteria (*Streptomyces albus*); -, negative control. Lanes 1-11: samples from Marion Island terrestrial habitats MI 5.3, MI 5.1, MI 1.2; MI 6.5; MI 6.2; MI 5.2; MI 3.3; MI 1.1; MI 6.4; MI 6.3 and MI 4.3 respectively.

These were suitable for nested PCR-DGGE (Section 3.6) and cloning into plasmid vectors for metagenomic clone libraries (chapter 4, Section 4.3).

3.4.2 Amplification of the actinomycete 16S rRNA gene

Primer F243 was recommended as an additional primer for increasing coverage of actinomycete diversity. This primer is more specific for actinomycetes and not the other groups of actinobacteria (Heuer *et al.,* 1997). In this study, the primer was used in combination with the reverse primer U1510R, which is specific for bacteria (Reysenbach & Pace, 1995a). The PCR amplification resulted in 1267bp DNA fragments in all samples, but products varied in concentration (Fig. 3.3).

The products were adjusted to the same DNA concentrations in subsequent studies to avoid distortions. These products were also found to be suitable for PCR-DGGE (Section 3.6) and cloning into plasmid vectors for metagenomic libraries (Chapter 4, Section 4.3).

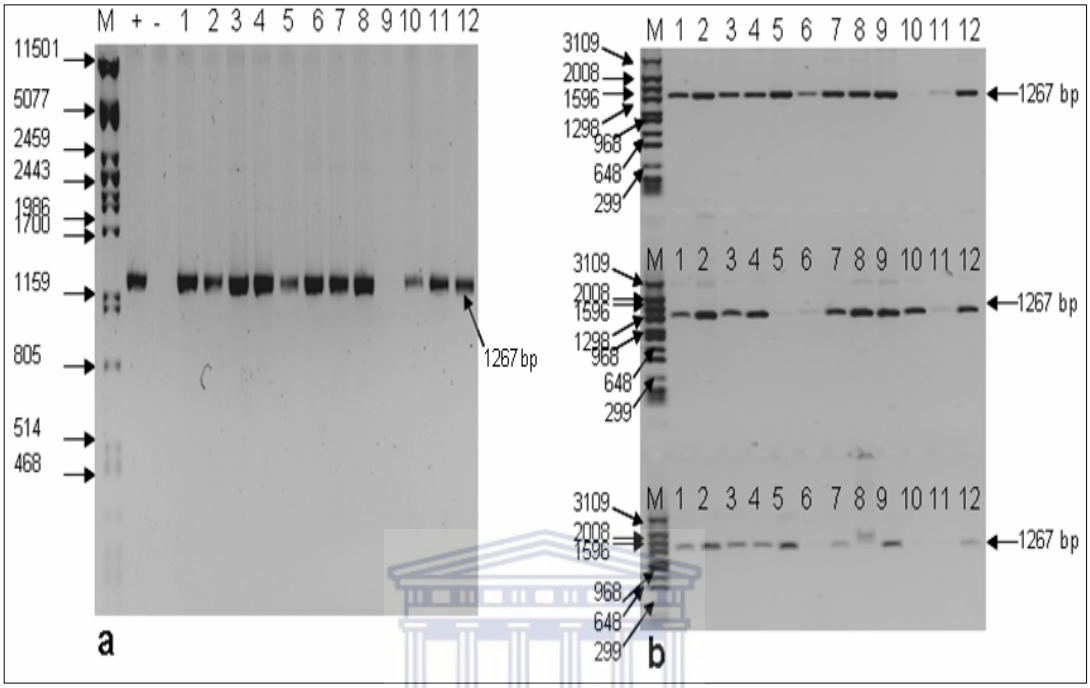


Fig. 3.3: Agarose gel (1%) of PCR products of the16S rRNA gene amplified using the actinomycete-specific primers F243 and U1510R (a) Unpurified (b) Gel-purified PCR product for each sample in triplicate. Lanes: M, molecular weight marker (bp); + and 12, positive control for actinomycetes (*Streptomyces albus*); -, negative control; 1-11, samples from Marion Island terrestrial habitats MI 5.3, MI 5.1, MI 1.2; MI 6.5; MI 6.2; MI 5.2; MI 3.3; MI 1.1; MI 6.4; MI 6.3 and MI 4.3 respectively.

3.4.3 Amplification of the bacterial 16S rRNA gene

The primer set 341F-GC and 534R (Muyzer *et al.,* 1993) was used to directly amplify the bacterial 16S rRNA gene from metagenomic DNA. This resulted in a 196bp product, suitable for DGGE analysis (Fig. 3.4).

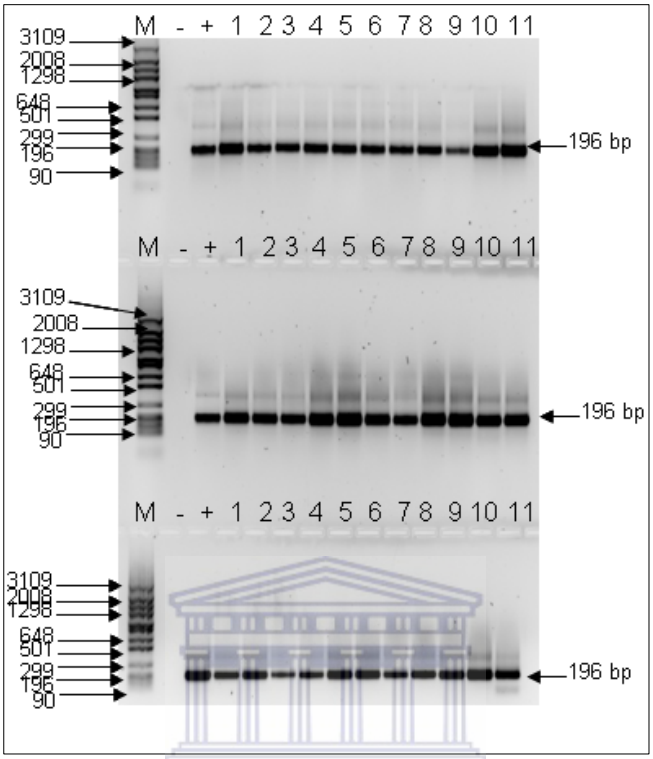


Fig. 3.4: Agarose gel (2%) of PCR products of the 16S rRNA gene amplified using the of bacterial-specific primers 341F GC and 534R. Each sample was independently amplified in triplicate. Lanes: M, molecular weight marker (bp); +, positive control for bacteria (*Streptomyces albus*); -, negative control; 1-11, samples from Marion Island terrestrial habitats MI 5.3, MI 5.1, MI 1.2; MI 6.5; MI 6.2; MI 5.2; MI 3.3; MI 1.1; MI 6.4; MI 6.3 and MI 4.3 respectively.

Initially, the primers E9F (Farrelly *et al.,* 1995) and U1510R (Reysenbach & Pace, 1995b) were used to amplify the full length bacterial 16S rRNA gene. This resulted in a product of the expected size (1501bp). However, the product was

not suitable for nested PCR-DGGE (section 3.5), hence primers 341F-GC and 534R were used.

3.5 Evaluating primers for producing suitable PCR-DGGE templates

The primary PCR products obtained using the specific primers were evaluated for suitability as templates for nested PCR-DGGE. They were expected to produce a product that could be reliably used for DGGE fingerprinting. The nested PCR-DGGE products obtained using templates generated from metagenomic DNA amplification resulted in 196bp fragments as expected (Fig. 3.5a). However, PCR-DGGE of templates initially generated from the amplification of metagenomic DNA using primer set E9F and U1510R produced two fragments. One of the fragments was slightly larger than 200bp and the other, approximately 1700bp. These products were probably PCR artifacts, possibly chimeras. They may have resulted from reannealing of incompletely transcribed 3'-ends to primers or other products (Heuer *et al.,* 2001; Yeates *et al.,* 1997). They were therefore, unsuitable for community analysis because they produced more than one DNA fragment on DGGE (Fig. 3.5b).

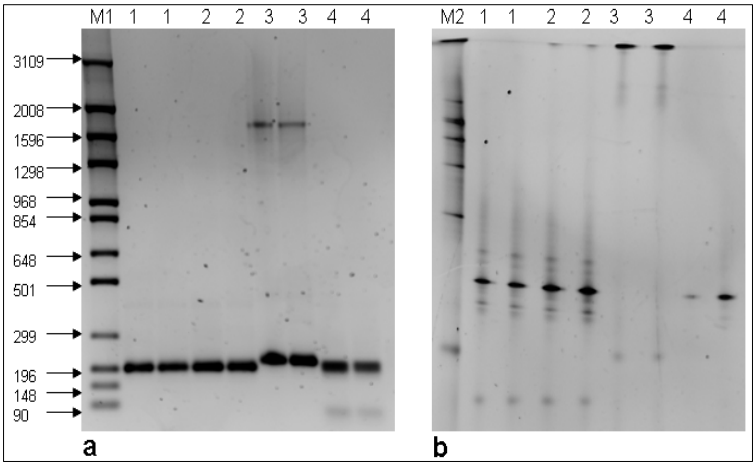


Fig. 3.5: PCR-DGGE products generated by primer sets specific for the actinobacterial, actinomycete and bacterial 16S rRNA genes, using *Streptomyces albus* as the positive control (a) on agarose gel (2%), and (b) on DGGE polyacrylamide gel (30-80% denaturing gradient). Lanes: M1, molecular weight marker (bp); M2, DGGE marker; 1, straight metagenomic DNA amplified using primers 341F GC and 534R for bacteria; 1-4: Nested PCR-DGGE products using primers 341F GC and 534R of PCR products. amplified from the metagenome using primers: 2, S-C-Act-235-a-S-20 and S-C-Act-878-a-S-19; 3, E9F and U1510R; and 4, F243 and U1510R.

The direct PCR-DGGE of metagenomic DNA using the universal bacterial 341F GC and 534R primers did not show such artifacts. Similarly, PCR-DGGE on products generated from the amplification of metagenomic DNA using all the other primer sets were suitable for community analysis.

3.6 Nested PCR-DGGE of metagenomic 16S rRNA genes

The PCR products amplified from metagenomic DNA were diluted to an equal concentration (40*p*g/µl) and 80*p*g used for PCR-DGGE. The nested primer set (341F-GC and 534R) was used for nested PCR-DGGE amplification. In all cases, fragments of the expected size (196bp) obtained (Fig. 3.6) were used for DGGE microbial profiling (Muyzer *et al.,* 1993).

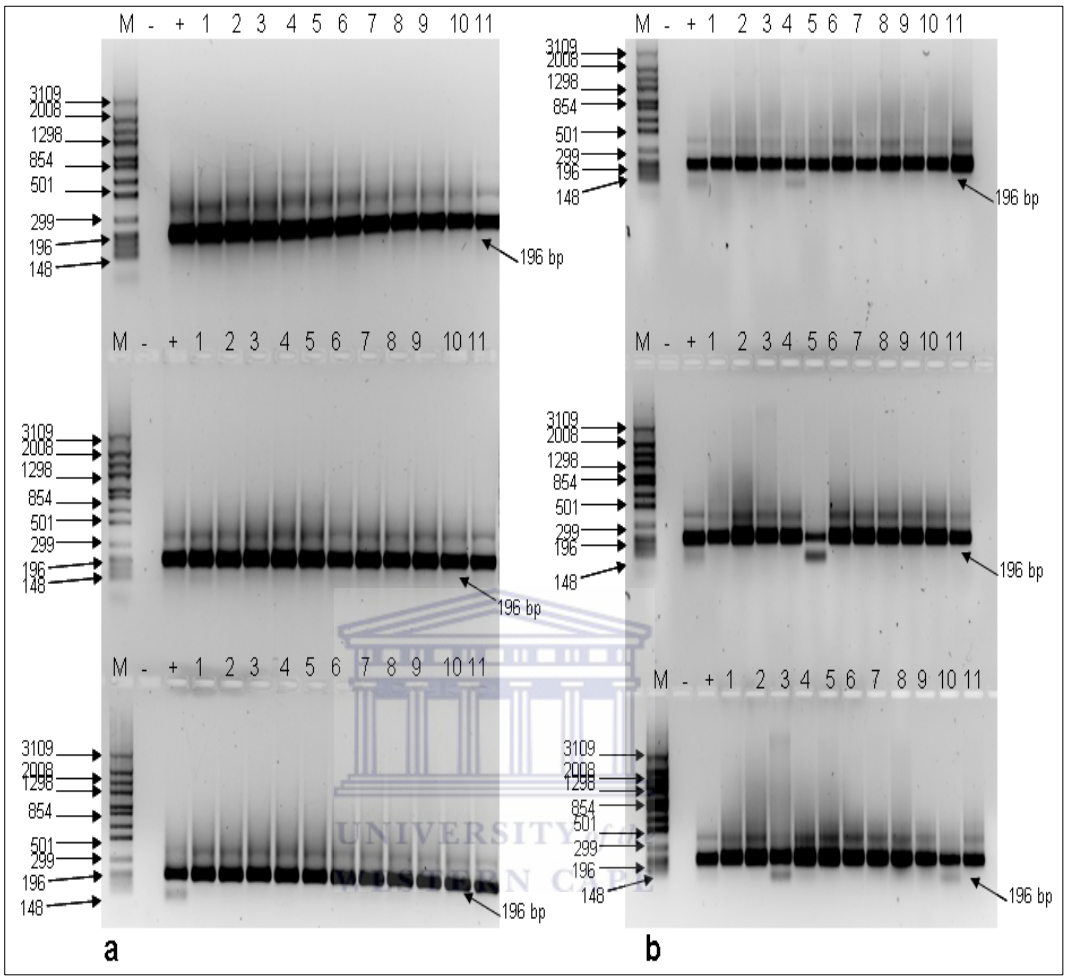


Fig. 3.6: Agarose gel (2%) of nested PCR-DGGE products amplified using the primers 341F GC and 534R shown in triplicate for each sample (a) nested PCR of actinobacterial 16S rRNA gene generated by primers S-C-Act-235-a-S-20 and S-C-Act-878-a-S-19 and (b) of actinomycete 16S rRNA gene generated by primers F243 and U1510R. Lanes: M, molecular weight marker (bp); +, positive control (*Streptomyces albus*); -, negative control, 1-11, samples from Marion Island terrestrial habitats MI 5.3, MI 5.1, MI 1.2; MI 6.5; MI 6.2; MI 5.2; MI 3.3; MI 1.1; MI 6.4; MI 6.3 and MI 4.3 respectively.

3.7 Analysis of habitat soil characteristics

In this study, the Marion Island soil characteristics (Smith, *et al.,* 2001) (Appendix A2) were statistically transformed in order to identify the physiochemical relationships amongst the habitats and their influence on the microbiology. The analysis included use of PCA, MDS, BIOENV, SIMPER and hierarchical clustering.

3.7.1 Relating habitats based on soil and plant characteristics

The data on soil physiochemical properties and the contributions of each group of plants to percentage surface cover were analyzed in order to identify related habitats. The findings were used to explain the factors that influenced the distribution of microorganisms in these habitats. MDS, SIMPER and hierarchical clustering were used for this analysis.

3.7.2 Habitat relatedness using soil physiochemical variables

Hierarchical clustering of the soil characteristics dataset produced 4 habitat clusters (Fig. 3.7). The habitats from the Mire and Coastal salt-Spray Complexes clustered together as expected. Habitats MI 1.1 and MI 1.2 formed an independent cluster (Cluster hab A) that was well distinct from other habitats.

Habitat MI 5.1 closely related to these habitats, but formed separate and independent cluster (Cluster hab B1). Habitats MI 6.2, MI 6.3 and MI 6.5 (Cluster hab B2) and MI 3.3, MI 6.4, MI 5.2, MI 5.3 and MI 4.3 (Cluster hab B3) belonged to the same major branch.

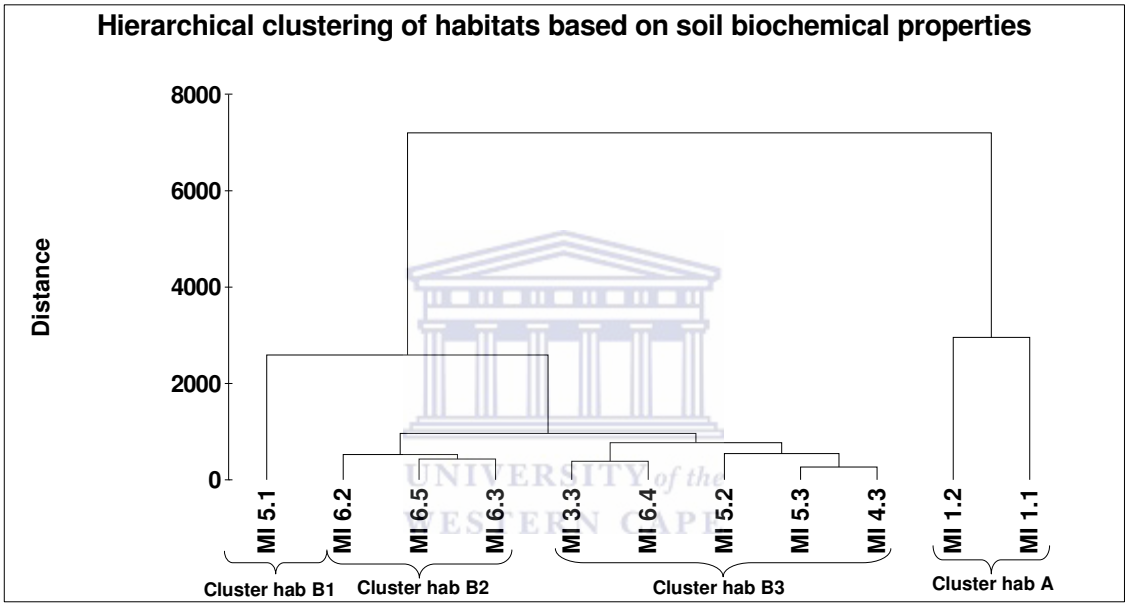


Fig. 3.7: Hierarchical clustering of Marion Island terrestrial habitats based on soil physiochemical properties (Smith, *et al.,* 2001). Dendogram drawn based on the Hierarchical clustering, average/UPGMA (Unpaired Group Mean Weight Average) and Euclidian distance methods using PRIMER5.

The clusters identified using hierarchical clustering of soil characteristics (Cluster hab A, (Cluster hab A and Cluster hab B or Cluster hab B1, Cluster hab B2 and Cluster hab B3) were superimposed onto MDS ordination plots (Fig. 3.8).

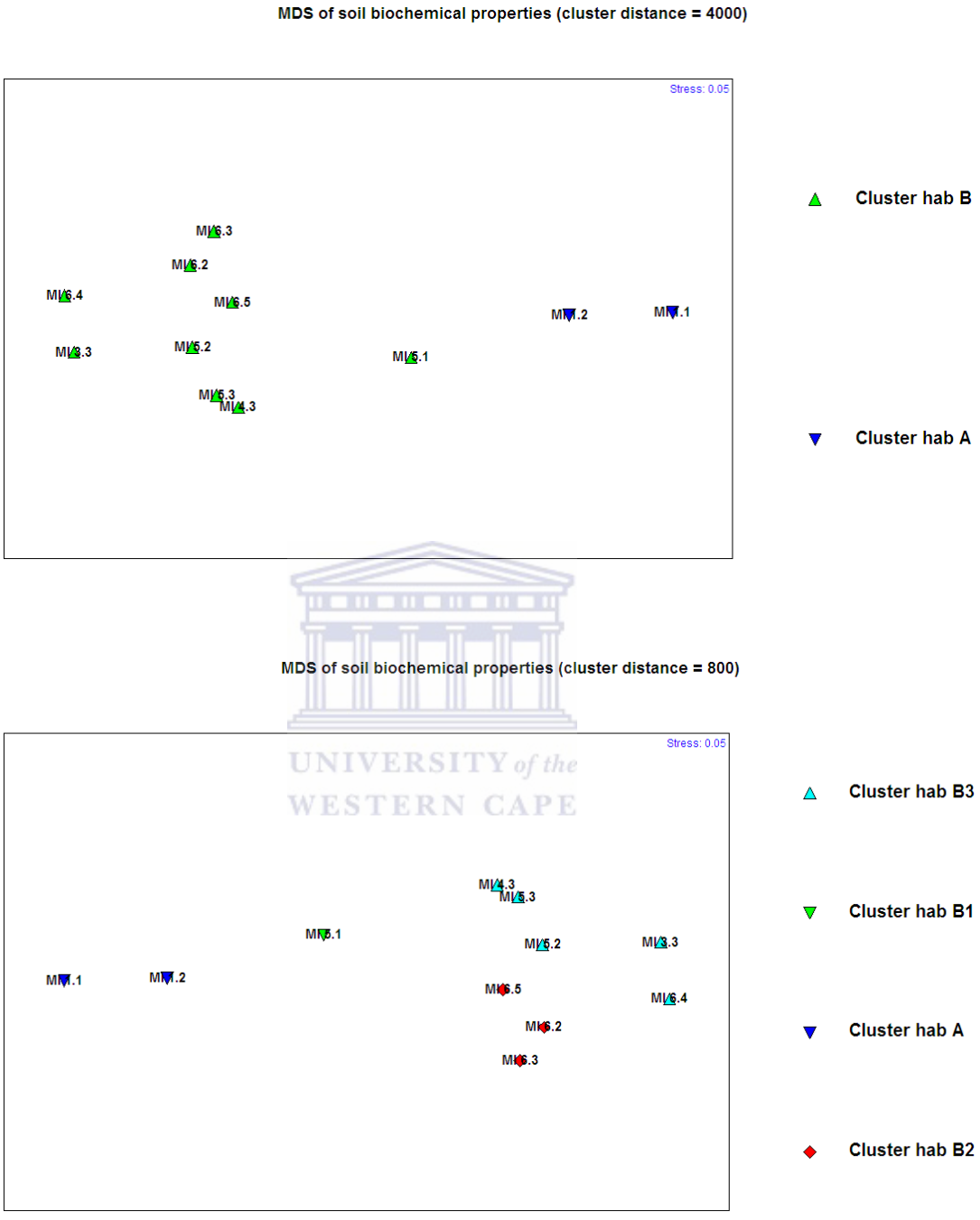


Fig. 3.8: MDS ordinations of relationships amongst Marion Island terrestrial habitats based on the average absolute values of soil biochemical parameters (Smith, *et al.,* 2001). Cluster hab A, B1, B2 and B3 are superimposed hierarchical clusters.

MDS showed that even though habitats MI 1.1 and MI 1.2 were similar in soil characteristics, they were distant from each. They separated from a major group of related habitats that comprised of the rest of the habitats, except for habitat MI 5.1.

SIMPER analysis showed that the clusters identified consisted of habitats with similarities in soil characteristics ranging between 72 and 86% (Appendix A4). Moisture and soil solution sodium contributed up to 65% of the similarity within Cluster hab B3. Solution sodium contributed to 80% of the similarity of habitats in Cluster hab A. Moisture contributed 46%, and up to 86% similarity of habitats in Cluster hab B2 together with soil solution sodium and solution potassium. In addition, Cluster hab A and Cluster hab B2 showed the highest dissimilarity (66%) compared to the other pairs of habitat clusters.

The dissimilarities between the pairs of habitat clusters were arising from their differences in soil solution sodium and moisture. Soil solution sodium accounted for 35% of the dissimilarity between the soil characteristics of habitats in Cluster hab B1 and B3, 61% between Cluster hab A and B3, 32% between Cluster hab A and B1, 28% between Cluster hab B1 and B2, and 55% between Cluster hab A and B2. Moisture accounted for 55% of the dissimilarity between Cluster hab A and Cluster hab B2.

3.7.3 Habitat relatedness based on plant species (percentage cover)

Hierarchical clustering based on plant percentage cover produced two major habitat clusters, Plant cluster AB and Plant cluster CD. These produced 4 habitat clusters at a lower measure of similarity distance (Fig. 3.9).

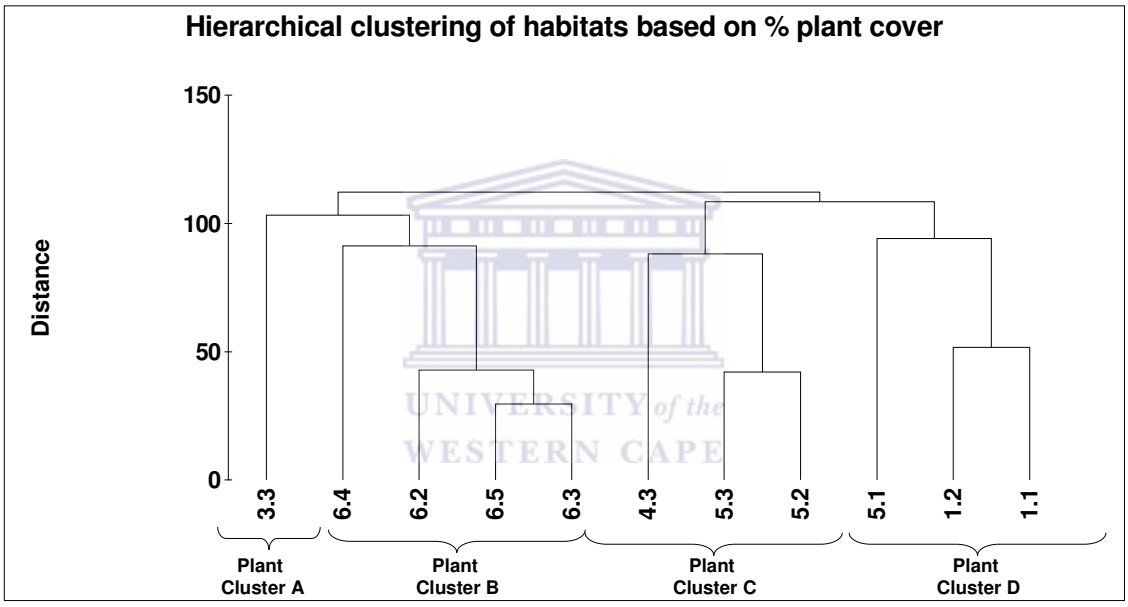


Fig. 3.9: Hierarchical clustering of Marion Island terrestrial habitats based on percentage plant cover (Smith, *et al.,* 2001). Dendogram drawn based on the Hierarchical clustering, average/UPGMA (Unpaired Group Mean Weight Average) and Euclidian distance methods using PRIMER5.

Similarly, as the clusters obtained using the soil physiochemical variables, habitats from the Mire Complex clustered together as expected (Plant cluster B).

Habitats MI 1.1, MI 1.2 and MI 5.1 belonged to the same cluster (Plant cluster D). Habitat MI 3.3 formed an independent cluster (Plant cluster A) whilst habitats MI 4.3, MI 5.2 and MI 5.3 formed the other cluster (Plant cluster C). These clusters resembled the relationships obtained using soil physiochemical variables.

MDS ordination based on the superimposed hierarchical clusters (Plant cluster AB and Plant cluster CD or Plant cluster A, Plant cluster B, Plant cluster C and Plant cluster D) showed that the habitats in Plant cluster CD were more scattered compared to those in Plant cluster AB (Fig. 3.10). This showed that the habitats of the Mire Complex were very similar in plant composition as in soil physiochemical properties. The habitats in Plant cluster CD resulted in the subcluster Plant cluster C in which habitats MI 4.3, MI 5.2 and MI 5.3 were not closely clustered. Plant cluster B and Plant cluster D identified using hierarchical clustering each consisted of habitats that grouped together on MDS ordination.

SIMPER analysis showed that Plant cluster AB consisted of habitats that were 67% similar in plant characteristics (Appendix A5). Plant cluster CD consisted of habitats that had 53% similarity in plant characteristics. Total plant cover contributed 79% of the similarity whilst the presence of Mat Dicots and Rosette

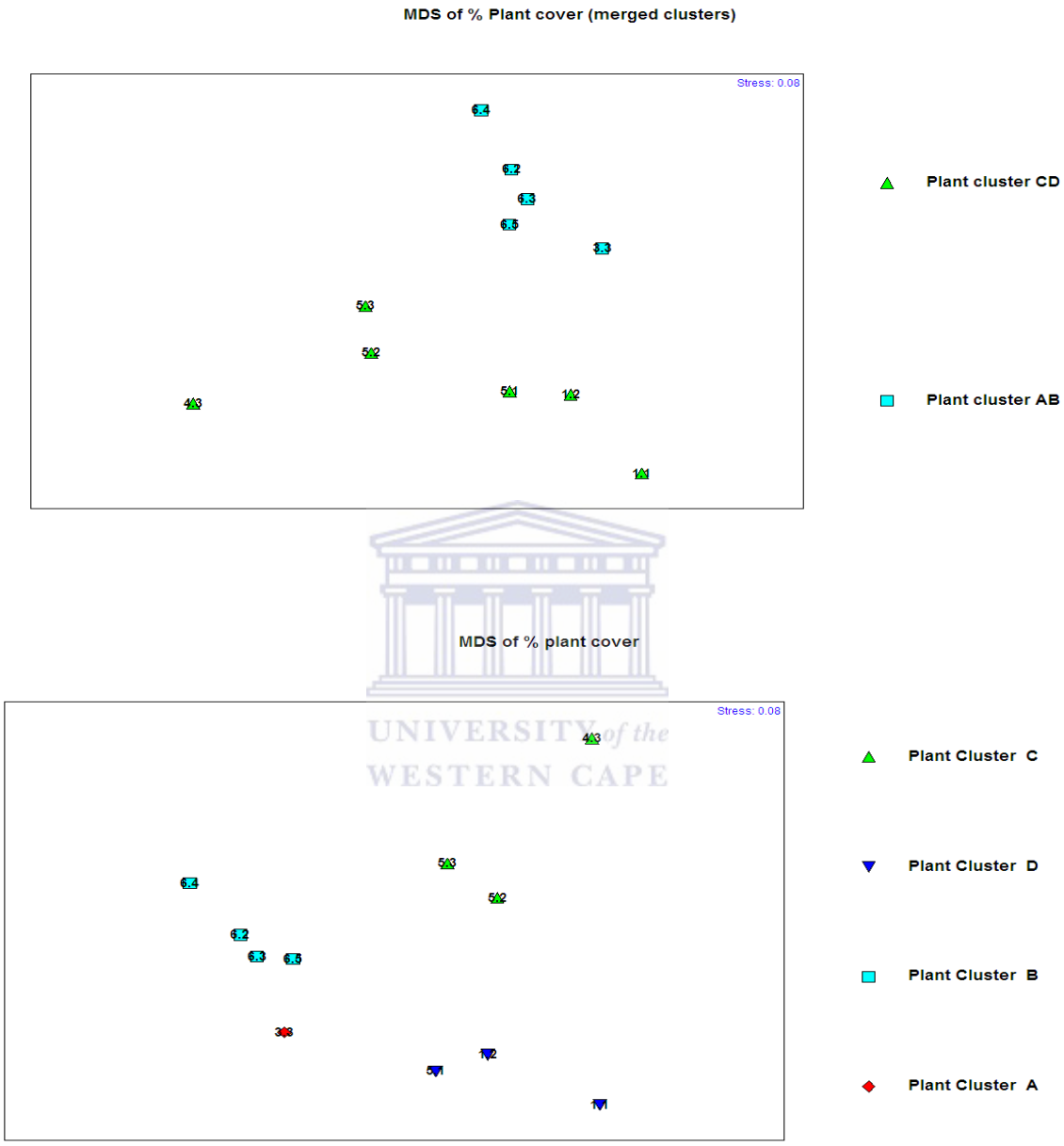


Fig. 3.10: MDS ordinations of relationships amongst Marion Island terrestrial habitats based on percentage plant cover (Smith, *et al.,* 2001). Plant Cluster A, B, C, D, AB and CD are superimposed hierarchical clusters.

Dicots also made notable marginal contributions to similarity (10% and 5% respectively). The habitats the Plant clusters B, C and D showed 64% to 74% similarities in characteristics.

Total plant cover was the most important factor in determining similarities amongst habitats within same clusters. Total plant cover and the presence of the Mat Dicot contributed to 93% of the similarity in Plant cluster C. The total plant cover (67% similarity), Mire Bryophytes (20% similarity) and Mire graminoids (12% similarity) contributed a total 99% similarity of habitats in Plant cluster B. Total plant cover (77%), Erect Dicot (12%) and Rose Dicot (11%) contributed a total 99% similarity in plant characteristics of habitats in Plant cluster D.

The dissimilarities between pairs of habitat clusters ranged between the pairs of habitat clusters based on plant characteristics varied and were caused by different plant species. The dissimilarities between Plant Clusters AB and CD was due to the mire bryophytes (10%), between Plant Cluster C and D to mat dicots (18%) and erect dicots (11%) and between Plant Cluster C and B to mire bryophytes (13%) and erect dicots (10%). The dissimilarities between Plant Cluster B and D were due to mire bryophytes (13%) and erect dicots (10%), between Plant Cluster A and C to pteridophytes (19%) and mat dicots (17%), between Plant Cluster D and A to pteridophytes (19%), and between Plant Cluster A and B to pteridophytes (15%).

3.7.4 Identifying the major determinants of soil variability

PCA analysis resulted in the identification of the major physiochemical parameters that were responsible for most of the variability observed in the data set of soil physiochemical properties. The contributions of each component to variability and eigenvalue loadings (Appendix A2) were used to construct the biplots. The first three components explained 77% of the variability, which is more than the 70-75% cumulative variability recommended for capturing most of the variability in a dataset. The dimensions used for the biplots were based on the first principal component because it explains the maximum variability of the data (42%), shown by maximum separation of the eigenvalues. Moisture and nutrients (organic and inorganic) showed negative eigenvalues whilst pH, C.E.C, bulk density and all the salts showed positive eigenvalues in the first principal components. A biplot of the first two principal components (PCA 1 vs PCA 2) showed distinct separation of habitats. The major groups were defined by high levels of different forms of all salinity indicators (habitats MI 1.1 and MI 1.2), high concentrations of nutrients (habitats MI 5.1, MI 5.2, MI 5.3 and MI 4.3b), high moisture content (habitats MI 6.2, MI 6.3 and MI 6.5) and high cation exchange capacity (habitats MI 3.3 and MI 6.4) (Fig. 3.11). The high salinity indicators resulted in the maximum separation of habitats MI 1.1 and MI 1.2 from the rest of the other habitats. In addition, habitat MI 5.1 showed a combination of high salinity content and high concentrations of nutrients, particularly phosphates. The PCA analysis explained the hierarchical clustering of the habitats.

A biplot of PCA 1 vs PCA 3 and PCA 1 vs PCA 3 showed similar results, but different combinations of nutrients were important in different habitats (Fig. 3.12 and Fig. 3.12). The presence of high concentration of nitrates and ammonium nitrogen defined habitats MI 4.3, MI 5.2, MI 5.3, MI 6.3, MI 6.4 and MI 6.5. Furthermore, habitats MI 6.2, MI 6.3 and MI 6.5 (the Mire Complex) had high levels of nitrites, and organic carbon and low levels of phosphates in addition to moisture and high C.E.C values. Habitat MI 5.1 was also rich in all the different forms of potassium.

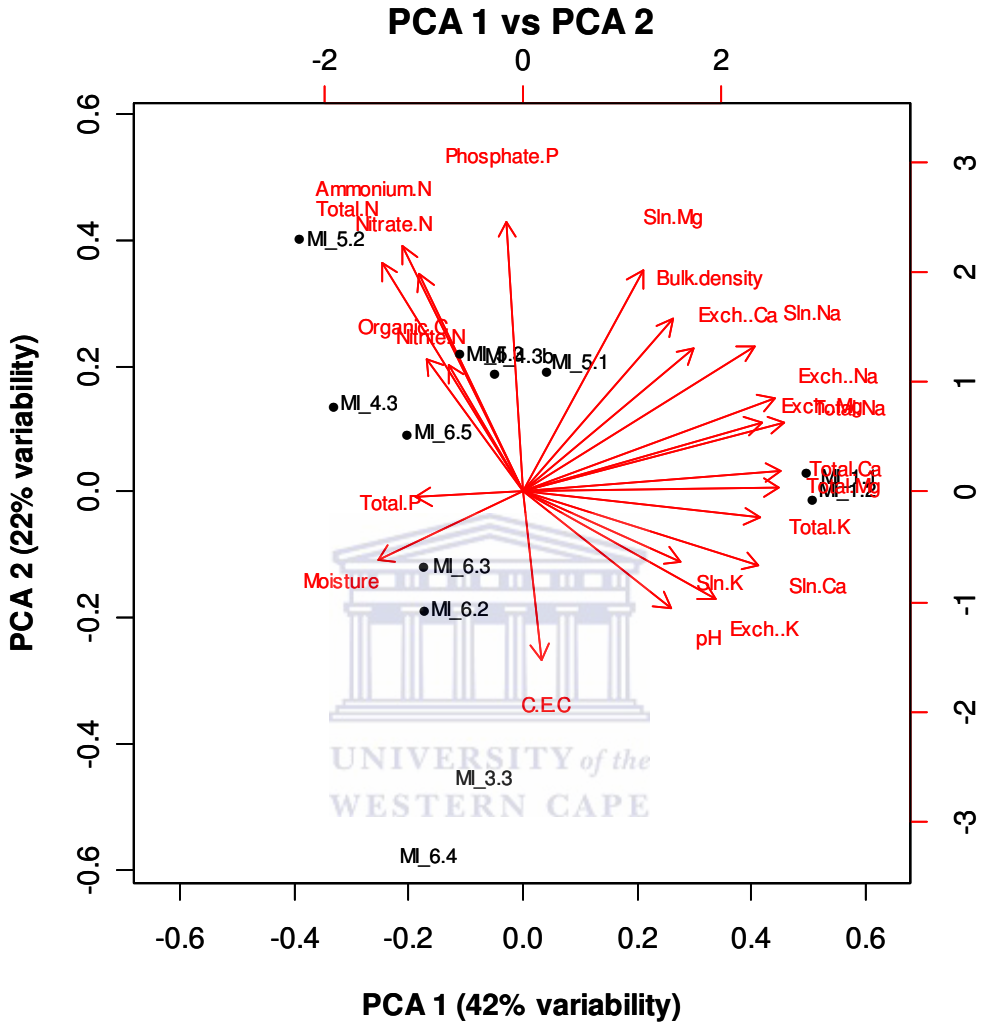


Fig. 3.11: Biplot of PCA 1 and PCA 2, showing the major causes of variability amongst the soil biochemical parameters in Marion Island terrestrial habitats. Arrows show the direction of variable and angles between arrows, correlation between the variables. The length of the arrows shows the strength of each variable.

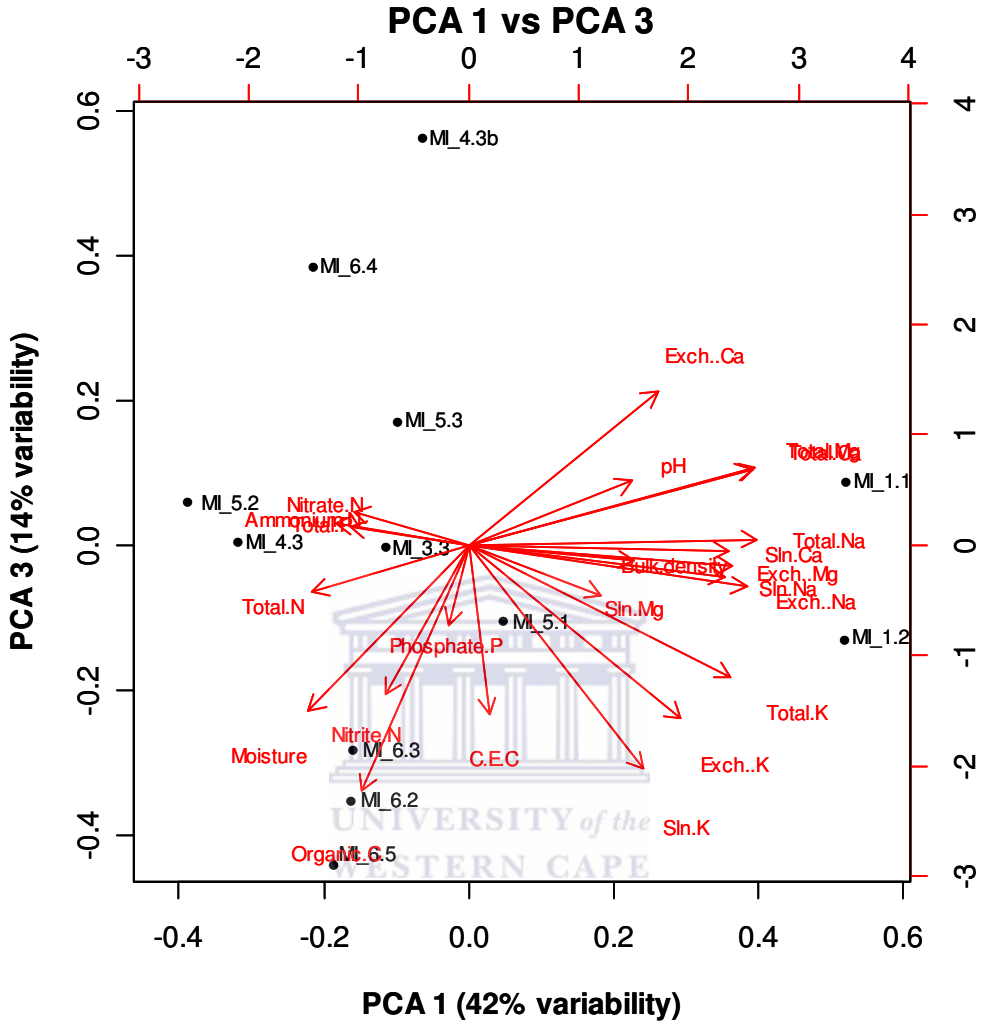


Fig. 3.12: Biplot of PCA 1 and PCA 3, showing the major causes of variability amongst the soil biochemical parameters in Marion Island terrestrial habitats. Arrows show the direction of variable and angles between arrows, correlation between the variables. The length of the arrows shows the strength of each variable.

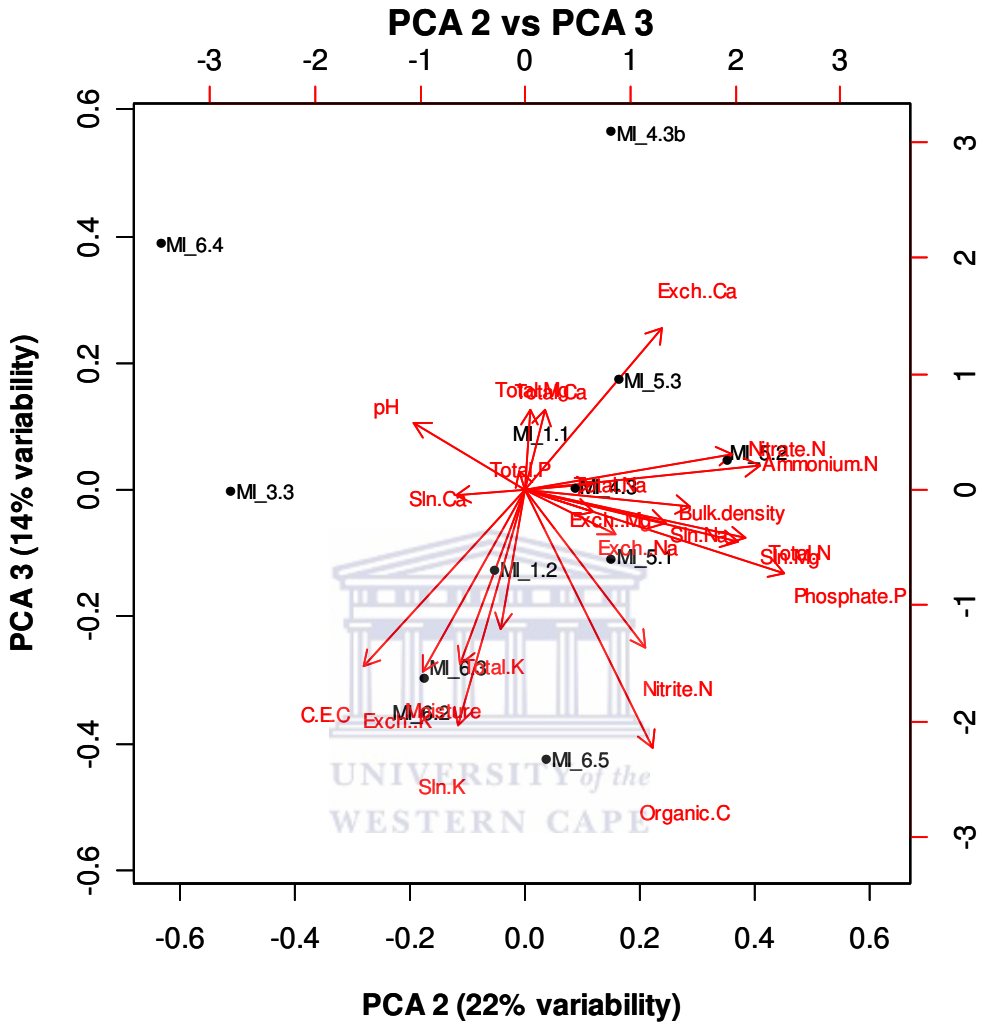


Fig. 3.13: Biplot of PCA 2 and PCA 3, showing the major causes of variability amongst the soil biochemical parameters in Marion Island terrestrial habitats. Arrows show the direction of variable and angles between arrows, correlation between the variables. The length of the arrows shows the strength of each variable.

Even though the plot of PCA 2 vs PCA 4 did not explain the maximum separation, it showed that habitat MI 6.3 had both high moisture and pH (Fig. 3.13). Habitats MI 5.1, MI 5.2 and MI 5.3 had high concentrations of exchangeable sodium, ammonium nitrogen and nitrites. In all cases of PCA combinations, habitats MI 3.3, MI 6.4, MI 4.3 and 4.3b were characterised by low concentrations of nutrients and salts. The important characteristics of habitats identified using PCA were used to explain the results obtained using hierarchical clustering and MDS of microbiological diversity. Thus, the analysis of plant and soil characteristics resulted in the identification of clusters that were consistent in habitat composition (Table 3.3).

Table 3.3: Description of characteristics and similarities between the habitat clusters identified based on the analysis soil and plant properties.

Habitat and MDS clusters	**Important PCA soil variables**	**Similarities within cluster (SIMPER)**
MI 1.1; MI 1.2, MI 5.1	High salinity indices High phosphate concentration in 5.1	Solution sodium concentrations Erect Dicot Rose Dicot
MI 4.3; MI 5.2; MI 5.3	High nutrient concentrations (nitrates and ammonium nitrogen);	Solution sodium concentrations Moisture content Mat Dicots
MI 6.2; MI 6.3; MI 6.5	High moisture content High C.E.C values High concentrations of nutrients: nitrates, nitrites and organic carbon	Solution sodium concentrations Solution potassium concentration Moisture content Mire Graminoids Mire Bryophytes

3.8 DGGE analysis

Assuming each different DNA fragment on the DGGE polyacrylamide gel represented a different bacterial genospecies within a community (Heuer *et al.,* 2001; Muyzer & Smalla, 1998). In this study, the community profiles of actinomycete, actinobacterial and bacterial diversities were analyzed based on their 16S rRNA genes. Since DGGE analysis is limited by the inability to completely resolve the migrating fragments (Jackson *et al.,* 2000), the denaturing gradients were optimized to improve separation. The PCR products of bacterial and actinomycete 16S rRNA gene amplifications were therefore, separated on 30-80% and 45-80% denaturing gradient gels respectively

3.8.1 Establishing the reliability of DGGE profiles

Soil type and sample size affects the quality and reliability of genetic fingerprints when characterizing microbiological communities (Ranjard *et al.,* 2003). In this study, the amount of soil used for metagenomic DNA extraction (0.5g) was twice the quantity recommended by Kang & Mills (2006) for silt loam soils with moderate organic matter (Kang & Mills, 2006). Each sample was prepared in triplicate, and treated independently throughout the study. In most cases, the DGGE patterns of migrating DNA fragments produced the same profiles and major genospecies from each independent treatment of the same samples (Fig. 3.14 and Fig. 3.15). The fragments showing more intensity were assumed to

represent the dominant genospecies. However, the more intense fragments may have also resulted from preferential cell lysis, PCR primer bias, differential amplification PCR templates, PCR drift and the occurrence of more than one gene copy number in some microorganisms than from their abundance (Farrelly *et al.,* 1995; Hugenholtz & Goebel, 2001; Yeates *et al.,* 1997).

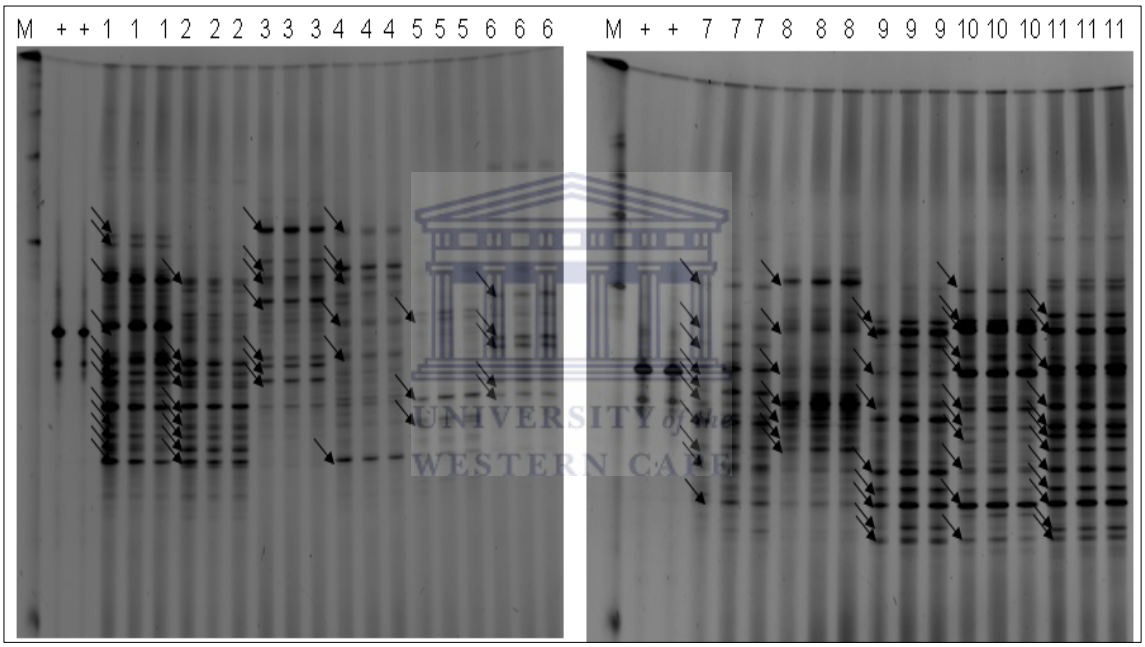


Fig.3.14: DGGE (45-80%) polyacrylamide gels (9%) showing the diversity of actinobacteria in selected Marion Island terrestrial habitats. The arrows indicate the dominant genospecies. Lanes: M, DGGE marker; +, positive control (*Streptomyces albus*) at different concentrations; 1-11, samples from Marion Island terrestrial habitats MI 5.3, MI 5.1, MI 1.2; MI 6.5; MI 6.2; MI 5.2; MI 3.3; MI 1.1; MI 6.4; MI 6.3 and MI 4.3 respectively.

The procedures used in this study were consistent and produced DGGE fingerprints that were reliable. It is therefore, concluded that the samples and procedures used in this study were suitable for microbiological community analysis.

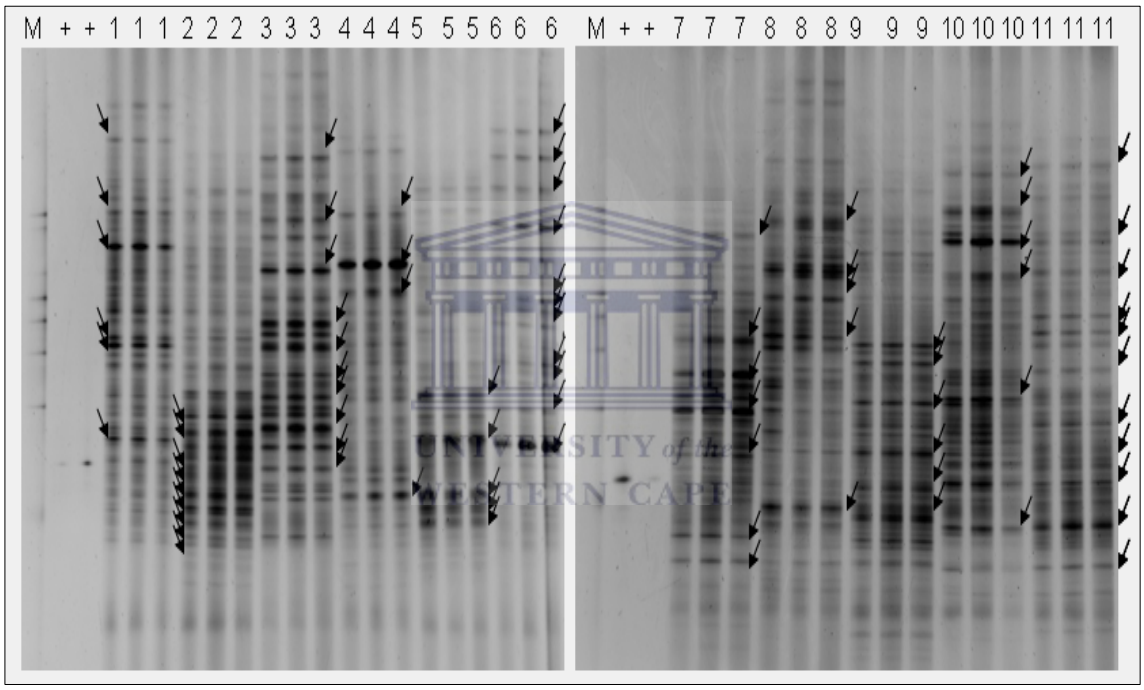


Fig. 3.15: DGGE (30-80%) polyacrylamide gels (9%) showing the diversity of bacteria in selected Marion Island terrestrial habitats. The arrows indicate the dominant genospecies. Lanes: M, DGGE marker; +, positive control (*Streptomyces albus*) at different concentrations; 1-11, samples from Marion Island terrestrial habitats MI 5.3, MI 5.1, MI 1.2; MI 6.5; MI 6.2; MI 5.2; MI 3.3; MI 1.1; MI 6.4; MI 6.3 and MI 4.3 respectively

3.8.2 Comparing the actinomycete and actinobacterial profiles

The primer sets used for PCR are normally biased because of their limitations in completely covering the community diversity in environmental samples (Baker *et al.,* 2003; Forney *et al.,* 2004; Hugenholtz & Goebel, 2001). The retrieval of less common sequences can be facilitated by the use of additional primer sets (Amann *et al.,* 1995)

The DGGE profiles showed that primers targeted for actinomycete (F243 and U1510R) had more diversity coverage compared to the actinobacterial primers (Fig. 3.16). This was unexpected because the actinomycetes are a subset of actinobacteria (Stackebrandt *et al.,* 1997). However, it was possible that there could have been unknown groups of actinomycetes in Marion Island soils. These may have resulted in more coverage with F243 and U1510R than expected. A more likely explanation is that the specificity of the F243 and U1510R was not specific for actinomycetes alone because U1510R primer is not specific for actinomycetes, but universal for bacteria. Fragments that were not common in both samples (for example regions marked “a”) were therefore, thought to be genospecies exclusively specific to each primer set. The co-migration of fragments from the same samples when different primer sets were used for PCR amplification showed that the primers managed to amplify similar genospecies. These genospecies were most likely actinomycetes since they amplified with both primer sets.

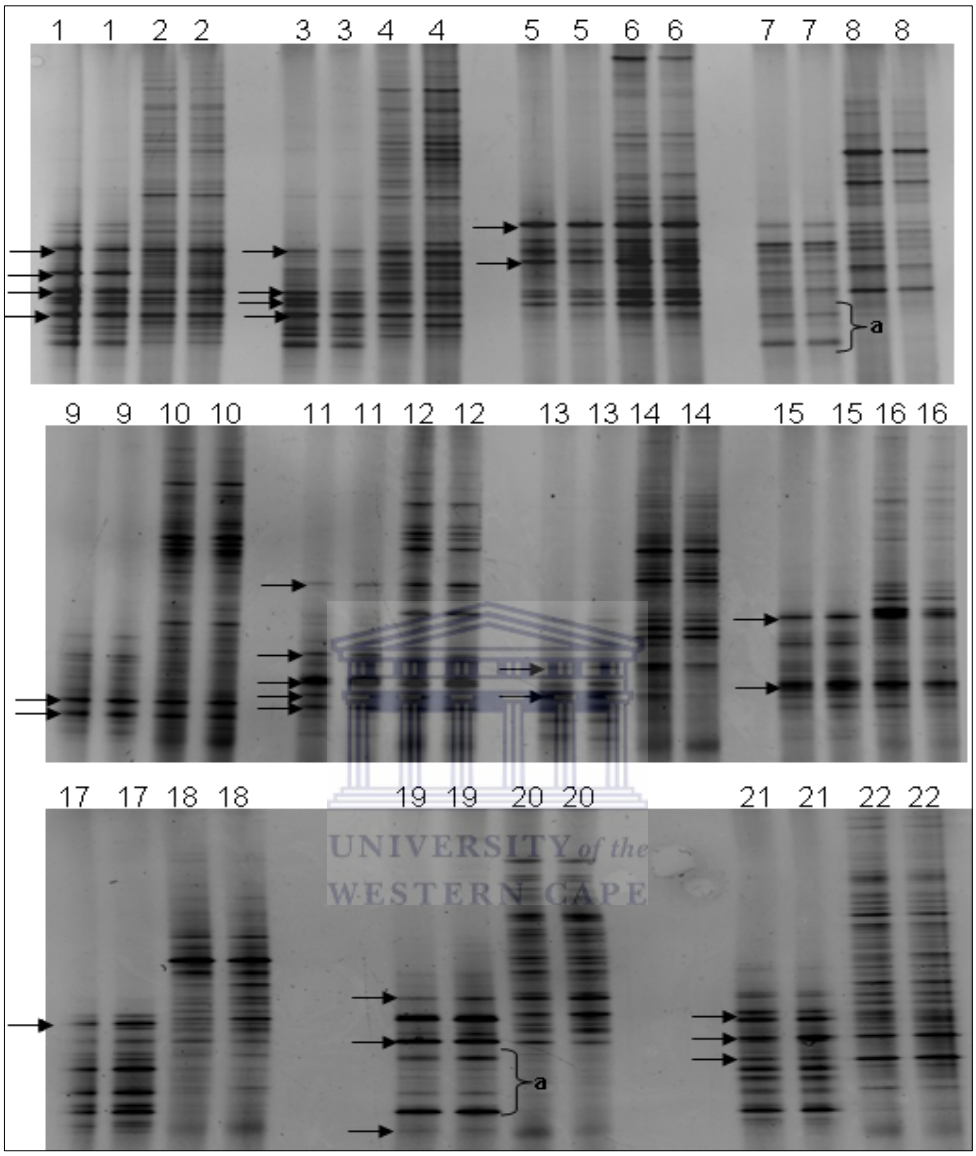


Fig. 3.16: Denaturing gradient (30-80%) polyacrylamide gels (9%) comparing the primer pairs S-C-Act-235-a-S-20 & S-C-Act-878-a-S-19 (actinobacterial-specific) with F243 & U1510R (actinomycete-specific) respectively in Marion Island terrestrial habitats. Lanes: 1-22 Marion Island samples 1 & 2, MI 5.3; 3 & 4, MI 5.1; 5 & 6, MI 1.2; 7 & 8, MI 6.5; 9 & 10; MI 6.2; 11 & 12, MI 5.2; 13 & 14, MI 3.3; 15 & 16, MI 1.1; 17 & 18, MI 6.4; 19 & 20, MI 6.3 and 21 & 22, MI 4.3 respectively. The arrows indicate the dominant genospecies.

In both samples, the intensities of DNA fragments were also relatively similar irrespective of the primer set used. In this study, the intensity of the DNA fragments was determined by visual inspection. The most intense fragments were assumed to represent dominant genospecies (shown by arrows). Even though the primer set F243 and U1510R was initially thought to have more coverage of the diversity, it was later found not to be very stringent in specificity (Chapter 4). Primer F243 is not very stringent in specificity and targets a few other groups of bacteria (Heuer *et al.,* 1997).

3.8.3. Comparing diversity amongst habitats

The composition of bacteria and actinobacteria genospecies in defined Marion Island terrestrial habitats was determined by using DGGE analysis. The diversity was found to be broadly similar for the independent treatments of each sample (Fig. 3.17 and Fig. 3.18) indicating good intra-sampling replication.

3.8.3.1 Statistical analysis of DGGE patterns

Statistical analysis simplifies the complex DGGE patterns that are difficult to characterize by visual inspection (Heuer *et al.,* 2001; Muyzer & Smalla, 1998). In this study, the DGGE patterns were first translated into numerical data by identifying, naming and coding the DNA fragments with similar electrophoretic mobility using the AlphaEase software (Alphaimager®, Imaging System,

AlphaInnotech). This enabled the diversity to be statistically analyzed (Chapter 2, Section 2.14.1) and to relate microbiological diversity to environmental factors. This was achieved using hierarchical clustering, MDS, SIMPER and BIOENV analyses.

3.8.3.2 Relative abundance of actinobacterial genospecies

The actinobacterial diversity differed across the habitats (Fig. 3.17). Some genospecies were qualitatively abundant only in specific habitats (genospecies type k, b, f, and H) and others in all habitats (genospecies type s). There were also genospecies that were unique to specific habitats (genospecies type a).

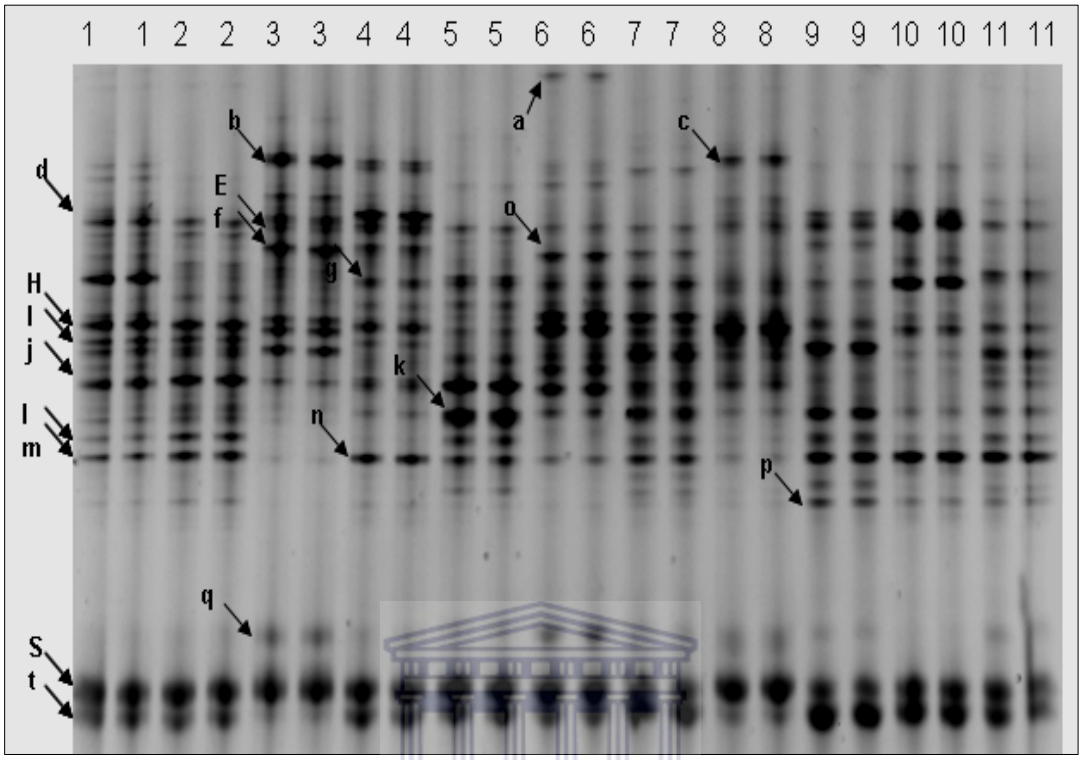


Fig. 3.17: DGGE polyacrylamide gel (9%) comparing the distribution of actinobacterial diversity in selected Marion Island terrestrial habitats. Fragments of 16S rRNA genes separated on a (45-80%) denaturing gradient. The arrows indicate the dominant genospecies, shown by fragments with high intensities. Lanes 1-11, duplicate of Marion Island samples MI 5.3, MI 5.1, MI 1.2; MI 6.5; MI 6.2; MI 5.2; MI 3.3; MI 1.1; MI 6.4; MI 6.3 and MI 4.3 respectively.

3.8.3.3 Relative abundance of bacterial genospecies

As expected, there were qualitatively more bacterial compared to actinobacterial genospecies as shown by the presence of more DNA fragments in each sample (Fig. 3.18, Table 3.4). Similarly, to the actinobacteria DGGE profiles, some

bacterial genospecies were either unique to specific habitats (genospecies type z1) or common to all habitats. For example, genospecies type's j and l were most abundant in specific habitats. The genospecies types d and e abundantly occurred in all habitats. Habitats MI 1.1, MI 1.2, MI 5.1, MI 6.2 and MI 6.3 consisted of high proportions of biotypes that were dominant.

Using the numbers of fragments observed in the DGGE profiles as a measure of the number of genospecies, it was calculated that the actinobacteria occurred in proportions ranging between 50 and 52% of the total bacteria in all habitats (Table 3.3). However, these proportions were limited by the extent of coverage of the primers used and the limitations of PCR amplification. The remarkably different abundance of actinobacterial genotypes given similar overall proportions in all habitats suggests adaptation to different environments.

Table 3.4: Proportions of actinobacterial compared to total bacterial genospecies occurring in Marion Island terrestrial habitats. Numbers were determined from the DNA fragments on DGGE.

Habitat	**MI 5.3**	**MI 5.1**	**MI 1.2**	**MI 6.5**	**MI 6.2**	**MI 5.2**	**MI 3.3**	**MI 1.1**	**MI 6.4**	**MI 6.3**	**MI 4.3**
Actinobacterial genospecies	35	38	41	37	31	34	33	36	29	36	31
Bacterial genospecies	70	76	81	74	62	65	66	70	58	72	62
Proportion of actinobacteria (%)	50	50	51	50	50	52	50	51	50	50	50

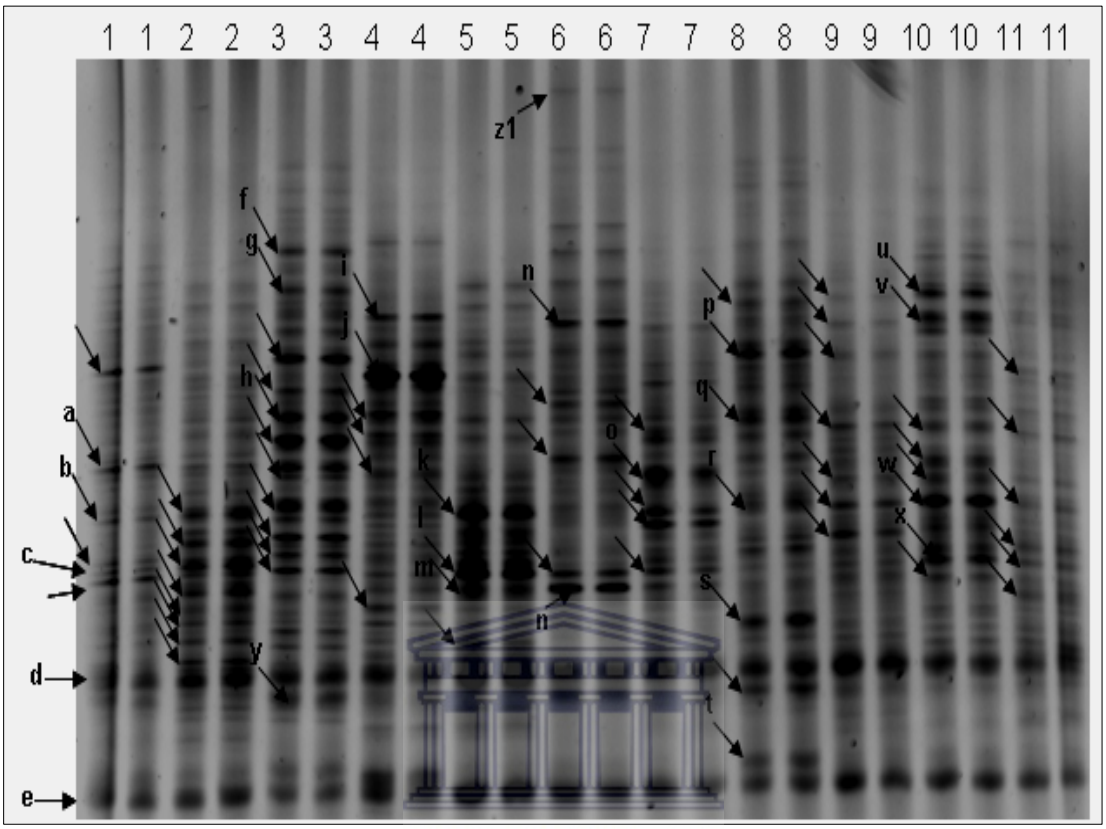


Fig. 3.18: DGGE polyacrylamide gel (9%) comparing the bacterial diversity in selected Marion Island terrestrial habitats. Fragments of 16S rRNA genes separated on a (30-80%) denaturing gradient. The arrows indicate the dominant genospecies. Lanes 1-11, duplicate of Marion Island samples MI 5.3, MI 5.1, MI 1.2; MI 6.5; MI 6.2; MI 5.2; MI 3.3; MI 1.1; MI 6.4; MI 6.3 and MI 4.3 respectively.

3.8.4 Comparison of habitats based on actinobacterial diversity

The actinobacterial diversity was hierarchically clustered based on a measure of the Bray-Curtis similarity of their occurrence in Marion Island terrestrial habitats (Fig. 3.19).

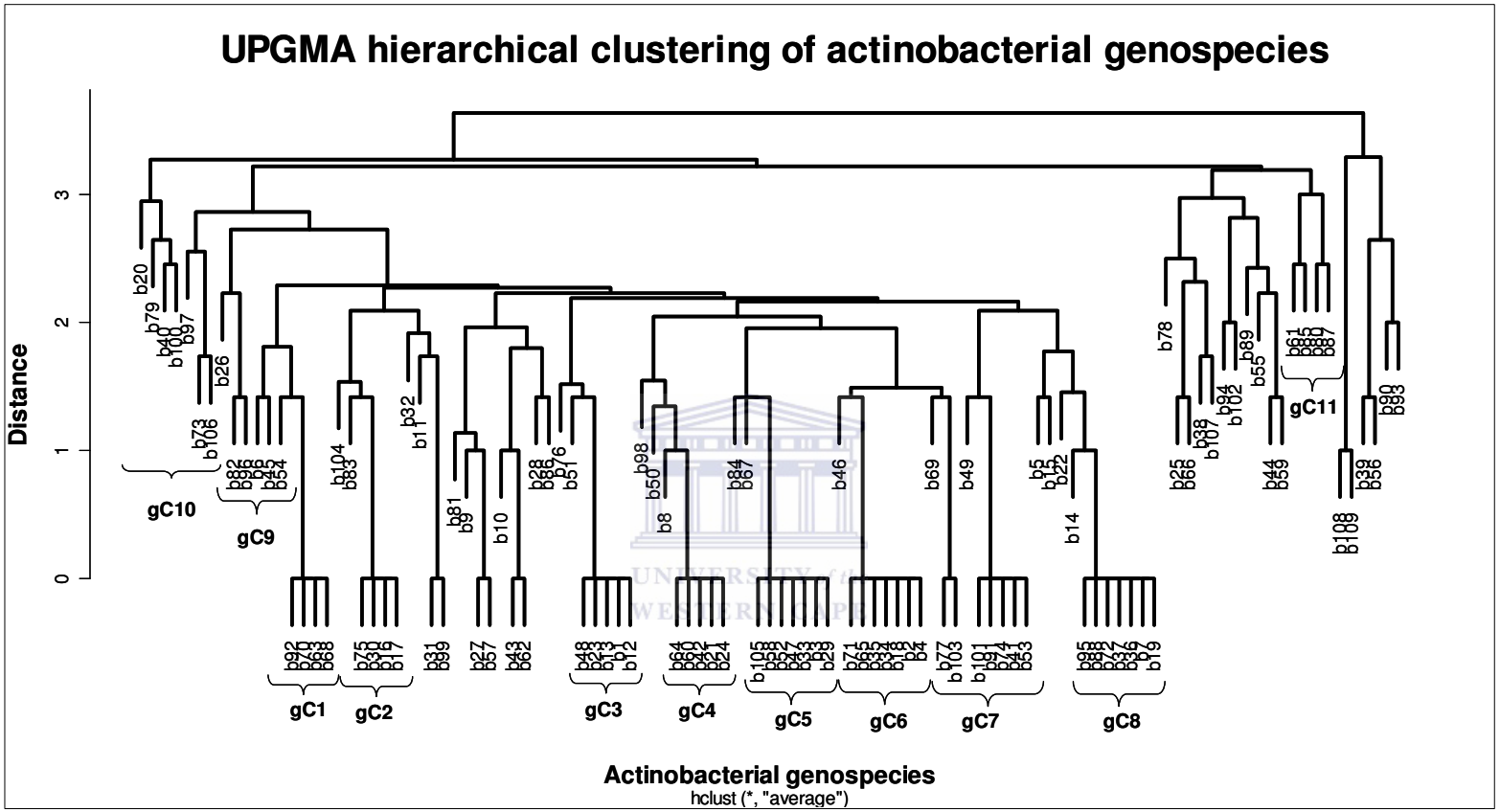


Fig. 3.19: The clustering of actinobacterial genospecies based on their occurrence in selected Marion Island terrestrial habitats using data from the analysis of patterns obtained from the separation of DNA fragments on DGGE. Dendogram drawn based on the Hierarchical clustering, average/ UPGMA (Unpaired Group Mean Weight Average. The representative DNA fragments are named b1 to b109.

Eleven well-defined clusters of actinobacterial genospecies (gC1-gC11) were identified, which were similarly occurring within the habitats. These genospecies were characterised by equal pair-wise distances. The DNA fragments were numbered according to their increasing electrophoretic mobility, according to the increasing percentage of molecular G+C nucleotide composition. Hierarchical clustering was also used to cluster the habitats based on the Bray-Curtis similarity of actinobacterial diversity amongst the habitats (Fig. 3.20).

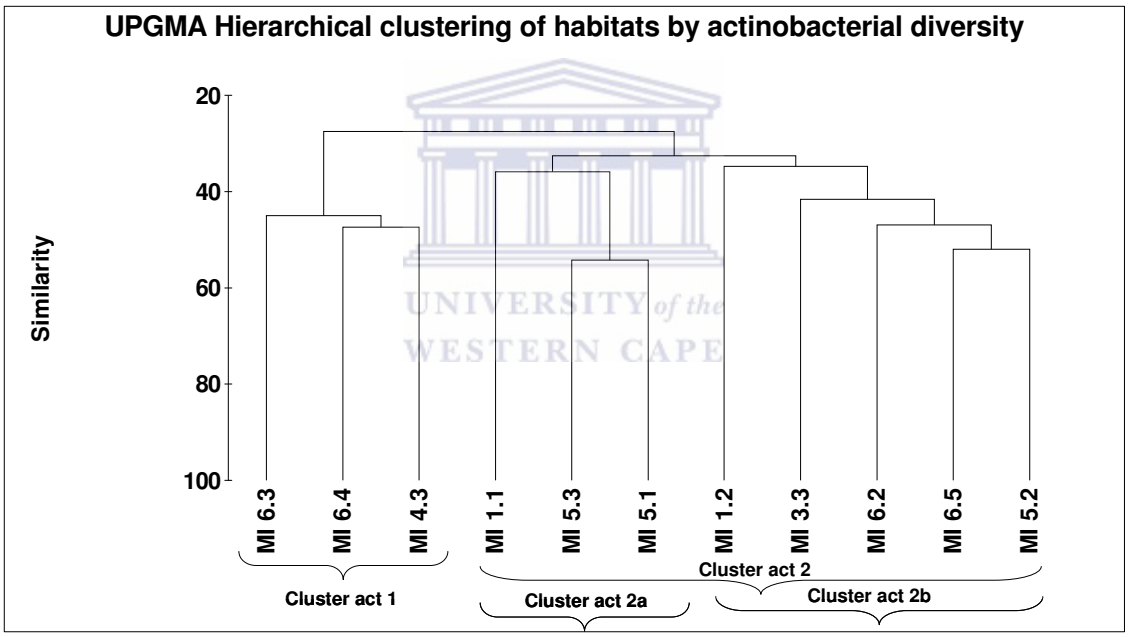


Fig. 3.20: Relationships amogst selected Marion Island terrestrial habitats based on the DGGE analysis of community patterns generated from actinobacterial 16S rRNA gene fragments. Dendogram drawn based on the Hierarchical clustering, average/ UPGMA, Bray-Curtis similarity matrix.

Three habitat clusters were identified consisting of habitats MI 6.3, MI 6.4 and MI 4.3 (Cluster act 1), MI 1.1, MI 5.1 and MI 5.3 (Cluster act 2a) and MI 1.2, MI 3.3, MI .5.2, MI 6.2 and MI 6.5 (Cluster act 2b) These habitat clusters were different from those identified based on the soil or plant characteristics. However, habitats from the same complexes belonged to similar clusters. These included habitats MI 6.3 and MI 6.4 in Cluster act 1 (Mire Complex), MI 5.1 and MI 5.3 in cluster act 2a (Biotic Herbfield Complex) and MI 6.2 and MI 6.5 in Cluster act 2b (Mire complex).

SIMPER analysis (Appendix A7) showed that the actinobacteria in Cluster act 1 had 45% similarity in genospecies composition whilst those in Cluster act 2 had 36% similarity. The habitats in Cluster act 1, Cluster act 2a and Cluster act 2b contained actinobacteria that were 41 to 46% similar in genospecies composition. These low similarities showed that actinobacterial diversity was different across Marion Island terrestrial habitats. However, the analysis of species contributions to the similarities was uninformative since the data was analyzed based on the presence or absence of a genospesies, but not their abundance. The average dissimilarities between the pairs of habitat clusters ranged between 68 and 75% showing that the clusters contained distinct actinobacterial genospecies.

BIOENV analysis was used to determine the environmental variables that best explained the correlations in actinobacterial diversity using the Spearman rank correlation (Appendix A7). The actinobacterial presence-absence Bray-Curtis similarity matrix was compared to the soil and plant datasets. The soil pH accounted for 35% of the correlations between the soil physiochemical characteristics and the distribution of actinobacteria within the habitats. A combination of soil pH, organic carbon, total potassium and exchangeable magnesium explained a total correlation of 26.4% in the distribution of actinobacteria in habitats. All these were positive correlations. The tussock graminoids, *Poa Annua*, mire bryophytes and *Bryum/Breutelia* plants accounted for 32% of the correlation between plant composition and cover and actinobacterial diversity. All plants showed positive correlations except for *Bryum/Breutelia*. In some instances, cushion bryophytes, lichens and tussock graminoids contributed to this correlation together with any two of the plant groups mentioned above.

The hierarchical clusters (Cluster act 1 and Cluster act 2 or Cluster act 1, Cluster act 2a and Cluster act 2b) were superimposed onto the MDS ordination plots (Fig. 3.21). MDS analysis showed that most of the habitats were scattered, except for the two clusters, one consisting of MI 5.2 and MI 3.3, and the other of MI 5.1 and MI 5.3.

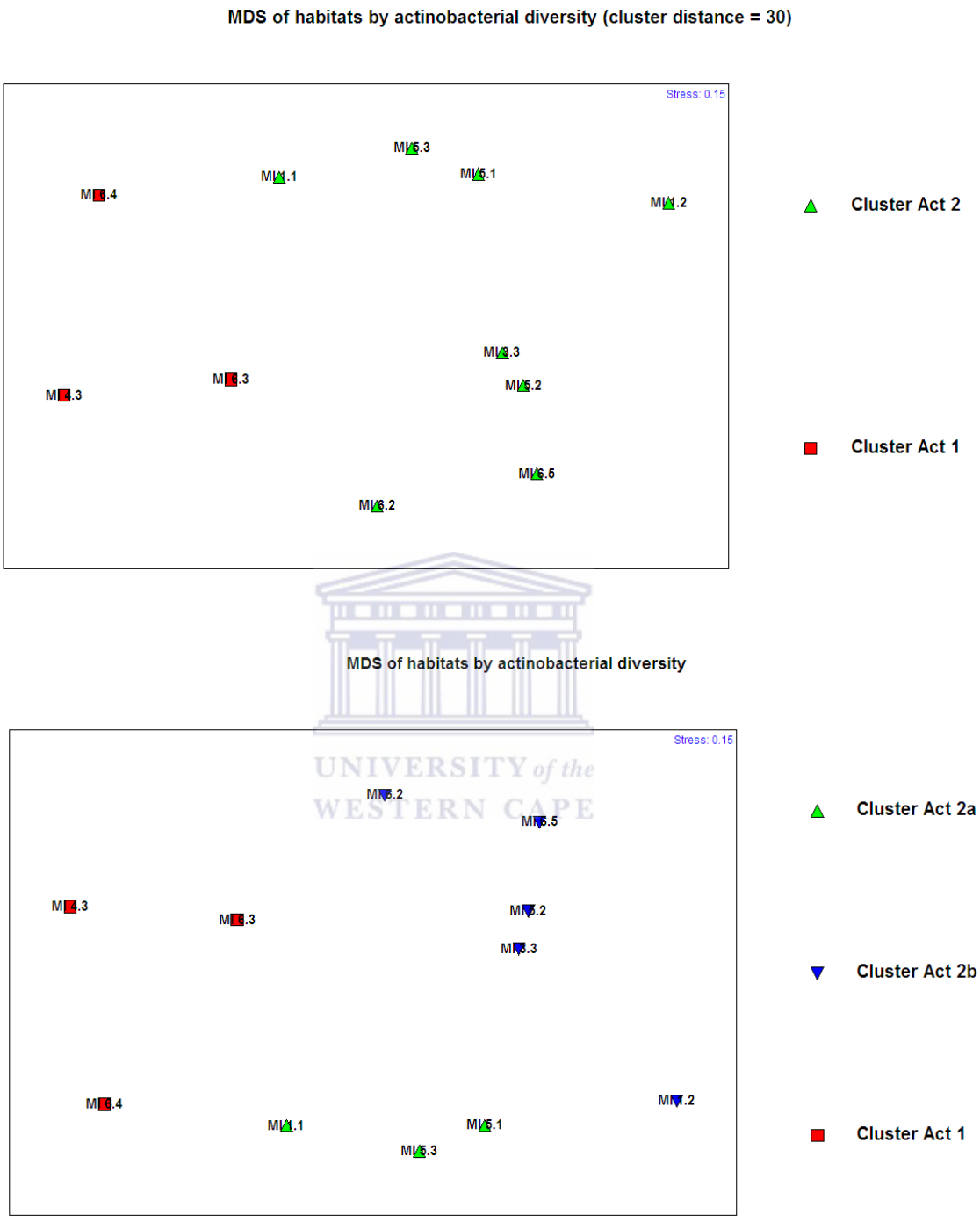


Fig. 3.21: MDS ordinations showing relationships amongst Marion Island terrestrial habitats based on the presence of actinobacterial genospecies. Cluster Act 1, 2, 2a and 2b are superimposed hierarchical clusters.

The rest of the habitats were therefore, not as closely related as shown by hierarchical clustering. The low stress value of 0.15 used for the MDS plot is an indication of high reliability. Thus, the actinobacterial diversity in Marion island terrestrial habitats was not cluster according to the corresponding plant or soil characteristics. There seemed to be differences in actinobacterial diversity in habitats except for habitats MI 5.1 and MI 5.3, which belonged to the same complex, and habitats MI 5.2 and MI 3.3. The information obtained from two-way clustering was used to identify habitats with unique actinobacterial diversity (Fig. 3.19 and Fig. 3.20).

This enabled the classification of Marion Island terrestrial habitats according to actinobacterial diversity. Some of the genospecies could not be assigned to specific clusters because they were commonly found in all habitats (genospecies unspecific to habitat). A number of clusters (gC1, gC2, gC4, gC5, gC6, gC7 and gC8) contained genospecies unique to specific habitats. Habitat MI 1.2 comprised of a cluster of unique genospecies in addition to those commonly found in most habitats. Most of the habitats were characterised by the presence of unique genospecies clusters except for those in Cluster act 1 (MI 4.3, MI 6.3 and MI 6.4). Habitats MI 3.3 and MI 6.5 (Cluster act 2b) had relatively fewer numbers of actinobacterial genospecies unique to one or two habitats compared to the rest of the habitats in that cluster.

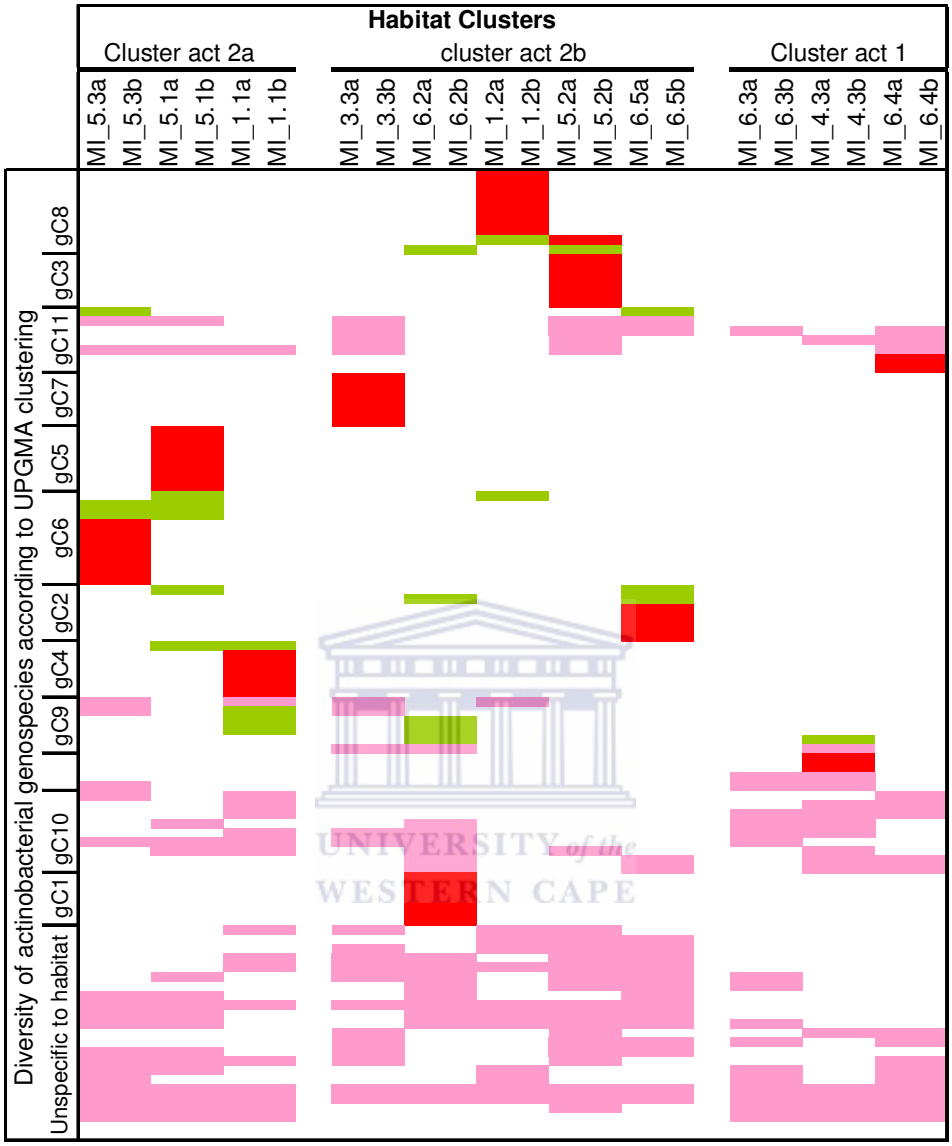


Fig. 3.22: The occurrance of actinobacterial genospecies in habitat clusters of Marion Island terrestrial habitats MI 5.3, MI 5.1, MI 1.1, MI 3.3, MI 6.2, MI 1.2, MI 5.2, MI 6.5, MI 6.3, MI 4.3 and MI 6.4. The 11 clusters of actinobacterial genospecies are indicated gC1 to gC11 and habitats hC1 to hC4. Key: , genospecies unique to one habitat; , genospecies occurring in two habitats; , genospecies occurring in more that two habitats.

These results showed that the Mire habitats generally contained less unique actinobacterial diversity compared to the other Marion Island terrestrial habitats. All habitats in Cluster act 2a contained unique actinobacterial genospecies.

3.8.5 Comparison of habitats based on bacterial diversity

Hierarchical clustering was used to relate bacterial genospecies according to their occurance in habitats based on the Bray-Curtis similarity matrix of the species presence-absence data (Fig. 3.23). This resulted in two distinct bacterial clusters in which the major cluster consisted of 11 sub-clusters of genospecies. Compared to actinobacterial diversity, most clusters did not consist of unique genospecies except for gC1 to gC5.

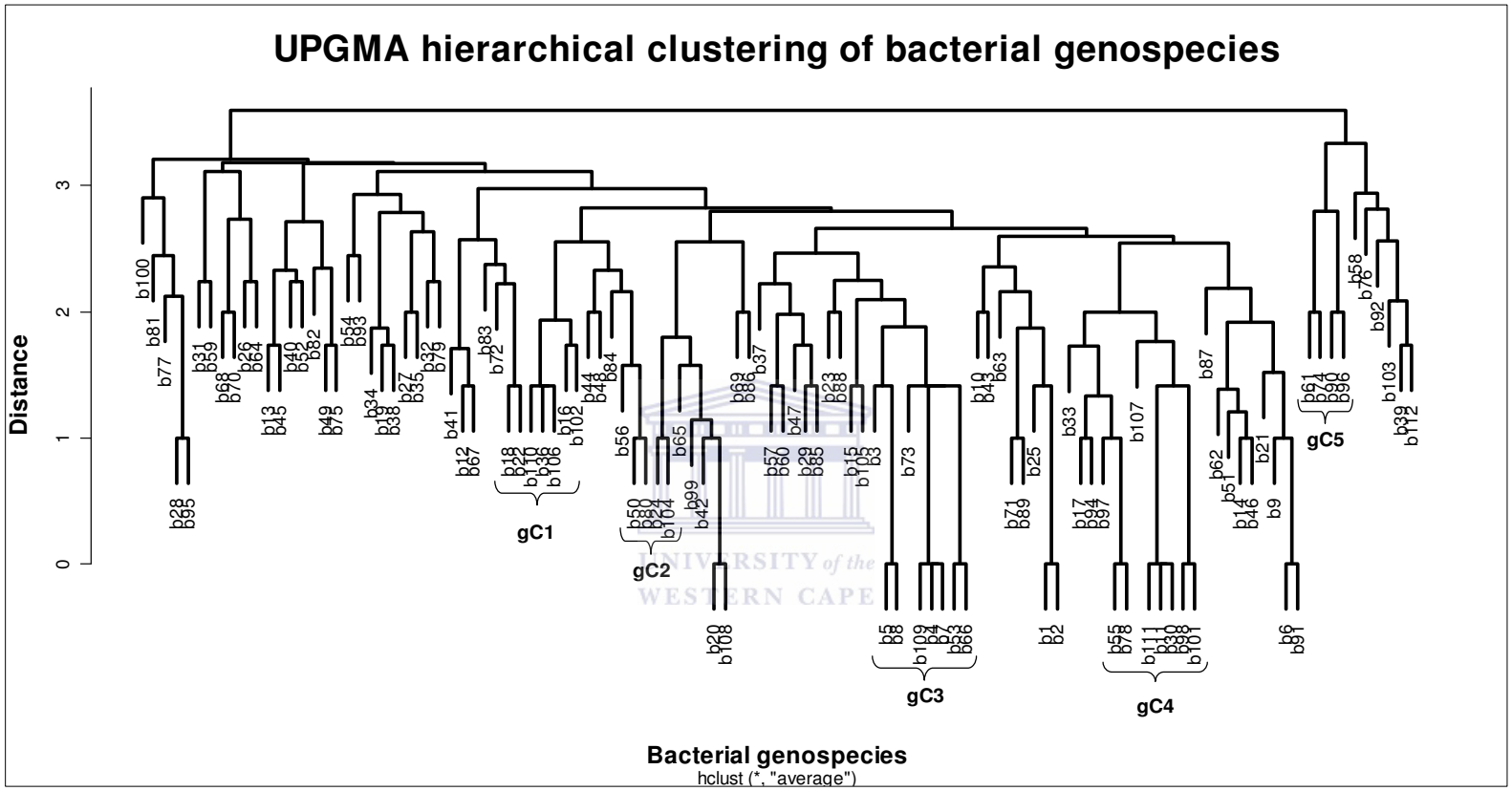


Fig. 3.23: Clustering of bacterial genospecies based on their distribution across selected Marion Island terrestrial habitats according the analysis of patterns obtained from the separation of DNA fragments on DGGE. Dendogram drawn based on the Hierarchical clustering, average/ UPGMA (Unpaired Group Mean Weight Average), Euclidian distance methods. The representative DNA fragments are numbered b1 to b112.

Hierarchical clustering was also used to classify the Marion Island terrestrial habitats based on their similarity measures of actinobacterial diversity (Fig. 3.24). Three habitat clusters (Cluster bact A, cluster bact B and Cluster bact C) were obtained. Some of the habitat clusters contained habitats from the same complexes. These included habitats MI 6.3 and MI 6.3 in Cluster bact A, MI 6.2 and MI 6.5 in Cluster bact B and MI 1.1 and MI 1.2 in Cluster bact C.

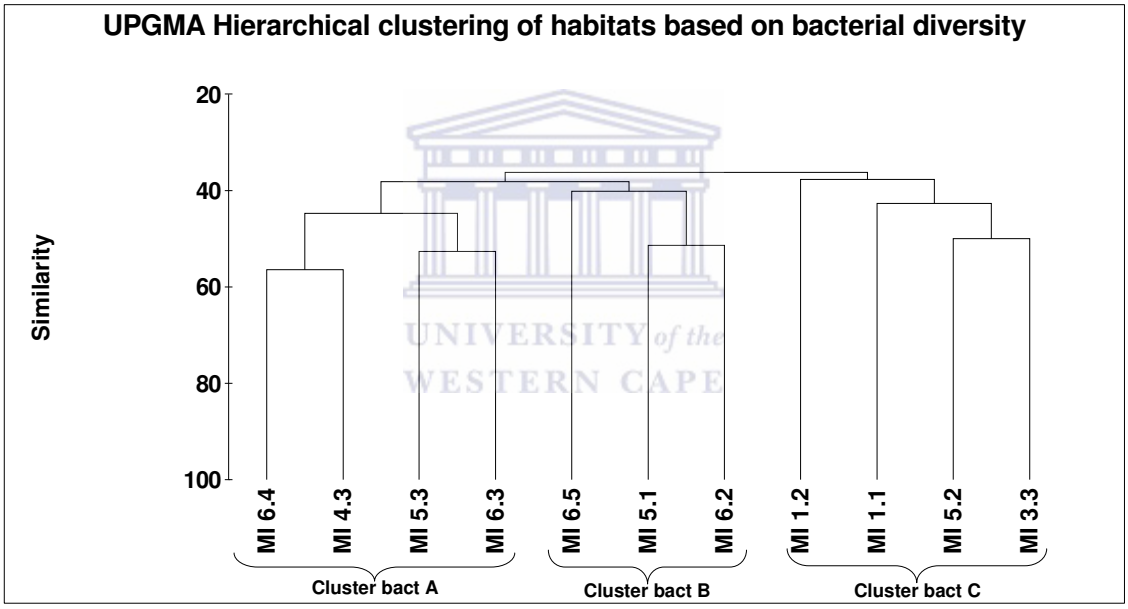


Fig. 3.24: Relationships amongst selected Marion Island terrestrial habitats based on the DGGE analysis of community patterns generated from bacterial 16S rRNA gene fragments. Dendogram drawn based on the Hierarchical clustering, average/ UPGMA, Bray-Curtis similarity matrix.

SIMPER analysis showed that each habitat cluster contained habitats that were 41-47% similar in composition of bacterial genospecies (Appendix A8). Thus, the habitats were not very similar in bacteria genospecies composition. The average dissimilarities between the pairs of these habitat clusters were relatively high (62 and 63%) showing that the clusters were distinct. The species contributions to similarities of actinobacterial diversity within each habitat cluster were uninformative because the abundance of the species was not taken into account. The data used for analysis was only based on the presence or absence of a genospecies.

BIOENV analysis was used to identify the soil and plant characteristic that best explained the distribution of bacteria in Marion Island terrestrial habitats using the Spearman rank correlation (Appendix A9). In all permutations of the best five soil variables, the salinity indicators showed the highest correlations (35-36%) with bacterial diversity. These included total calcium, exchangeable calcium, soil solution calcium, soil solution magnesium, soil solution potassium, total sodium and exchangeable sodium. All the correlations obtained using the soil or plant data were positive. Amongst the plants, *P. annua* resulted in the highest correlation (21%) that best explained the distribution of bacteria in all habitats. A combination of *P. annua* and lichens, epiphytic graminoids or brachythecium mosses resulted in a correlation of 21%. A combination of *P. annua* with lichens and brachythecium mosses, epiphytic graminoids and brachythecium mosses or brachythecium mosses and epiphytic graminoids also accounted for 21% of the

correlations. The habitat clusters identified using hierarchical clustering of the bacterial diversity (Cluster bact A, Cluster bact B and Cluster bact C) were superimposed onto the MDS ordination plots (Fig. 3.25).

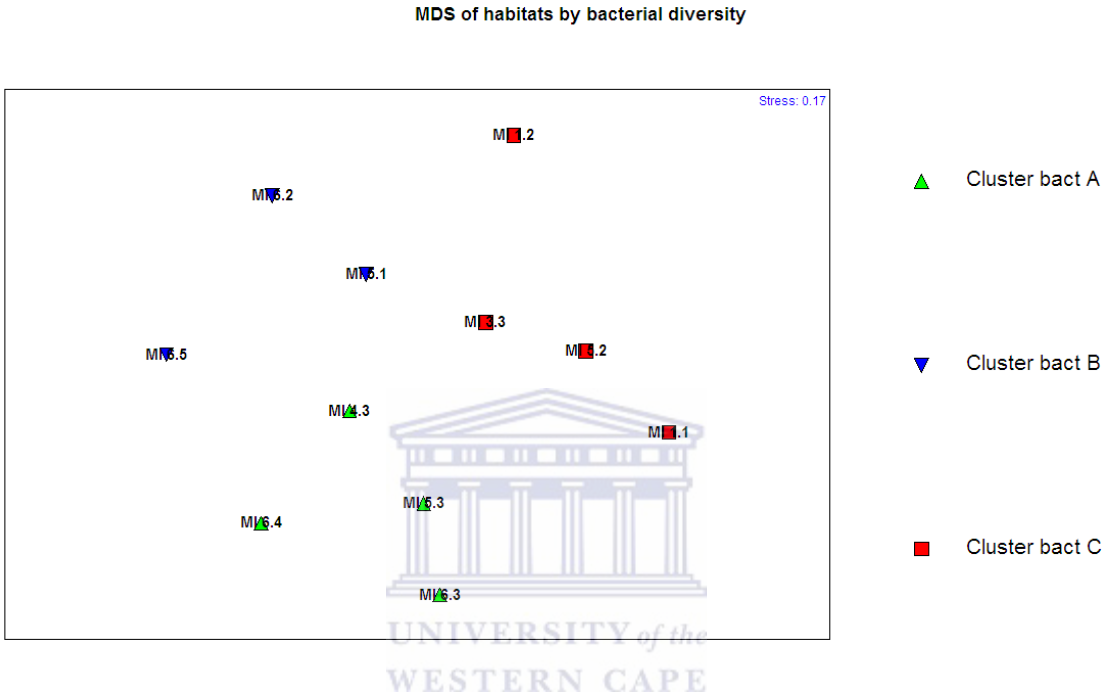


Fig. 3.25: MDS ordination showing relationships amongst Marion Island terrestrial habitats based on the presence of bacterial genospecies. Cluster bact A, B and C are superimposed hierarchical clusters.

The MDS analysis was based on the Bray-Curtis similarity matrix of genospecies presence-absence data. MDS analysis (at a stress level of 0.17) showed that the habitats did not form distinct clusters based on bacterial diversity. Thus, the habitats contained bacteria that widely varied in diversity. The results confirmed the low similarities within the habitat clusters that were identified using SIMPER.

The two-dimensional clustering of Marion Island terrestrial habitats according to bacterial diversity (Fig. 3.23 and Fig. 3.24) resulted in a classification scheme that was also used to identify habitats with unique genospecies (Fig. 3.26).

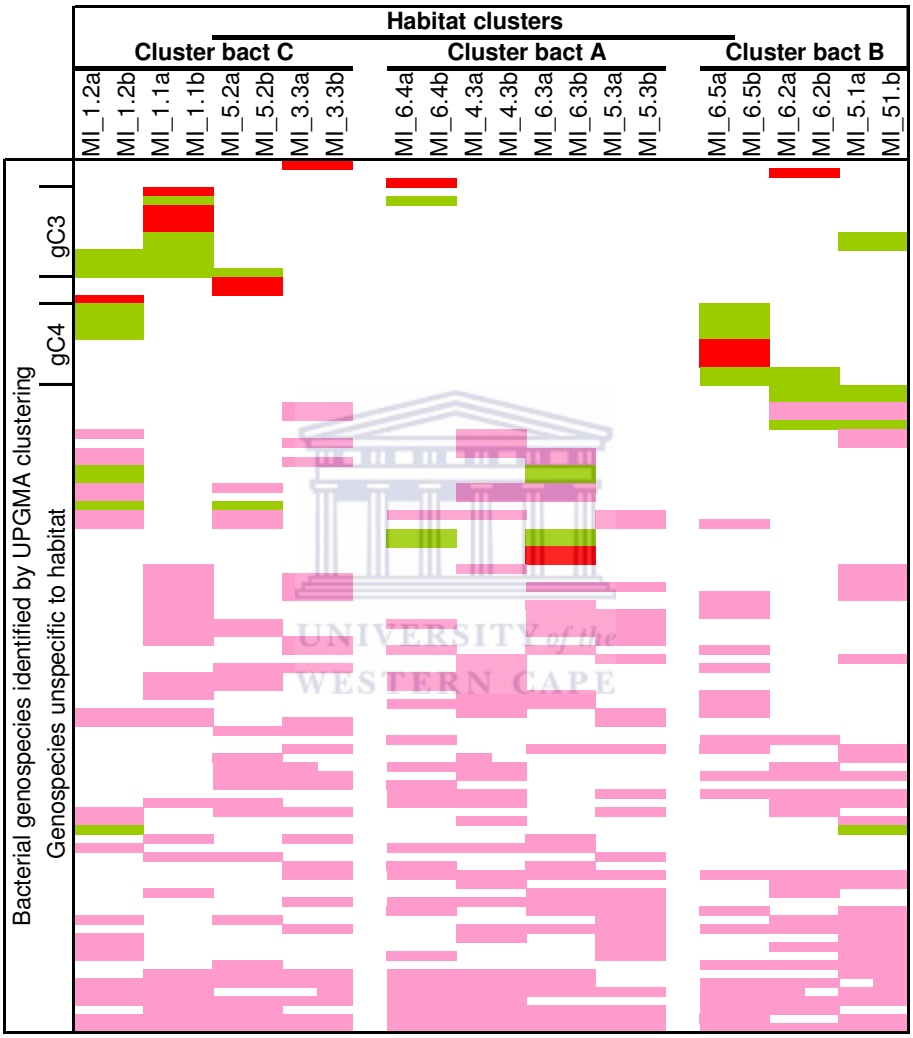


Fig. 3.26: The occurrance of bacterial genospecies in habitat clusters of Marion Island terrestrial habitats MI 1.2, MI 1.1, MI 5.2, MI 3.3, MI 6.4, MI 4.3, MI 6.3, MI 5.3, MI 6.5, MI6.2 and MI 5.1. Only two clusters of bacterial genospecies are indicated C3 and C4. Key: , genospecies unique to one habitat; , genospecies occurring in two habitats; , genospecies occurring in more that two habitats.

Some of the genospecies belonged to clusters that were unique to one habitat or that also occurred in only one other habitat (genospecies clusters gC3 and gC4). These genospecies were characterised by similar pair-wise distances. Habitat MI 1.1 was distinct and comprised of an additional genospecies (cluster gC3). Some of the genospecies in this cluster also occurred only in habitat MI 5.1. The genospecies were probably adapted to high salinity, which is characteristic to soils that were found in both habitats. In contrast, a proportion of those genospecies that were most commonly found in other habitats were absent from habitats MI 5.1 and MI 6.2. These habitats therefore, formed another distinct sub-cluster. The habitats MI 1.1, MI 5.1 and MI 6.2 contained genospecies (occurring near genospecies clusters gC3 and gC4) that were not found in other habitats.

3.9 Classification of Marion Island habitats based on microbial diversity

Table 3.5 shows a comparison of the clusters identified according to hierarchical clustering of actinobacterial and bacterial diversities. In both cases, three habitat clusters were obtained. Habitats MI MI 4.3, MI 6.3 and MI 6.4 clustered together using either bacterial or actinobacterial diversity. Similarly, habitats MI 1.2, MI 3.3 and MI 5.2 also clustered together in both instances. However, habitats MI 1.1, MI 5.1, MI 6.3 and MI 6.5 did not consistently belong to the same clusters if the different groups of microorganisms were analyzed.

Table 3.5: Comparison of habitat clusters identified using actinobacterial and bacterial diversities. Habitats occurring in similar clusters irrespective of the diversity group are underlined.

No. of clusters	Actinobacteria	Bacteria
1	**Cluster act 1** MI 4.3, MI 6.3, MI 6.4 No unique genospecies were identified in these habitats.	**Cluster bact A** MI 4.3, MI 6.3, MI 6.4, MI 5.3
2	**Cluster act 2a** MI 5.1, MI 1.1, MI 5.3	**Cluster bact B** MI 5.1, MI 6.2, MI 6.5
3	**Cluster act 2b** MI 1.2, MI 3.3, MI 5.2, MI 6.5	**Cluster bact C** MI 1.2, MI 3.3, MI 5.2, MI 1.1

However, these habitat clusters consistently identified using hierarchical clustering of both bacterial and actinobacteria diversities were not congruent to those identified based on plant or soil physiochemical properties. These results, supported by MDS showed that the similarities in habitat characteristics do not necessarily predict similarities in microbiological diversity in Marion Island terrestrial habitats.

3.10 Discussion

The clusters identified based on soil characteristics consisted of habitats that were very similar (72 and 86%). The habitats from the Mire and Coastal salt-Spray Complexes clustered together as expected. The dissimilarities between the habitats clusters were due to soil solution sodium (32-55% in most pairs of habitat clusters) and moisture content (55% between only two habitat clusters).

Moisture, soil solution sodium and solution potassium contributed between 46% and up to 86% similarity within the habitats.

The four habitat clusters identified based on plant cover characteristics related by similarities ranging between 64% and 74%. The similarities within the habitat clusters were mainly due to total plant cover (67-79%). The differences in plant composition were also an important factor in determining similarities within the habitats and dissimilarities between the habitat clusters.

The first principal component explained 42% of the variability in soil physiochemical properties. PCA separated habitats into main groups characterised by high levels of different salinity indicators, high concentrations of nutrients, high moisture content and high cation exchange capacity. The salinity indicators showed maximum separation from other variables. This resulted in habitats MI 1.1 and MI 1.2 being isolated from the rest of the other habitats. In a related study, the analysis of database sequences from environmental samples showed that salinity was the most important factor in determining the composition of microbiological communities (Lozupone & Knight, 2007). This was concluded after reviewing the bacterial diversity from a wide range of environmental samples. The samples varied in physical and chemical parameters and magnitudes. These included soil, water, seawater, hotsprings, hydrothermal vents, marine ice, hypersaline basins, hypersaline lakes, hypersaline mats, acid

springs and rocks, alkaline lakes, oligotrophic caves and common soil environments amongst others.

In this study, the two habitats MI 1.1 and MI 1.2 were identified as having similar characteristics using hierarchical clustering. PCA analysis showed that different combinations of nutrients were important in different habitats. A combination of high nutrients and salinity resulted in the independent clustering of habitat MI 5.1, closer to MI 1.1 and MI 1.2. This habitat contained high phosphate concentrations. These three habitats are located near the coast and influenced by sea salt-spray and by manuring from marine birds and mammals (Smith e*t al.,* 2001; Smith & Steenkamp, 2001).

Two similar habitat clusters were consistently identified using hierarchical clustering of both bacterial and actinobacteria diversities. However, these habitat clusters were not congruent to those identified based on plant or soil characteristics. MDS analysis showed that most of the habitats were not as closely related to form identifiable clusters based on microbiological diversity. The results showed that the similarities in habitat characteristics do not necessarily predict similarities in microbiological diversity in Marion Island terrestrial habitats. The actinobacterial clusters showed 68-75% dissimilarities and contained genospecies that were 36-45% similar in composition. The habitat clusters identified using bacterial diversity showed 62-63% dissimilarities between the pairs of habitat clusters, and similarities of 41-47% in genospecies

composition within the clusters. The low similarities within the clusters confirmed the scattering of these habitats on MDS ordination, that the habitats were not closely relates even though they clustered together.

The soil pH accounted for 35% of the positive correlations between the soil characteristics and distribution of actinobacteria, and 26.4% in combination with organic carbon, total potassium and exchangeable magnesium. The tussock graminoids, *Poa Annua*, mire bryophytes and *Bryum/Breutelia* plants accounted for 32% of the correlation, the latter showing a negative correlation. In some cases, cushion bryophytes, lichens and tussock graminoids also contributed to the positive correlations.

Total calcium, exchangeable calcium, soil solution calcium, magnesium, and potassium, total sodium and exchangeable sodium showed the highest correlations (35-36%, positive) with bacterial diversity. The *P. annua* resulted in the highest correlation (21%, positive) that best explained the distribution of bacteria in all habitats. *P. annua* also showed the positive correlation of 21% in combination with lichens, epiphytic graminoids or brachythecium mosses.

Two-way hierarchical clustering resulted in the identification of habitats with unique microorganisms. Most of the habitats were characterised by the presence of unique genospecies clusters except for MI 4.3, MI 6.3 and MI 6.4. Habitats MI

3.3 and MI 6.5 had relatively fewer numbers of unique actinobacterial genospecies.

This study showed that the soil physiochemical characteristics were important determinants of microbiological diversity. The consistent occurance of same habitats in similar clusters as determined by both bacteria and actinobacteria showed that common environmental characteristics govern distribution patterns. However, the clusters of habitats obtained using environmental variables do not directly explain those obtained using microbiological diversity. Bacteria consist of other groups that are not as phyologenetically related as actinobacteria. Actinomycetes, which form the majority of the actinobacteria adapt to a broad range of environmental factors due to their ability to form vegetative structures and to assimilate complex substrates (Goodfellow & Williams, 1983; Lechevalier & Lechevalier, 1967; Paul & Clark, 1996). Actinomycetes thrive in environments with lower nutrients and moisture content compared to other bacteria. In addition, actinomycetes are strictly aerobic or microaerophilic compared to other bacteria, some, which are anaerobic (Goodfellow & Williams, 1983; Paul & Clark, 1996). These differences show that actinomycetes and other bacteria thrive under different conditions. This could have resulted in the differences observed in the classifications using bacterial and actinobacterial diversities and their environmental requirements.

3.11 Summary

In this chapter DGGE, analysis was used to compare the composition of the actinobacterial and bacterial diversity in Marion Island terrestrial habitats. The DGGE patterns were analyzed using multivariate statistical techniques (MDS, PCA, SIMPER and BIOENV). Different soil variables and plant species were correlated with microbiological diversity and their contributions determined in different habitat clusters. Salinity, nutrient availability and moisture were the most important determinants (principal components) of habitat characteristics amongst the environmental factors studied. Hierarchical clustering was also used to identify habitats containing unique bacterial and actinobacterial genospecies.

CHAPTER 4

Phylogenetic analysis of actinobacterial diversity in three habitats

4.1 Aims

The spatial distribution of microbial taxa is influenced by their genetic traits and adaptation to environmental factors (Green *et al.,* 2008; Kassen and Rainey, 2004). This results in microorganisms that are endemic to particular environments (Martiny *et al.,* 2006). This study was aimed at comparing the distribution of actinobacterial taxonomic groups in three Marion Island terrestrial habitats based on the 16S rRNA sequences. These habitats had different plant and soil characteristics, and contained unique actinobacterial genospecies based on DGGE analysis (Chapter 3, Fig. 3.22 and Fig. 3.26). The actinobacteria were also compared to those found in other habitats. The culture-independent approach was based on cloning the 16SrRNA genes amplified using PCR, sequencing and phylogenetic analysis. The study was also aimed at phylogenetic placement of the actinobacterial phylotypes. The samples used were from habitats MI 1.2 (Coastal Fellfield habitat), MI 5.1 (Cotula herbfield habitat) and MI 6.3 (Wet Mire Habitat).

4.2 Background and literature review

Metagenomic studies on different environmental samples indicate that most microorganisms are yet to be cultured (Amann *et al.,* 1995). These constitute more than 99% of the total bacterial genotypes (Sharma *et al.,* 2005). The search for microbiological novelty is currently focused on remote environments, such as the cold-desert mineral soils (Smith *et al.,* 2006) and other habitats of the Antarctica and sub-Antarctic regions (De la Torre *et al.,* 2003; Nichols *et al.,* 2002). Most novel actinobacteria recently isolated from cold habitats were from the marine environments (Bull *et al.,* 2000; Colquhoun *et al.,* 1998a; Maldonado *et al.,* 2005a; Shivaji *et al.,* 2004; Stach *et al.,* 2003a) whereas the terrestrial Antarctic and sub-Antarctic actinobacterial diversity remains unexplored.

The two main culture-independent approaches are based on assessing the community phylogenetic diversity of microorganisms using the rRNA genes as molecular markers. The shotgun approach involves cloning community metagenomic DNA and hybridization screening of the resulting library using species-specific rRNA gene probes. PCR-based methods target and amplify the rRNA genes directly from the metagenomic DNA templates. This is followed by cloning, screening and identification through homology searches against known 16S rRNA genes in databases (Amann *et al.,* 1995). In both cases, the process begins with the direct extraction of community DNA. This involves either beginning with cell enrichment or direct DNA extraction from source samples (Amann *et al.,* 1995; Cowan *et al.,* 2005; Tsai & Rochelle, 2001). The genotypes

are identified by analyzing the sequences of the cloned rRNA genes, which are used for constructing phylogenetic trees.

The tree construction algorithms select the final phylogenetic tree with the smallest amount of total evolutionary changes. Distance methods continuously grouping two most similar sequences stepwise manner based on the shortest distance. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) is suitable for datasets consisting lineages with relatively constant rates of evolution. The Neighbor joining (NJ) involves grouping OTUs with shortest pair-wise-distance of branch length and is suitable for dataset consisting of varying rates of evolution (Saitou & Nei, 1987). Both UPGMA and NJ are relatively fast and suitable for analyzing large data sets (Nei, 1996).

The Maximum likelihood and Maximum parsimony methods emphasize more on the evolutionary origin of species and assume common ancestry by mutation and selected processes without hybridization or other horizontal gene transfers. These methods are best for comparing trees and emphasize on informative sites based on nucleotide alignments at that position. The Maximum parsimony method assumes that trees with the minimum number of evolutionary changes are the most preferable trees by minimizing the total number of evolutionary steps required to explain a given set of data (Sourdis & Nei, 1988). The Maximum likelihood method constructs phylogeny based on statistical modeling of evolution. This involves fitting a mathematical model to a data set and

choosing the evolutionary tree with the highest probability of fitting the observed data. This is the most accurate method, which gives rise to few errors and can be used to test existing trees (Tateno *et al.*, 1994).

Culture-independent studies showed that actinobacteria make up the majority of the microorganisms identified from the Antarctica and sub-Antarctica terrestrial environments (Chapter 1) (Kochkina *et al.,* 2001; Moncheva *et al.,* 2000-2002; Nichols *et al.,* 2002; Sjöling & Cowan, 2003; Smith *et al.,* 2006). Similar results were also obtained using culture-dependent studies (Bagatzevska, 2000-2002; Brambilla *et al.,* 2001; Giudice *et al.,* 2007; Shivaji *et al.,* 2004) This suggests that they contribute to important ecosystem functions in low temperature environments (Chapter 1, Section 1.8). The lower temperatures are unfavorable for the proliferation of bacteria in general (Paul & Clark, 1996; Vorobyova *et al.,* 1997). The harsh environmental conditions are therefore, expected to exert a selective pressure that favours the evolution of unique and adapted actinobacteria.

The sub-Antarctic Marion Island was chosen as a suitable habitat for the present study because not much is known about the microbiology of the island. In addition, the milder climate of the sub-Antarctica is supportive to life compared to the Antarctica. In order to increase the coverage in metagenomic libraries, more than one set of PCR primers was used in this study.

4.3 Construction of 16S rRNA gene libraries

The habitats that contained soils with different major soil characteristics and showed the potential for novelty in actinobacterial diversity (Chapter 3) were selected for this study. Three representative habitats, MI 1.2 (Coastal Fellfield Habitat), MI 5.1 (Cotula Herbfield Habitat) and MI 6.3 (Wet Mire Habitat) were selected for comparative studies (Table 1.1, Section 1.2) (Smith, *et al.,* 2001). The actinobacterial and actinomycete-specific 16S rRNA genes were amplified using PCR (Chapter 3, Section 3.4) and gel-purified (Chapter 2, Section 2.13). The sizes and purity of the DNA fragments were verified on agarose gel before cloning (Fig. 4.1). Pure DNA fragments of expected sizes were recovered (approximately 648bp for actinobacterial and approximately 1267bp for actinomycete specific 16S rRNA genes). These fragments were suitable for cloning and developing metagenomic libraries. They were therefore, separately cloned into the plasmid vector PTZ57R/T (Chapter 2, Section 2.15), to generate metagenomic libraries.

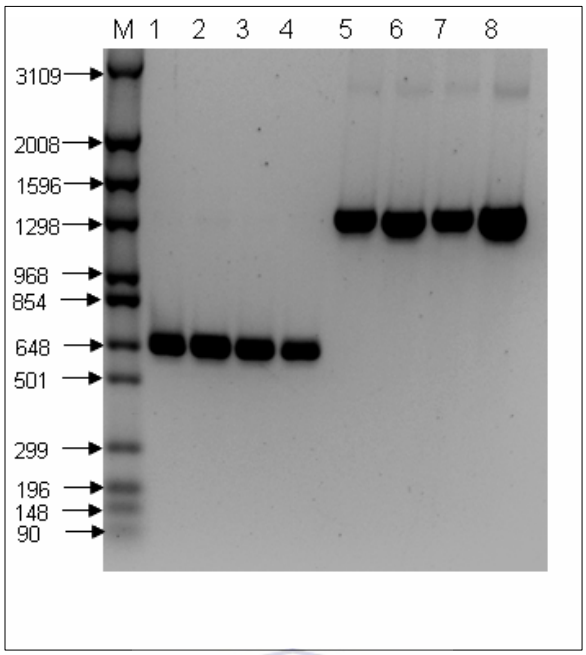


Fig. 4.1: Agarose electrophoresis gel (1%) showing the gel-purified PCR products amplified from metagenomic DNA using actinobacterial-specific primers (S-C-Act-235-a-S-20 and S-C-Act-878-a-S-19) (lanes 1-4) and actinomycete-specific primers (F243 and U1510R) (lanes 5-8) 16S rRNA genes for clone libraries. Lanes: M; Molecular weight marker (bp); 1 & 5, positive control (*Streptomyces albus*); 2-4, Marion Island samples from habitats MI 5.1, MI 1.2 and MI 6.3 respectively; 6-8, Marion Island samples from habitats MI 5.1, MI 1.2 and MI 6.3 respectively.

4.4 Screening of metagenomic libraries

For each resulting library, positive recombinants were randomly selected, and screened for the presence of correct sized DNA fragments (Chapter 2, Section 2.15). The M13 universal primers were used for colony PCR amplification. Fig. 4.2 and Fig. 4.3 show representative results of colony PCR screening from the

first 96 clones in the actinobacterial and actinomycete-specific 16S rRNA gene libraries. In both cases, PCR amplification resulted in DNA fragments of expected sizes, approximately 648bp and 1470bp for actinobacterial and actinomycete-specific 16s rRNA gene libraries respectively. About 99% of the clones were recombinants and contained the expected insert size. These clones were therefore, suitable for sequencing and phylogenetic analysis, because they had fragments of expected sizes.

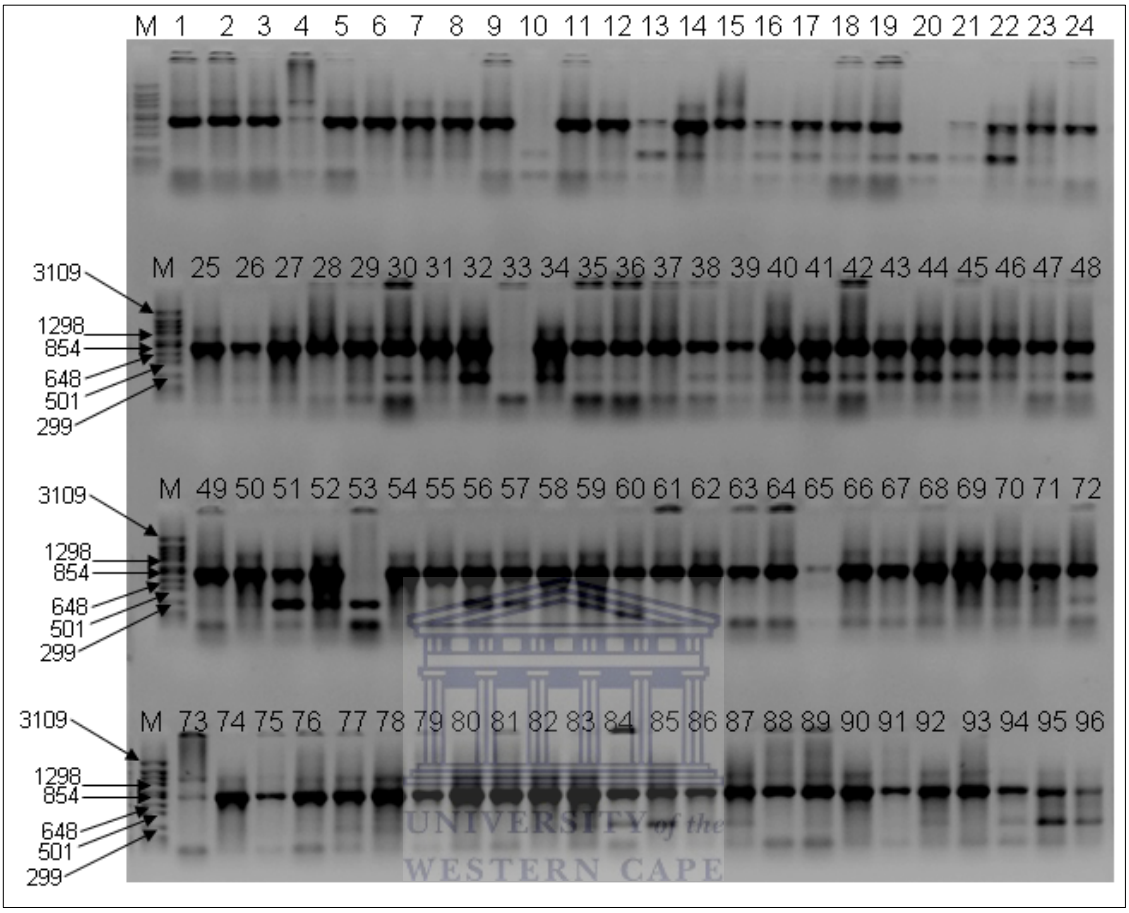


Fig. 4.2: Agarose gel (1%) showing colony PCR screening of an actinobacteria-specific library (amplified using primers S-C-Act-235-a-S-20 and S-C-Act-878-a-S-19) using the primers M13F and M13R. Lanes: M, molecular weight marker (bp); 1-96, individual clones from the metagenomic library.

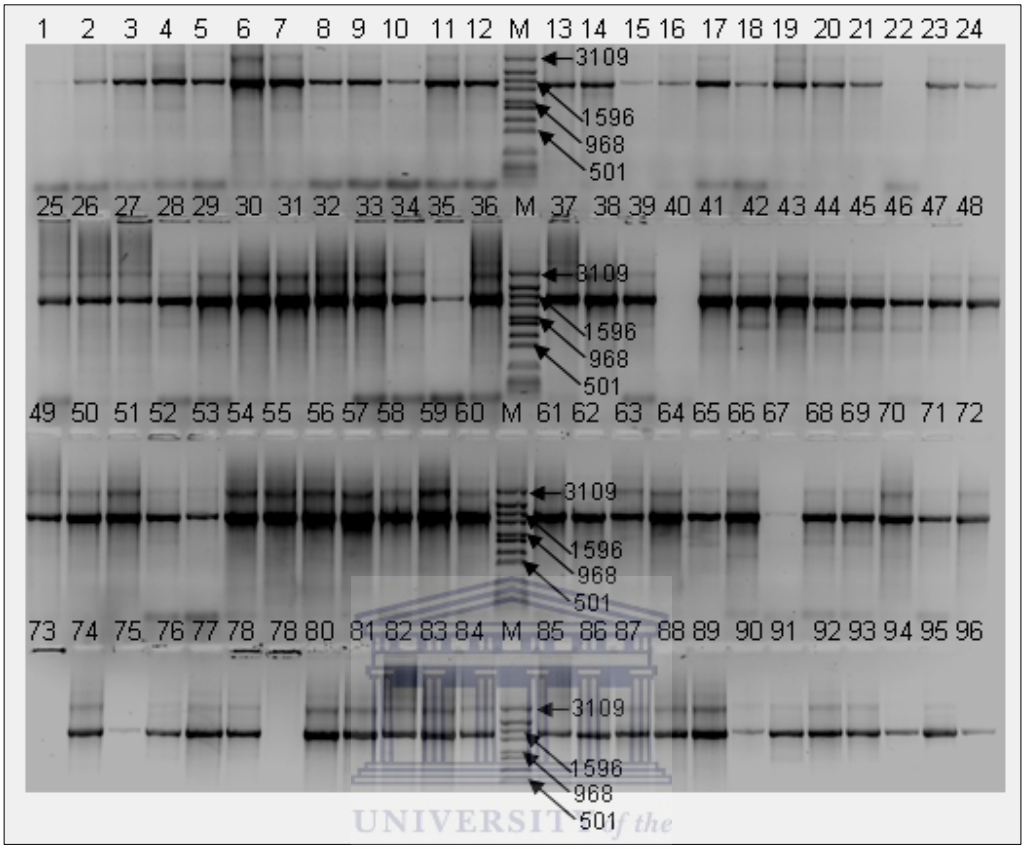


Fig. 4. 3: Agarose gel (1%) showing colony screening PCR of an actinomycete-specific library (amplified using primers F243 and U1510R) using the primers M13F and M13R. Lanes: M; molecular marker (bp); 1-96, individual clones from the metagenomic library.

Fingerprinting methods can be used to distinguish 16S rRNA genes from different microorganisms. Single Strand Conformation Polymorphism (SSCP) (Stach *et al.,* 2003a) and Amplified Ribosomal DNA Restriction Analysis (ARDRA) (Dunbar *et al.,* 1999; Sjöling & Cowan, 2003; Smith *et al.,* 2006; Zhou *et al.,* 1998) are frequently used to screen the diversity within metagenomic libraries. Randomly Amplified Polymorphic DNAs (RAPDs) have also been used

to differentiate between groups of bacteria based on the 16S rRNA gene (El-Fiky *et al.,* 2003; Williams *et al.,* 1990). DGGE is based on the same principle as SSCP and has been used to differentiate between different organisms based on the 16S rRNA gene (Anderson & Wellington, 2001).

In order to be able to reconcile the phylogenetic studies with the profiles established (Chapter 3, Section 3.8) DGGE was used to screen the metagenomic libraries.

4.4.1 Nested PCR-DGGE of the clones

The primer set 341F GC and 534R was used for nested PCR amplification of the cloned 16S rRNA gene fragments. This resulted in fragments of the expected size, 196bp as shown in Fig. 4.4.

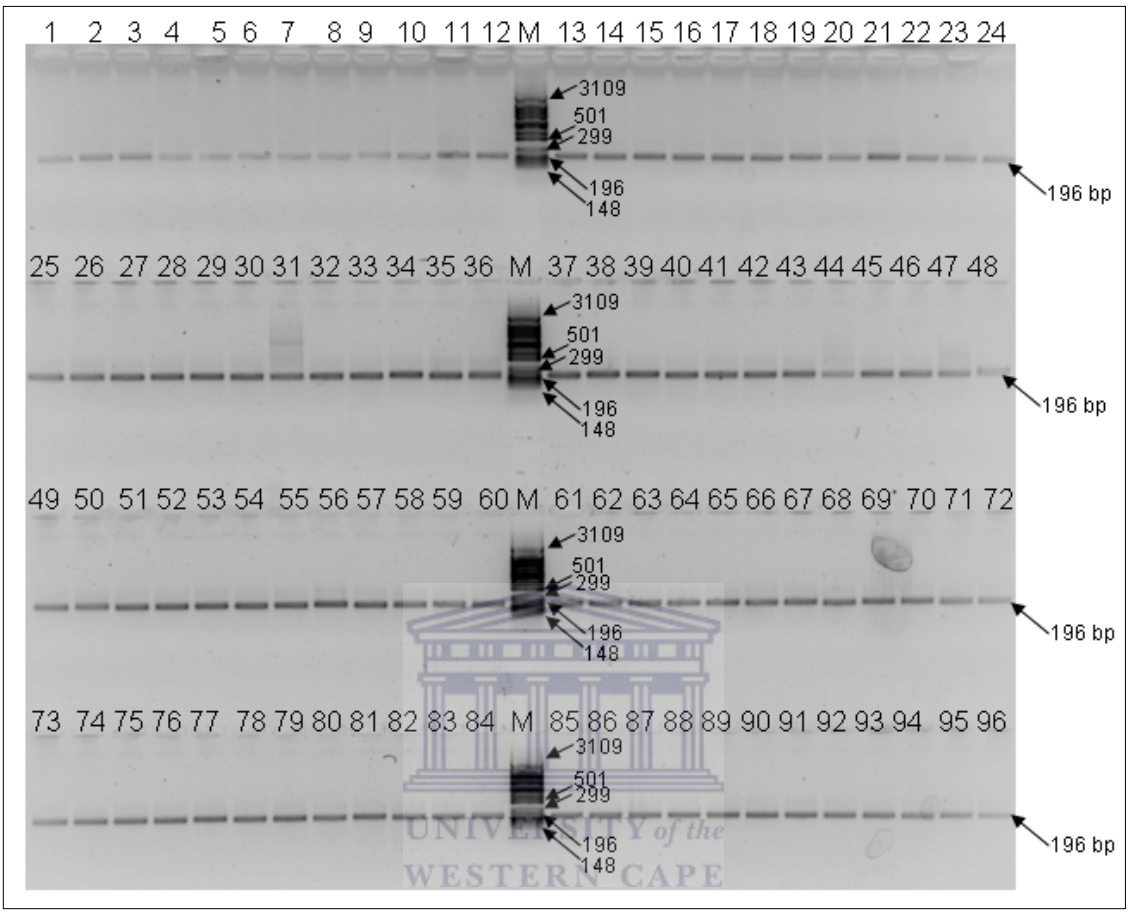


Fig. 4.4: Agarose gel (1%) of representative nested PCR products amplified from 16S rRNA clone libraries using the primers 341F GC and 534R. Lanes: M; Molecular weight marker (bp); 1-96, PCR products of individual 16S rRNA clones.

4.4.2 Screening actinobacterial 16S rRNA metagenomic libraries

The products from the PCR were diluted approximately 50 times to a concentration approximately 2ng/ul in order to be able to clearly separate the single DNA fragments on DGGE and minimize comigration.

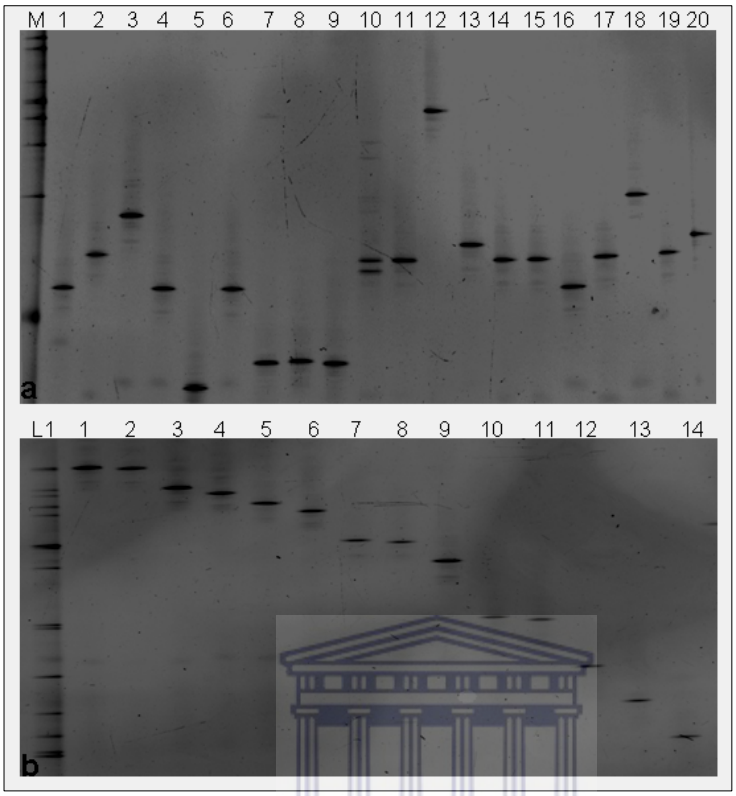


Fig. 4.5: DGGE of the actinobacterial 16S rRNA gene libraries using 45-80% denaturing gradient on a 9% polyacrylamide gel (a) Identification of different genotypes by random screening of the rRNA libraries and (b) Array generated to replicate and identify representative genotypes during further screening of the clone libraries. M; DGGE marker; lanes: L1, probe ladder from already identified clones; 1-20, nested PCR-DGGE products of metagenomic library clones using primers 341F GC and 534R.

The actinobacterial metagenomic libraries were screened using DGGE on a 45 to 80% denaturing gradient (Fig. 4.5). Clones resolving to the same positions were considered identical (replicates) and their frequency of occurrance noted (Fig. 4.5a).

Representative clones were verified by running the mixture of clones against an array of single clones arranged in order of increasing electrophoretic migration. The mixtures of clones were then used to construct reference ladders for further screening of the 16S rRNA libraries (Fig. 4.5b).

Rarefaction curves show the frequencies of occurrence of individual clones during the screening of metagenomic libraries (Fig. 4.6).

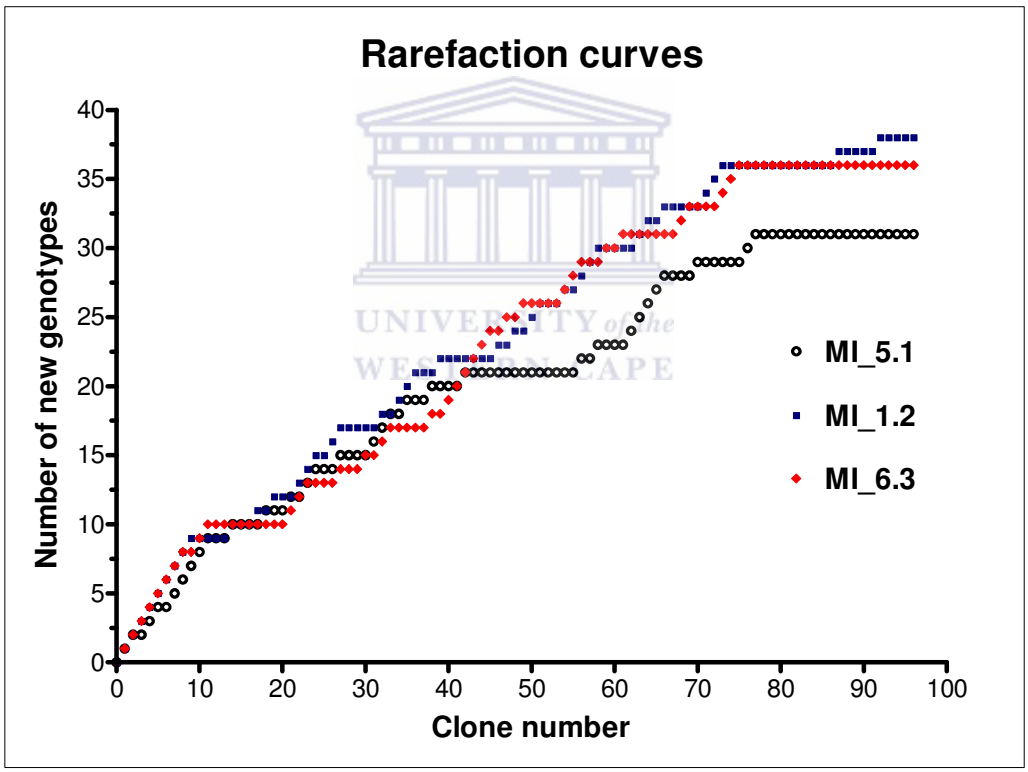


Fig. 4.6: Rarefaction curves showing the estimated diversity and the extent of coverage during the screening of three actinobacterial metagenomic clone libraries from Marion Island terrestrial habitats MI 1.2, MI 5.1, and MI 6.3 using DGGE analysis.

These are used to establish the extent of screening required to ensure full coverage of community diversity, and to determine the extent of the coverage (Stach *et al.,* 2003b). In this study, the rarefaction curves (collection curves) for each library enabled the coverage of each library to be determined.

The actinobacterial 16SrRNA gene libraries from habitats MI 5.1 and MI 6.3 were screened to almost full coverage as shown by the saturation of diversity in the non-randomized rarefaction curves (Fig. 4.6). The rarefaction curve for habitat MI 1.2 was almost leveling off, showing that the diversity of most genotypes was covered. In addition, the extent of diversity coverage was calculated at 85% for habitat MI 1.2, 88% for habitat MI 5.1 and 84% for habitat 6.3 using the Good estimate (Good, 1953). The total numbers of different actinobacterial genotypes (species diversity) were estimated at 32 in habitat MI 5.1, 40 in habitat MI 1.2 and 38 in habitat MI 6.3.

In addition, a composite of all unique 16S rRNA clones identified from each metagenomic library were compared to the corresponding environmental samples in order to further verify completeness of the coverage (Fig. 4.7). In all the actinobacterial libraries, most of the genotypes in the environmental samples could be identified against the clones in the ladders showing enough coverage for each library

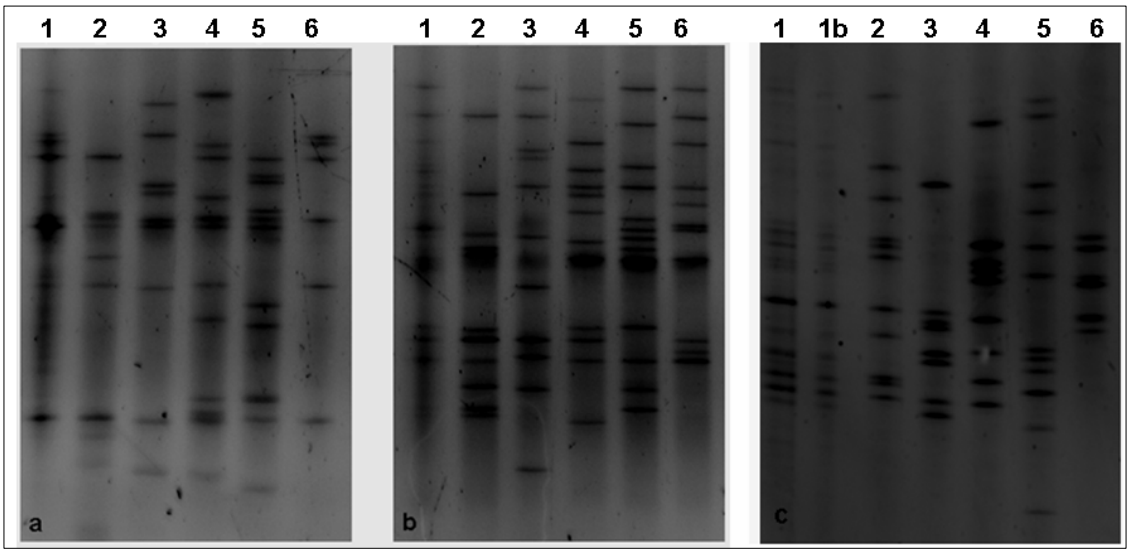


Fig. 4.7: DGGE to compare the composite of all genotypes recovered from the clone libraries against their environmental samples. PCR-DGGE used actinobacterial specific primers (S-C-Act-235-a-S-20 and S-C-Act-878-a-S-19) and products were analyzed using DGGE on a 45-80% denaturing gradient in 9% polyacrylamide gels. Lanes: 1, Environmental samples; 1b, diluted environmental sample; 2-5, composite clone ladders L1, L2, L3, L4, and L5 from metagenomic libraries from habitats (a) MI 6.3; (b) MI 1.2 , and (c) MI 5.1.

It is important to note that the majority of DNA fragments in environmental samples were well resolved thereby reducing the chances of analysing co-migrating bands consisting of more than one genotype.

4.4.3 Screening actinomycete 16S rRNA metagenomic libraries

Most of the metagenomic clones identified from habitat MI 5.1 that were targeted for actinomycetes using primer set F243 and U1510R belonged to the order

Verrucomicrobiales. Sequence analysis of the first 96 clones showed that only 14% were actinomycetes and the rest belonged to the order *Verrucomicrobiales*. The primer set was therefore, not very stringent even after optimization. The products were not suitable for developing metagenomic clone libraries for studying actinomycete diversity. Even though primer F243 was not very stringent, it is normally recommended as an additional primer, used for increasing the coverage of diversity (Heuer *et al.,* 1997). The primer was used because a single primer set will not amplify all the members of a given target group in environmental samples considering that some of the uncultured members are unknown (Baker *et al.,* 2003). However, the failure to amplify most actinomycetes as expected may also have been a result of the lower representation of actinomycetes in Marion Island soils compared to members of the order *Verrucomicrobiales*.

4.5 Diversity and phylogenetic analysis

The amplified DNA sequences were identified as fragments of the 16S rRNA genes through homology searches using BLAST against the NCBI non-redundant database. The closest matches were retrieved and aligned against the clone sequences from this study using BioEdit (Appendix A10). The alignments were subjected to cluster analysis using *Mega4* (Chapter 2, Section 2.17) resulting in a phylogenetic tree (Fig. 4.9).

Clusters generated in the phylogenetic tree were classified into suborders and families, based on the reliability provided by the bootstrap values and phylogenetic distances. The sequences from known cultured species were used as positive controls. Phylogenetic analysis showed the presence of a variety of actinobacterial groups in different habitats, resulting in different community structures. The following section compares the occurrence of actinobacterial phylogenetic groups in Marion Island terrestrial habitats.

4.5.1 Actinobacterial diversity indices in specific habitats

The Shannon-Wiener indices were used to compare the species richness, evenness and diversity within the habitats (Table 4.1). The indices were calculated using the frequencies of occurrence of clones that represented different species in metagenomic clone libraries, which were identified by sequencing.

Table 4.1: The diversity, richness and evenness of actinobacterial genospecies in Marion Island terrestrial habitats calculated using the Shannon-Wiener index. Frequencies obtained from screening of metagenomic clone libraries. ΣH, Shannon-Weinner diversity index; S, species richness (total number of species); n, number of species present, Pi, the proportion of clones from a species and E_H, evenness.

Genus	Species code	n	Pi	ln(Pi)	-H	S	lnS	EH
Habitat MI 5.1								
Acidimicrobium	MI 5.1 A10	4	0.04	-3.10	0.14			
Acidimicrobium	MI 5.1 A11	3	0.03	-3.39	0.11			
Acidimicrobium	MI 5.1 F8	1	0.01	-4.49	0.05			
Acidimicrobium	MI 5.1 H10	3	0.03	-3.39	0.11			
Acidimicrobium	MI 5.1 A9	1	0.01	-4.49	0.05			
Acidimicrobium	MI 5.1 D1	3	0.03	-3.39	0.11			
Acidimicrobium	MI 5.1 E12	3	0.03	-3.39	0.11			
Acidothermus	MI 5.1 B8	3	0.03	-3.39	0.11			
Acidothermus	MI 5.1 C11	2	0.02	-3.80	0.09			
Actinomadura	MI 5.1 A1	5	0.06	-2.88	0.16			
Actinomadura	MI 5.1 A3	5	0.06	-2.88	0.16			
Actinomadura	MI 5.1 A7	7	0.08	-2.54	0.20			
Actinomadura	MI 5.1 B4	5	0.06	-2.88	0.16			
Actinomadura	MI 5.1 B12	1	0.01	-4.49	0.05			
Actinomadura	MI 5.1 D6	2	0.02	-3.80	0.09			
Actinomadura	MI 5.1 F6	1	0.01	-4.49	0.05			
Blastococcus	MI 5.1 A8	16	0.18	-1.72	0.31			
Ferrimicrobium	MI 5.1 F9	2	0.02	-3.80	0.09			
Ferrimicrobium	MI 5.1 F7	1	0.01	-4.49	0.05			
Ferrimicrobium	MI 5.1 G9	1	0.01	-4.49	0.05			
Frankia	MI 5.1 F2	1	0.01	-4.49	0.05			
Frankia	MI 5.1 G8	2	0.02	-3.80	0.09			
Humicoccus	MI 5.1 D10	2	0.02	-3.80	0.09			
Kitasatospora	MI 5.1 C2	3	0.03	-3.39	0.11			
Modestobacter	MI 5.1 C12	5	0.06	-2.88	0.16			
Mycobacterium	MI 5.1 E11	1	0.01	-4.49	0.05			
Mycobacterium	MI 5.1 A5	1	0.01	-4.49	0.05			
Thermomonospora	MI 5.1 F10	1	0.01	-4.49	0.05			
Thermomonospora	MI 5.1 F3	1	0.01	-4.49	0.05			
Thermomonospora	MI 5.1 D3	3	0.03	-3.39	0.11			
Total		**89.00**	**1.00**	**0.00**	**3.08**	**31.00**	**3.43**	**0.90**

Table 4.1 continued

Genus	Species code	n	Pi	ln(Pi)	-H	S	lnS	EH
Habitat MI 1.2								
Acidothermus	MI 1.2 B1	1.00	0.01	-4.64	0.04			
Acidothermus	MI 1.2 A7	1.00	0.01	-4.64	0.04			
Acidothermus	MI 1.2 A7	2.00	0.02	-3.95	0.08			
Actinomadura	MI 1.2 A4	2.00	0.02	-3.95	0.08			
Actinomadura	MI 1.2 H10	1.00	0.01	-4.64	0.04			
Arthrobacter	MI 1.2 A11	7.00	0.07	-2.70	0.18			
Arthrobacter	MI 1.2 F9	1.00	0.01	-4.64	0.04			
Arthrobacter	MI 1.2 D11	1.00	0.01	-4.64	0.04			
Arthrobacter	MI 1.2 C1	6.00	0.06	-2.85	0.16			
Dermatophilus	MI 1.2 B9	1.00	0.01	-4.64	0.04			
Dermatophilus	MI 1.2 C6	3.00	0.03	-3.55	0.10			
Dermatophilus	MI 1.2 C6	3.00	0.03	-3.55	0.10			
Ferrimicrobium	MI 1.2 B4	5.00	0.05	-3.03	0.15			
Ferrimicrobium	MI 1.2 B6	2.00	0.02	-3.95	0.08			
Ferrimicrobium	MI 1.2 E6	1.00	0.01	-4.64	0.04			
Humicoccus	MI 1.2 F2	2.00	0.02	-3.95	0.08			
Humicoccus	MI 1.2 G3	1.00	0.01	-4.64	0.04			
Intrasporangium	MI 1.2 C5	1.00	0.01	-4.64	0.04			
Janibacter	MI 1.2 A1	2.00	0.02	-3.95	0.08			
Knoellia	MI 1.2 F1	2.00	0.02	-3.95	0.08			
Knoellia	MI 1.2 G11	1.00	0.01	-4.64	0.04			
Microbacterium	MI 1.2 E11	1.00	0.01	-4.64	0.04			
Mycobacterium	MI 1.2 B12	1.00	0.01	-4.64	0.04			
Nocardioides	MI 1.2 A5	2.00	0.02	-3.95	0.08			
Oerskovia	MI 1.2 E4	1.00	0.01	-4.64	0.04			
Propionicicella	MI 1.2 F8	2.00	0.02	-3.95	0.08			
Rhodococcus	MI 1.2 C3	9.00	0.09	-2.45	0.21			
Rhodococcus	MI 1.2 E12	4.00	0.04	-3.26	0.13			
Streptosporangium	MI 1.2 A8	5.00	0.05	-3.03	0.15			
Terracoccus	MI 1.2 A2	3.00	0.03	-3.55	0.10			
Terracoccus	MI 1.2 A3	9.00	0.09	-2.45	0.21			
Tessaracoccus	MI 1.2 D7	3.00	0.03	-3.55	0.10			
Tessaracoccus	MI 1.2 E5	3.00	0.03	-3.55	0.10			
Tessaracoccus	MI 1.2 B11	6.00	0.06	-2.85	0.16			
Tessaracoccus	MI 1.2 C2	1.00	0.01	-4.64	0.04			
Tessaracoccus	MI 1.2 E9	1.00	0.01	-4.64	0.04			
Thermomonospora	MI 1.2 B10	6.00	0.06	-2.85	0.16			
Thermomonospora	MI 1.2 F3	1.00	0.01	-4.64	0.04			
Total		**104.00**	**1.00**	**0.00**	**3.35**	**39.00**	**3.66**	**0.91**

Table 4.1 continued

Genus	Species code	n	Pi	ln(Pi)	-H	S	lnS	EH
Habitat MI 6.3								
Acidimicrobium	MI 6.3 A3	5	0.05	-2.92	0.16			
Acidimicrobium	MI 6.3 C3	3	0.03	-3.43	0.11			
Acidimicrobium	MI 6.3 C6	7	0.08	-2.59	0.19			
Acidimicrobium	MI 6.3 C8	1	0.01	-4.53	0.05			
Acidimicrobium	MI 6.3 D11	2	0.02	-3.84	0.08			
Acidimicrobium	MI 6.3 E6	1	0.01	-4.53	0.05			
Acidimicrobium	MI 6.3 F1	10	0.11	-2.23	0.24			
Acidimicrobium	MI 6.3 F9	1	0.01	-4.53	0.05			
Acidimicrobium	MI 6.3 G2	1	0.01	-4.53	0.05			
Acidimicrobium	MI 6.3 D9	1	0.01	-4.53	0.05			
Acidimicrobium	MI 6.3 E11	1	0.01	-4.53	0.05			
Acidothermus	MI 6.3 G1	1	0.01	-4.53	0.05			
Actinomadura	MI 6.3 A1	1	0.01	-4.53	0.05			
Aestuariimicrobium	MI 6.3 E7	2	0.02	-3.84	0.08			
Blastococcus	MI 6.3 D7	3	0.03	-3.43	0.11			
Blastococcus	MI 6.3 A8	2	0.02	-3.84	0.08			
Ferrimicrobium	MI 6.3 A10	12	0.13	-2.05	0.26			
Ferrimicrobium	MI 6.3 B10	2	0.02	-3.84	0.08			
Ferrimicrobium	MI 6.3 G4	2	0.02	-3.84	0.08			
Ferrimicrobium	MI 6.3 G5	1	0.01	-4.53	0.05			
Ferrimicrobium	MI 6.3 G9	2	0.02	-3.84	0.08			
Ferrimicrobium	MI 6.3 A4	2	0.02	-3.84	0.08			
Ferrimicrobium	MI 6.3 A7	3	0.03	-3.43	0.11			
Frankia	MI 6.3 A9	1	0.01	-4.53	0.05			
Kineosporia	MI 6.3 D6	2	0.02	-3.84	0.08			
Kineosporia	MI 6.3 D8	2	0.02	-3.84	0.08			
Kineospora	MI 6.3 C9	1	0.01	-4.53	0.05			
Mycobacterium	MI 6.3 E1	1	0.01	-4.53	0.05			
Mycobacterium	MI 6.3 A2	3	0.03	-3.43	0.11			
Mycobacterium	MI 6.3 B11	5	0.05	-2.92	0.16			
Mycobacterium	MI 6.3 D4	1	0.01	-4.53	0.05			
Mycobacterium	MI 6.3 E8	1	0.01	-4.53	0.05			
Mycobacterium	MI 6.3 G3	2	0.02	-3.84	0.08			
Nostocoida	MI 6.3 D2	1	0.01	-4.53	0.05			
Sporichthya	MI 6.3 A6	2	0.02	-3.84	0.08			
Streptomyces	MI 6.3 A5	5	0.05	-2.92	0.16			
		93.00	**1.00**	**0.00**	**3.25**	**38.00**	**3.64**	**0.89**

The highest diversity indices for actinobacterial genospecies were obtained in habitats MI 1.2 (H=3.4) and MI 5.1 (H=3.4) compared to MI 6.3 (H=3.3). This showed that habitats MI 1.2 and MI 5.1 contained higher actinobacterial diversity, and possibly more unique species compared to MI 6.3. Habitat MI 1.2 was also characterised by the highest species richness of actinobacteria (S=39) compared to habitats MI 5.1 (S=34) and MI 6.3 (S=38). Similarly, habitat MI 1.2 had relatively higher equitability of species as shown by the highest value of evenness (E_H=0.91) compared to habitats MI 5.1 (E_H=0.90) and MI 6.3 (E_H=0.89). The equitability of species distribution was however, not very different amongst the three habitats. The values for species evenness were generally high in all the three habitats. They normally range from zero to one and an E_H value of one shows complete evenness. Lozupone & Knight (2007) showed that saline environments had significantly higher phylogenetic diversity of bacteria compared to other environments (Lozupone & Knight, 2007).

4.5.1.1 Proportions of actinobacterial suborders

The values of diversity index were generally low in all the three habitats (ranging between 3.1 and 3.4) because not all the actinobacterial diversity (Chapter 1, Fig. 1.2) was identified from the metagenomic clone libraries. Instead, most of the actinomycetes identified in this study belonged to few suborders. The Shannon-Weiner diversity index increases by either having more unique species or by having greater species evenness. A total of 286 clones were identified as members of the class actinobacteria, contributing 98% of all the clones sampled.

These clones comprised of 111 actinobacterial genotypes. Few of the clones were from non-actinobacteria, including members of the *Gemmatimonadetes* and a *Gammaproteobacteria* (Appendix A10).

Amongst the total actinobacterial clones sampled (Appendix A10), 31.1% (89 clones) were from habitat MI 5.1, 36.4% (104 clones) from habitat MI 1.2 and 32.5% (93 clones) from habitat MI 6.3. None of the actinobacterial orders *Rubrobacterales, Coriobacterales, Sphaerobacterales* or *Bifidobacterales* was represented amongst the clone libraries from all three habitats. Most clones belonged to the order *Actinomycetales* (72.7%), except for members of the order *Acidimicrobiales* (27.3%). None of the actinomycete genotypes belonged to the suborders *Actinomycineae, Micromonosporineae, Pseudonocardineae* or *Glycomycineae*. The proportions of members of the actinobacterial suborders were not equally represented within specific habitats (Fig. 4.8).

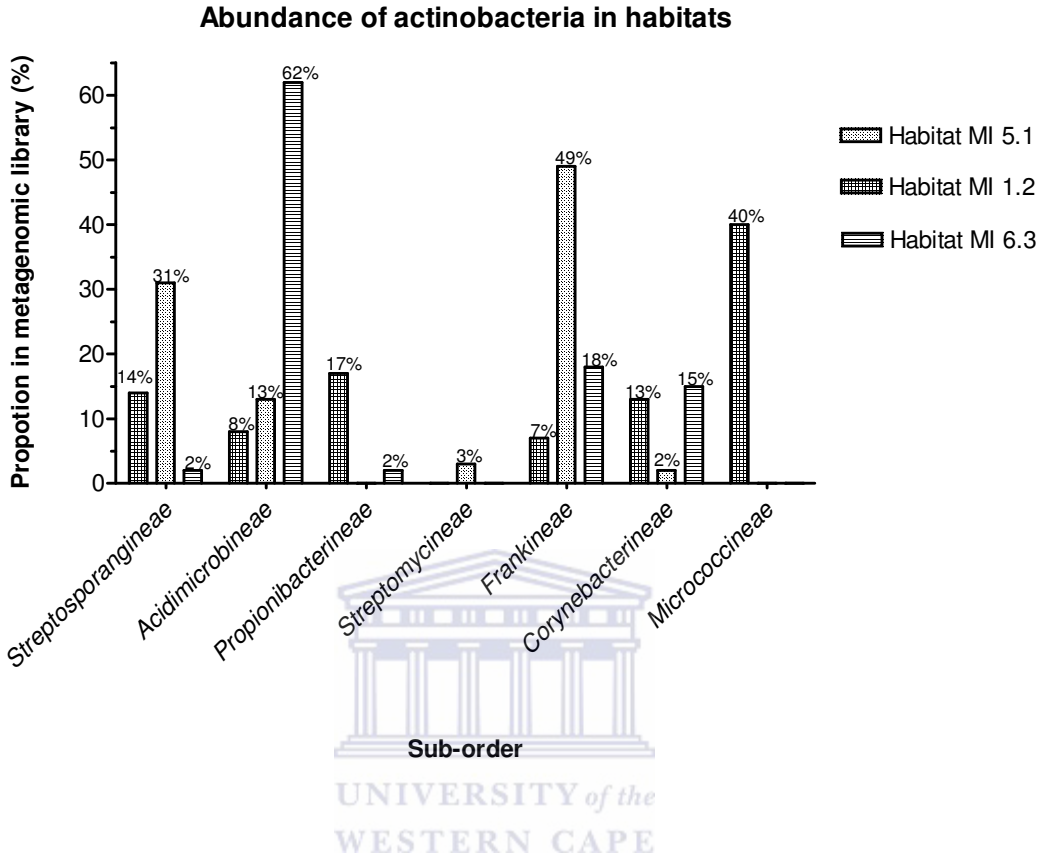


Fig. 4.8: Comparison of the distribution of genotypes belonging to actinobacterial suborders *Streptosporangineae, Acidimicrobineae,, Propionibacterineae, Streptomycineae, Frankineae, Corynebacterineae* and *Micrococcineae* as a proportion of the total actinobacterial clones in metagenomic libraries from Marion Island terrestrial habitats MI 5.1, MI 1.2 and MI 6.3.

The members *Streptosporangineae* and *Frankineae* dominated in habitat MI 5.1 (31%, 11 genotypes and 49%, 13 genotypes respectively). Whilst the *Acidimicrobineae* occurred in all habitats, they dominated in habitat MI 6.3 (62%, 21 genotypes). The *Micrococcineae* occurred only in habitat MI 1.2, forming the

dominant group of actinobacteria (40%, 17 genotypes). The members from other suborders occurred in low proportions and genetic diversity in all habitats.

Even though the *Propionibacterineae* were not in high proportions, they were almost exclusive to habitat MI 1.2, making up 17% of the actinobacteria and consisting of seven genotypes. Members of the genus *Streptomyces* are the most frequently isolated bacterial group from soil habitats. Surprisingly, *Streptomycineae* constituted only 1% of the total actinobacterial clones, occurring only in habitat MI 5.1 (3%). This may be a true reflection of their occurrence in the habitats, or a result of bias arising during the cell lysis step of metagenomic DNA extraction. Most members of *Streptomyces* are filamentous and spore forming, which could make them resistant to lysis. However, primers used here are known to amplify *Streptomycineae* 16S rRNA genes in environmental samples (Stach *et al.,* 2003a; Stach *et al.,* 2003b). In this study, the modified Miller bead beating procedure, consisting of chemical and physical lysis should have ensured efficient cell lysis. The *Corynebacterineae* consisted of no more than 15% of the actinobacteria in each habitat.

Frostegård *et al.,* (1999) reported the differential representation of the members of the actinomycetes in a study involving the *in situ* cell lysis followed by DNA extraction. A combination of chemical or enzymatic lysis, accompanied by mechanical methods such as grinding or bead beating was therefore, recommended. These methods were found to be more efficient for the lysis of

microorganisms with spores and resistant cell walls from soil matrices (Frostegård *et al.,* 1999).

4.5.1.2 Species richness and genetic diversity

The members of suborders that occurred in high abundance (phylogenetic diversity) (Fig. 4.8) did not proportionately consist of high numbers of different species (genetic diversity) and vice versa (Fig. 4.10, Table 4.1). Most bacterial species usually occur in low abundance in complex microbial communities, which makes the complete coverage of all bacterial species (genetic diversity) difficult. The species richness (S) estimates obtained in metagenomic studies therefore; represent a fraction of the true species richness in these ecosystems. In addition, the diversity estimates depend on the size of the clone libraries (Dunbar *et al.,* 2002; Youssef & Elshahed, 2008).

In this study, the highest numbers of single actinobacterial species that occurred in habitat MI 1.2 were from the genera *Tessaracoccus* (7), *Arthrobacter* (4), *Dermatophilus* (3) and *Ferrimicrobium* (3). The rest of the genera occurred as one or two species. The highest proportions were from the two species of the genera *Thermomonospora* (6 and 7% respectively), a *Rhodococcus* (9%) and a *Terrasacoccus* (9%). The highest numbers of single species occurring in habitat MI 5.1 belonged to the genera *Acidimicrobium* (8), *Actinomadura* (7) *Ferrimicrobium* (3) and *Thermomonospora* (3). Most actinobacterial species occurred in proportions ranging between 1 and 3% except for an *Actinomadura*

(8%), two species of *Actinomadura* (6% respectively), a *Blastococcus* (18%) and a *Modestobacter* (6%). In habitat MI 6.3, the highest numbers of a single species were from the genera *Acidimicrobium* (11), *Ferrimicrobium* (7) and *Mycobacterium* (6). Most actinobacterial species occurred in proportions ranging between 1 and 3% except for three *Acidimicrobium* (5%, 8% and 11% respectively), a *Ferrimicrobium* (13%), a *Mycobacterium* (5%) and a *Streptomyces* (5%).

The problems of diversity estimation could be improved by increasing the sampling coverage (Youssef & Elshahed, 2008). The problem of underestimating the genetic diversity of actinobacteria communities using species richness estimations was also reported for actinobacterial communities (Stach *et al.,* 2003b). The species richness and abundance reported in this study does not therefore, completely reflect the actual genetic diversity of actinobacterial communities. However, these could be improved by further screening effort, which is expensive.

4.5.2 Actinobacterial diversity in habitats

Phylogenetic analysis of the 16S rRNA gene sequences showed that most of the actinobacterial genotypes clustered into suborders and families according to habitats of origin (Fig. 4.9). In addition, some genotypes belonged to suborders that were only found in specific habitats, some that were over-represented, and others under-represented in other habitats (Fig. 4.10). Greater confidence in the

phylogenetic placement of the actinobacterial genotypes could be obtained if several algorithms were compared for the construction of the phylogenetic trees. These include Unweighted Pair Group Method with Arithmetic Mean (UPGMA), Neighbor joining (NJ), Maximum likelihood (ML) and Maximum parsimony methods (MP) (Section 4.2). The NJ method was selected in this study because it is relatively fast and suitable for analyzing large data sets with varying rates of evolution (Kumar *et al.*, 2004; Saitou & Nei, 1987).

The following section describes the community structures of each habitat based on the diversity of the genotypes identified. Most of the DNA sequences showed sequence identities ranging between 89 and 100 % to sequences of genotypes in the NCBI database (Table 4.2; Appendix A10). Actinobacterial genotypes that showed less than 99% identities in 16S rRNA gene sequence may belong to different species. This is because they usually show less than 70% DNA-DNA homology (Stach *et al.,* 2003b; Stackebrandt & Goebel, 1994). For other bacteria, a 16S rRNA gene sequence identity of less than 97% represents a new species (Wayne *et al.,* 1987).

Only one genotype showed 100% sequence identity to a known cultured species, which was identified as an *Arthrobacter* in habitat MI 1.2. The rest of the genotypes showed closest identities to uncultured sequences from other environmental surveys. This was a proportion of 0.9% cultured representatives out of the 111 actinobacterial genotypes identified from the metagenomic

libraries. This finding supports the claims that only less than 1% of genotypes identified from environmental metagenomic clone libraries are cultured species (Amann *et al.,* 1995).

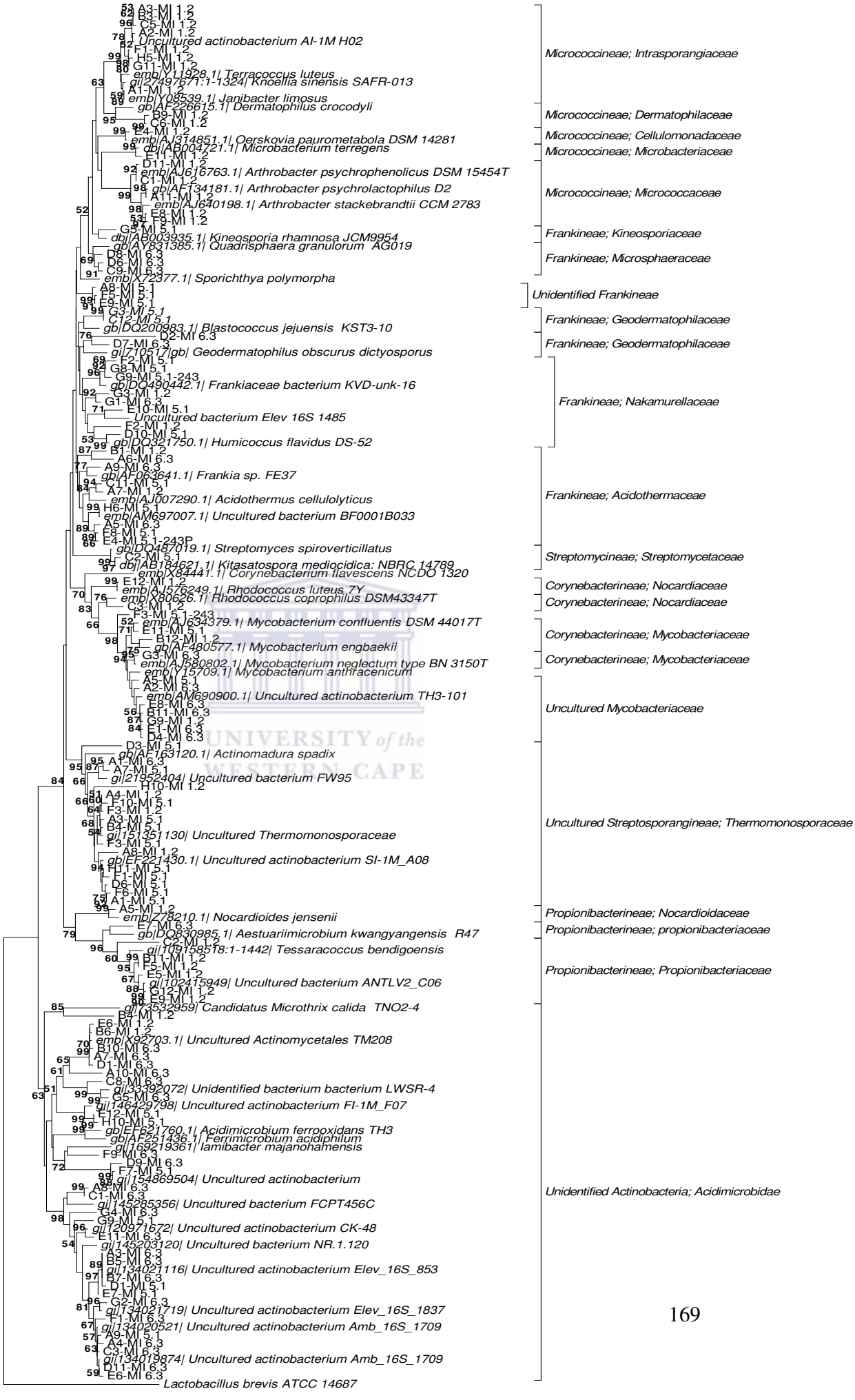


Fig. 4. 9: Phylogenetic relationships amongst the actinobacteria from Marion Island terrestrial habitats MI 5.1, MI 1.2 and MI 6.3. Tree drawn based on 550bp alignment using *MEGA 4* (Tamura *et al.,* 2007), Neighbor-Joining method, 1000 bootstrap replicates, Maximum Composite Likelihood substitution model with pairwise deletion of gaps, substitutions include transitions and transversions, and pattern among lineages assumed homogeneous (Felsenstein, 1985; Saitou & Nei, 1987; Tamura *et al.,* 2004). Scale shows units of the number of base substitutions per site.

In each habitat, most of the clones showed sequence homologies ranging between 89 to 96% to those from the databases, suggesting the presence of abundantly uncultured actinobacteria in environmental samples. Most of these may belong to new genera and species (Table 4.2 and Appendix A10).

The genotypes showing less than 97% 16S rRNA sequence identities to database sequences comprised 75% of the total clones identified in habitat MI 5.1, 69.8% in MI 6.3 and 40% in habitat MI 1.2. Amongst these, genotypes showing sequence identities between 89 and 94% to database sequences were 60.5% from habitat MI 6.3, 27.8% from MI 5.1 and only 15.4% from MI 1.2. The details of the BLAST results are shown in Appendix A10.

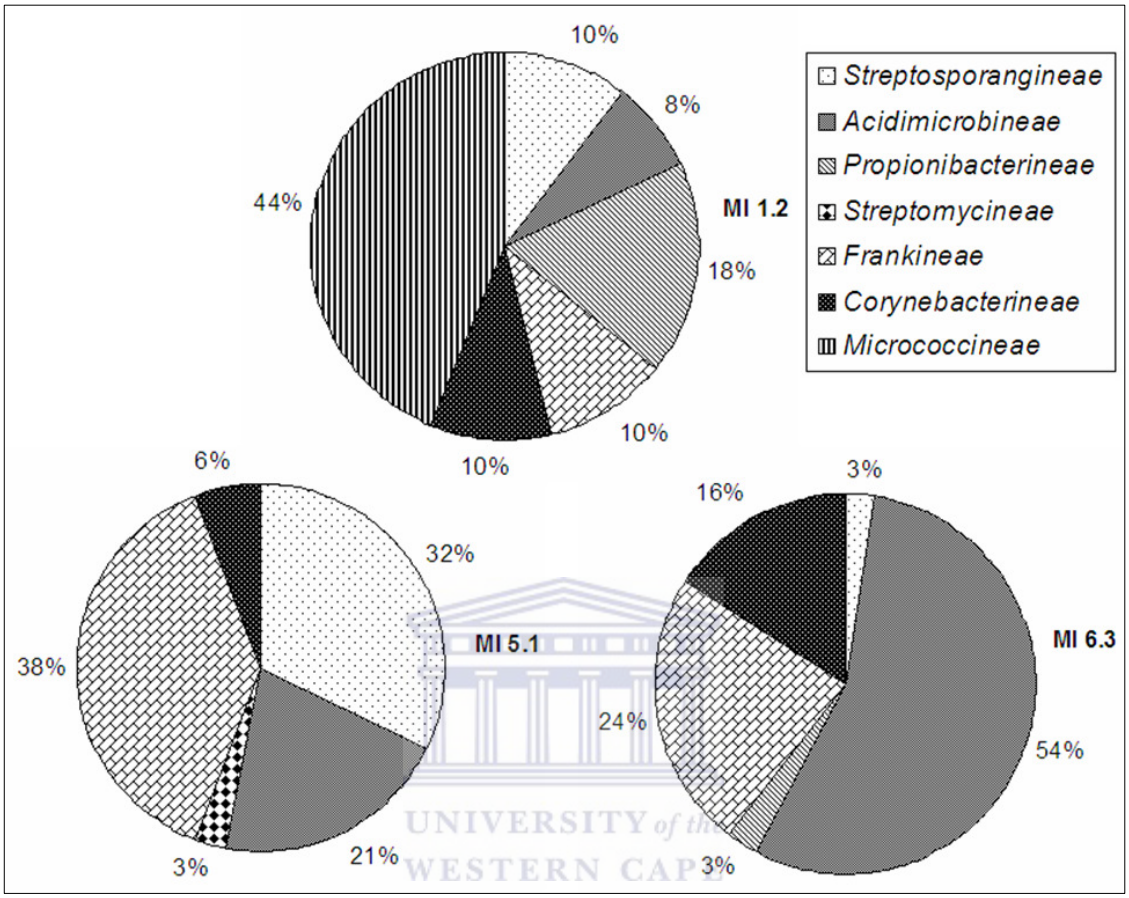


Fig. 4. 10: Actinobacterial diversity in Marion Island terrestrial habitats MI 1.2, MI 5.1 and MI 6.3 based on the proportions of representative genotypes from suborders identified in each habitat.

Table 4.2: The proportions of representative actinobacterial genotypes that were present in clone libraries from Marion Island terrestrial habitats and their sequence identities to known closest relatives. Proportions are indicated as a percentage of the total actinobacterial genotypes identified using BLAST of the NCBI database (http://www.ncbi.nlm.nih.gov/) in each habitat.

	Sequence identity (%)				
Habitat	**100**	**97-99**	**95-96**	**89-94**	**89-96**
MI 5.1	0.0	25.0	47.2	27.8	75.0
MI 1.2	2.6	56.4	25.6	15.4	41.0
MI 6.3	0.0	30.2	9.3	60.5	69.8

4.5.2.1 Actinobacterial diversity in habitat MI 5.1

Out of the total 34 genotypes, the actinobacteria consisted of members from the suborders *Frankineae* (38%), *Streptosporangineae* (32%), *Acidimicrobineae* (21%), *Corynebacterineae* (6%) and *Streptomycineae* (3%). However, the *Micrococcineae* and *Propionibacterineae* were not present in habitat MI 5.1 (Fig. 4.10). Thus, the three suborders, *Frankineae, Acidimicrobineae* and *Streptosporangineae*, comprised 91% of the actinobacterial diversity occurring in habitat MI 5.1. The *Frankineae* occurring in this habitat were mostly unknown, uncultured members. Some of these were related to *Sporichthya polymorpha* (Fig. 4.9). Other *Frankineae* belonged to the families *Kineosporaceae* (genus *Kineospora*), *Geodermatophilaceae* (genus *Geodermatophilus*) and *Acidothermaceae* (genus *Acidothermus*). The *Streptosporangineae* were all uncultured members of the family *Thermomonosporaceae*, related to *Actinomadura spadix,* and were predominantly found in this habitat. Similarly, to

habitat MI 6.3, the *Acidimicrobineae* were all uncultured and related to *Acidimicrobium ferooxidans, Iamibacter majanohamensis* and *Ferrimicrobium acidiphilum.* The *Corynebacterineae* were all members of the family *Corynebacteriaceae* (genus *Corynebacterium*). Only a single *Streptomycineae* (family *Streptomycetaceae,* genus *Streptomyces*) genotype occurred in this habitat.

4.5.2.2 Actinobacterial diversity in habitat MI 1.2

The 39 actinobacterial genotypes identified were from the suborders *Micrococcineae* (44%), *Propionibacterineae* (18%), *Frankineae* (10%), *Streptosporangineae* (10%), *Corynebacterineae* (10%) and *Acidimicrobineae* (8%) (Fig. 4.10). The members of *Streptomycineae* were absent from habitat MI 1.2. Thus, two suborders, *Micrococcineae* and *Propionibacterineae* dominated habitat MI 1.2, comprising 62% of the actinobacterial diversity, and were almost entirely absent from habitats MI 5.1 and MI 6.3. In addition, the members of other suborders that were found in habitat MI 1.2 were more represented (10% each) than in other habitats. Most of the *Micrococcineae* in habitat MI 1.2 were uncultured members as shown by pronounced phylogenetic distances separating the clades from the neighboring groups and high bootstrap values (at least 90%) (Fig. 4.9). The *Micrococcineae* included members that belonged to the families *Intrasporangiaceae* (genera *Terracoccus* and *Knoellia*), *Dermatophilaceae* (genera *Dermatophilus* and *Janibacter*), *Microbacteriaceae* and *Micrococcaceae* (genus *Arthrobacter*).

Both families of the suborder *Propionibacterineae*, *Nocardioidaceae* (genus *Nocardioides*) and *Propionibacteriaceae* (Uncultured *Tessaracoccus* genera), were also found in this habitat. Amongst the *Frankineae,* only members of the families *Nakamurellaceae* (uncultured *Humicoccus* genera) and *Acidothermaceae* (uncultured *Acidothermus* genera) occurred in habitat MI 1.2.

Only two genotypes were of the suborder *Streptosporangineae* (*Actinomadura* genus), which consisted of uncultured members, were present in this habitat. The *Corynebacterineae* found in habitat MI 1.2 were novel and uncultured, belonging to the families *Nocardiaceae* (genera *Rhodococcus*) and *Mycobacteriaceae* (an uncultured *Mycobacterium*). All the *Acidimicrobineae* occurring in habitat MI 1.2 were uncultured and related to those found in habitats MI 5.1 and MI 6.3, but occurred in very low proportion in comparison (8%).

4.5.2.3 Actinobacterial diversity in habitat MI 6.3

The 38 genotypes identified in habitat MI 6.3 were from the suborders *Acidimicrobineae* (54%), *Frankineae* (24%), *Corynebacterineae* (16%), *Propionibacterineae* (3%) and *Streptosporangineae* (3%). The *Streptomycineae* and *Micrococcineae* were not present in this habitat (Fig. 4.10). Members from three suborders, *Acidimicrobineae, Frankineae* and *Corynebacterineae* dominated the diversity in this habitat, accounting for 94% of the actinobacterial diversity.

All members of the suborder *Acidimicrobineae* were uncultured, mostly showing sequence identities between 89 and 94% to *Acidimicrobium ferooxidans, Iamibacter majanohamensis* or *Ferrimicrobium acidiphilum* (Fig.4.10 and Table 4.2). The *Frankineae* occurring in this habitat belonged to the family *Microsphaeraceae* (genus *Quadrisphaera*). Uncultured *Frankineae* related to the family *Nakamurellaceae* (genus *Humicoccus*).

The *Corynebacterineae* belonged only to the family *Mycobacteriaceae,* consisting of both cultured and uncultured members of the genus *Mycobacterium.* The only *Propionibacterineae* genotype belonged to the family *Propionibacteriaceae* that related to the genus *Aestuariimicrobium*. Similarly, only one genotype, an uncultured member of the *Streptosporangineae* belonged to the family *Thermomonosporaceae* (genus Actinomadura) in habitat MI 6.3.

4.5.3 Identification of related habitats

It was also important to associate the identified genotypes to the natural ecological conditions inhabited by their closest relatives. This would provide an insight into the ecological functions of the different actinobacterial communities. The details of ecological conditions discussed in the following sections are based on the information gathered from the BLAST results summarized in Appendix A10.

4.5.3.1 Relatedness of actinobacteria from habitat MI 6.3

The *Acidimicrobiales*, represented by clone C8-MI 6.3, were all uncultured actinobacteria related to *Acidimicrobium Ferrooxidans* strain TH3 (90% identity). Some were related to *Ferrimicrobium acidiphilum* (89-91% identities), a Heterotrophic Iron-oxidizing and extremely acidophilic bacteria involved in bioleaching of sulfide minerals. Other *Acidimicrobiales* related to *Candidatus Microthrix calida* (89% identities), which was isolated from an industrial activated sludge wastewater treatment plant. Most *Acidimicrobiales* occurring in habitat MI 6.3 were related to genotypes identified in clone libraries from humic lake sediments (USA.), acid mine waters, cropland soils used for rice-wheat cultivation for centuries (China), trembling aspen rhizosphere under elevated CO_2 conditions, and Peat bog.

The *Propionibacterineae* were related to an uncultured bacterium isolated from permafrost soil and to *Aestuariimicrobium kwangyangensis* R47 (clone E7-MI 6.3) (94% identity), which was isolated from an enrichment of diesel oil.

The *Frankineae* were related to species associated with various root nodule specific hosts (clone A9-MI 6.3) (98% identity) and to *Kineosporia rhamnosa*, isolated from plant samples (clone D8-MI 6.3) (97% identity). Other *Frankineae* included an uncultured actinobacterium related to *Geodermatophilus obscurus dictyosporus* (90% identity), which was identified in soil from Angelo Coast Range Reserve (California). These were also related to a *Frankiaceae* (isolate

10; Namibia) isolated from dry soils, rocks, and monument surfaces in Namibia (97% identity). They were also related to an uncultured *Acidothermaceae* identified in a flooded anoxic rice paddy soil in South Korea, and to *Acidothermus cellulolyticus* (96% identity).

The *Corynebacterineae* related to an uncultured actinobacterium TH3-101 from a large, shallow, freshwater of Subtropical Taihu Lake in China (clone E1-MI 6.3, clone B11-MI 6.3) (99% identity). They also related to *Mycobacterium anthracenicum* that is associated with the degradation of vinyl chloride and polyaromatic hydrocarbons (98% identity). Some of the *Corynebacterineae* were related to *Mycobacterium saskatchewanense* MB54784, which was isolated from human clinical specimens (98% identity). Some phylotypes related to an uncultured *Mycobacterium,* isolated from a freshwater reservoir rich in metal particles in Colorado (A2-MI 6.3) (99% identity). Others related to *Mycobacterium neglectum* that was isolated from a drinking water biofilm in Germany (clone G3-MI 6.3) (99% identity).

The *Streptosporangineae* included a phylotype related to an uncultured *Thermomonosporaceae,* which was identified from a forested wetland (clone A1-MI 6.3) (95% identity). These were also related to *Actinomadura cremea* (95% identity). The *Micrococcineae* included a phylotype that was related to an uncultured *Intrasporangiaceae,* CrystalBog022D6 (D2-MI 6.3). This phylotype

was identified from humic lake samples (USA) and was related to *Nostocoida limicola* (92% identity), isolated from activated sludge.

4.5.3.2 Relatedness of actinobacteria from habitat MI 1.2

The *Micrococcineae* in habitat MI 1.2 related to a psychrotrophic *Janibacter* species, isolated from Antarctic seawater (Terra Nova Bay, Ross Sea) (clone A1-MI 1.2) (92% identity). Some of the *Micrococcinae* were related to an uncultured bacterium identified in Penguin droppings sediments from Ardley Island, Antarctica (Clone B9-MI 1.2) (99% identity). These were also related to *Dermatophilus crocodyli* that was isolated from *Crocodylus porosus* (saltwater crocodile) infected with 'brown spot' disease (95% identity).

A phylotype that clustered with the *Arthrobacter* (Clone F9-MI 1.2), was related to an uncultured actinobacterium identified from high Arctic permafrost soil from Spitsbergen (99% identity) and to *Arthrobacter stackebrandtii* (98% identity). Clone A11-MI 1.2 represented a phylotype that was related to an *Arthrobacter sp.* TSBY-69 that was also isolated from alpine permafrost frozen soil in the Tianshan Mountains, North-Western China. This phylotype closely clustered with *Arthrobacter psychrolactophilus* D2 (98-99% identities, 98% bootstraps). Clone D11-MI 1.2 represented a phylotype that was related to an uncultured bacterium clone identified from the aquatic environments of the high altitude, Andean Altiplano (Northern Chile) freshwaters (99% identity). This phylotype was also related to a cultured representative, *Arthrobacter psychrophenolicus* (92%

bootstraps), that was isolated from an alpine ice cave. An *Intrasporangiaceae* phylotype (Clone A3-MI 1.2) was related to an uncultured bacterium from soil environments under mosses *(Sanionia uncinata)* on Anchorage Island in Antarctica (99% identity), and to a soil actinomycete, *Terracoccus luteus* (97% identity). An uncultured bacterium, identified from Glacial and sub glacial ice environments (200 year-old glacial ice) from Guliya related to clone E4-MI 1.2 (99% identity) and to *Oerskovia paurometabola* (98% identity). Some of the *Intrasporangiaceae* phylotypes were related to an uncultured actinobacterium that was identified from rice-wheat growing soils under long-term manure and chemical fertilizer treatments in China (clone H5-MI 1.2). These clones were related to *Knoellia sinensis* amongst the cultured representatives, which was isolated from spacecraft assembly facilities (98% identities).

The *Streptosporangineae* phylotypes were related to an uncultured *Thermomonosporaceae* from Taiwan forest soils (93% identity) and *Streptosporangium vulgare* (clone A8-MI 1.2) (92% identity). Others were related to uncultured members identified in grasslands at the GASP KBS-LTER sampling site (USA: Michigan) (93% identity) and to *Actinomadura libanotica* (clone H10-MI 1.2) (90% identity).

The *Frankineae* were related to phylotypes from a wide variety of environments. The phylotypes represented by clone G3-MI 1.2 related to an uncultured bacterium identified from rice-wheat growing soils under long-term manure and

chemical fertilizer treatments in China (98% identity). These were also related to *Humicoccus flavidus,* a soil bacterium (96% identity). The phylotype represented by clone A7-MI 1.2 was related to an uncultured bacterium identified in soil environments under mosses *(Chorisodontium aciphyllum)* on Signy Island, Antarctica (98% identity). This phylotype was also related to *Acidothermus cellulolyticus* (97% identity).

The *Propionibacterineae* phylotypes represented by clone A5-MI 1.2, were related to an uncultured actinobacterium identified from rice-wheat growing soils under long-term manure and chemical fertilizer treatments (China, Jiangsu Province) (98% identity). These phylotypes were also related *Nocardioides jensenii* (98% identity) that was isolated from Antarctic sandstone. The *Propionibacterineae* phylotypes represented by clone C2-MI 1.2, were related to an uncultured bacterium identified from a Perennial ice cover of Lake Vida in Antarctica, McMurdo Dry Valleys (Southern Victoria Land Valley) (93% identity). These were also related to *Tessaracoccus bendigoensis*, which was isolated from wastewater generated in the production of stainless steel in a reactor (92% identity).

The *Acidimicrobineae* phylotypes from MI 1.2 were related to uncultured bacteria identified from a perennial ice cover of Lake Vida, Antarctica, Antarctica, Southern Victoria Land, Victoria Valley (clone B4-MI 1.2) (99% identity). These phylotypes also related to *Candidatus Microthrix* that was isolated from an

industrial activated sludge of a wastewater treatment plant (90% identity). A number of other clones of the suborder *Acidimicrobineae* from this habitat were also related to *Ferrimicrobium acidiphilum*, which was identified from a peat bog (Germany). This microorganism is associated with the oxidation of ferrous compounds and mineral sulphides (93% identity).

The *Propionibacterineae* phylotypes of the family *Propionibacteriaceae* were related uncultured bacteria from a perennial ice cover of Lake Vida, McMurdo Dry Valleys, Antarctica (clone B11-MI 1.2) (98% identity). These were also related to *Tessaracoccus bendigoensis* (97% identity), which is associated with wastewater generated in the production of stainless steel. The phylotype represented by clone F8-MI 1.2 was related an uncultured soil bacterium from a Romanian oil-polluted soil (98% identity). This phylotype was also related to *Propionicicella superfundia,* which is a chlorosolvent-tolerant propionate-forming, facultative anaerobic propionibacteria (96% identity). The *Propionibacterineae* phylotypes of the family *Nocardioidaceae* (clone A5-MI 1.2) were related an uncultured actinobacterium identified from rice-wheat growing soils under long-term manure and chemical fertilizer treatments (China, SuZhou, Jiangsu Province) (98% identity). Some of the phylotypes were related to *Nocardioides jensenii*, which was isolated from Antarctic sandstone (98% identity).

The *Corynebacterineae* phylotypes (clone C3-MI 1.2) were related to uncultured bacteria and a *Rhodococcus sp.* isolated from ornithogenic soil from Seabee

Hook, Cape Hallet, in Antarctica (98% identity). This *Rhodococcus* species is associated with hexadecane mineralization activity.

4.5.3.3 Relatedness of actinobacteria from habitat MI 5.1

Most members of *Acidimicrobineae* and *Frankineae* from habitat MI 5.1 were also identified in environments similar to those described for habitat MI 6.3 except those descried here. The *Frankineae* phylotypes represented by clone G8-MI 5.1, were related to an uncultured *Frankiaceae* bacterium identified from recent volcanic deposits (<300 yr old) (Kilauea volcano, Hawaiia, USA) (98% identity). A similar phylotype was identified using primer F243 (clone G9-MI 5.1-F243) in this study and also related to these clones (97% identity). In addition to the conditions described for closest relatives to those found in habitat MI 6.3, *Acidimicrobineae* from habitat MI 5.1 were identified under other ecological conditions. These include soil environments under *Empetrum rubrum* at the Falkland Islands (United Kingdom) (97% identity, clone E12 MI 5.1), geothermal sites in Yellowstone National Park and Nunnock River granitic landscape (Australia) (92% identity, clone H10 MI 5.1). Using primer F243, a phylotype represented by clone F5-MI 5.1-F243 was also identified as a *Kineococcus*-like bacterium (*Kineosporiaceae, Frankineae*) from similar environmental samples.

The *Streptosporangineae* phylotypes represented by clone F6-MI 5.1, were related to uncultured bacteria identified from Signy Island (Antarctica) terrestrial soils under mosses (*Chorisodontium aciphyllum*) (98% identity). The phylotype

represented by clone A7 MI 1.2 was also identified from the same habitat. Other related uncultured *Streptosporangineae* phylotypes were identified from grassland soils (GASP KBS-LTER sampling site, Michigan, USA) (clone B4-MI 5.1, 99% identity) and forest soils (Fushan Forest, Taiwan) (clone D6-MI 5.1, 98% identity). All these phylotypes were related to the *Thermomonosporaceae, Actinomadura spadix* (95% identity).

The *Frankineae* phylotypes belonging to the family *Sporichthyaceae* (clone G3-MI 5.1) related to an uncultured actinobacterium from unvegetated soil environments of Antarctic terrestrial habitats in Anchorage Island (99% identity). These were also related to *Blastococcus jejuensis* (97% identity). Other *Frankineae* phylotypes (clone C11-MI 5.1) were related to *Acidothermus cellulolyticus* (family *Acidothermaceae)* (97% identity). These also related to an unidentified clone from soil environments under mosses *(Chorisodontium aciphyllum)* on Signy Island, Antarctica (99% identity). Other *Frankineae* phylotypes such as F1-MI 5.1 related to *Actinomadura spadix* (94% identity). The phylotypes of the family *Sporichthyaceae* (clone F8-MI 5.1) were related an uncultured bacterium from an Italian rice paddy soil (99% identity) and to *Sporichthya polymorpha* (96% identity). A phylotype represented by clone E4-MI 5.1-F243 was also related to an uncultured bacterium (99% identity) identified from paddy rice soil using primer F243 in this study. Another phylotype, represented by clone D10-MI 5.1, was related to an uncultured *Frankineae* (family *Nakamurellaceae*) (99% identity) identified from polychlorinated biphenyls

(PCBs)-contaminated soil. This clone was also related to *Humicoccus flavidus* (98% identity), a soil actinobacterium, amongst the cultured representatives.

Some of the *Thermomonosporaceae* phylotypes (Clone A7-MI 5.1) were related an uncultured bacterium from a forested wetland affected by reject coal and to *Thermomonospora formosensis* (98% identity). The *Kineosporiaceae* (clone G5-MI 5.1) was related to a *Kineosporiaceae* identified as endemic to the Mojave Desert (97% identity), and to *Kineosporia rhamnosa* (97% identity). The phylotype represented by clone E10-MI 5.1 was related to an uncultured *Frankineae* from a trembling aspen rhizosphere under elevated CO_2 conditions (96% identity), and to a host-specific soil *Frankia sp*ecies *BCU110505* isolate (95% identity). Thus, *Frankia* phylotypes were associated with anaerobic conditions. The *Corynebacterineae* phylotypes (Clone A5-MI 5.1) were related to *Mycobacterium saskatchewanense*, a slowly growing species isolated from human clinical specimens.

4.6 Discussion

The phylogenetic tree (Fig. 4.9) showed that most of the phylogenetic groups identified were reliable considering the high bootstrap values obtained for most phylogenetic nodes (ranging between 51-99%). Hierarchical clustering techniques provide a reliable way of analyzing biodiversity microbial communities (Brendan *et al.,* 2003; Stach *et al.,* 2003a; Tamura *et al.,* 2004). The bootstrap

values show the confidence of delineating the phylogenetic groups (Felsenstein, 1985; Saitou & Nei, 1987).

The actinomycetes, which constitute most of the actinobacteria identified in this study, are related and quite often difficult to delineate on phylogenetic trees constructed using the 16S rRNA genes. This is because the members of class *Actinobacteria* evolved recently over a relatively short period compared to other groups of bacteria (Embley & Stackebrandt, 1994, 1994b). The small differences in the primary structure of the actinobacterial 16S rRNA gene makes it difficult to clearly delineate neighbouring clades, which results in the lower bootstrap values (Stackebrandt *et al.,* 1997). Actinobacteria usually belong to separate operational taxonomic units if they differ by identities of more than 1% in the 16S rRNA gene, based on evidence from DNA-DNA reassociation (Stach *et al.,* 2003b; Stackebrandt & Goebel, 1994). In this study, the phylogenetic tree was drawn using the Maximum Composite Likelihood model, based on the Jukes-Cantor correction, which is an acceptable method used for most phylogenetic studies involving actinomycetes (Embley & Stackebrandt, 1994).

In most community studies, the findings are distorted by lower primer specificity and coverage (Forney *et al.,* 2004). The Primer S-C-Act-0235-a-S-20 was selected for this study because it has more specificity, and coverage of up to 213% higher compared to other primers used for targeting actinobacteria (Stach *et al.,* 2003a). However, not all actinobacterial sub-orders were identified from

diversity of the three Marion Island terrestrial habitats. The diversity in each habitat was characterised by actinobacteria that belonged only to a few sub-orders. However, Stach *et al.,* (2003b) showed that these primers could amplify actinobacteria that belong to more sub-orders than those identified in this study. The Marion Island terrestrial habitats sampled in this study therefore, contained low actinobacterial diversities. As expected, lower temperatures on Marion Island reduce numbers and diversity of actinomycetes, which might lead to the suggestion of the low species richness.

Even though the libraries developed using the PCR primers F243 and U1510R were not screened to completion, most of the genotypes were *Verrucomicrobiales* and not actinomycetes. However, some of the actinomycetes identified using this primer set were divergent and could not align properly with other sequences included in the tree, These include clones F5-MI 5.1-F243 (97% identity to a *Frankineae*), C4-MI 5.1-F243 (unknown actinomycete identified through the Ribosomal Database Project) and H4-MI 5.1-F243 (99% identity to a *Mycobacterium*). This showed that primer 243 does amplify some of the actinomycetes that the actinobacterial primers would not be able to amplify. However, the stringency of this primer was low when used in combination with primer U1510R, universal for bacteria.

Even though Principal Component Analysis was used to identify the most important soil variables (Chapter 3), these may not have completely accounted

for the distribution of the actinobacteria identified in this study. According to Riesenfeld *et al.,* (2004), phylogenetic analysis provides information that can be useful in developing hypotheses about the functions and physiology of communities (Riesenfeld *et al.,* 2004). A comparison of the genotypes to database sequences showed that habitat MI 1.2 haboured a wealth of uncultured actinobacteria, mostly *Micrococcineae* and *Propionibacterineae.* Some of the phylotypes identified from habitats MI 5.1 and MI 1.2 were closely associated with Antarctica and sub-Antarctica environments. In addition, habitats MI 6.3 and MI 5.1 were characterised by high proportions of phylotypes associated with anaerobic and acidic soils compared to habitat MI 1.2. The phylotypes of the sub-orders *Frankineae* and *Acidimicrobineae* were dominant in both habitats.

According to Zenova & Zvyagintsev (2003), the distribution of actinomycetes in terrestrial ecosystems depends on the soil habitat factors. The *Streptosporangineae* of the genus *Streptosporangium* (suborder *Streptosporangineae*) occur in acid forest soils and *Actinomadura* (suborder *Streptosporangineae*) in neutral to slightly alkaline soils, with low moisture content, at early stages of decomposition, such as peats and forests (Zenova & Zvyagintsev, 2003). In our study, almost all *Streptosporangineae* occurred in habitat MI 5.1, but as expected, were much less, almost absent from habitat MI 6.3, which is also characterised by more acidic peats compared to MI 5.1. The conditions in which the *Streptosporangineae* thrive, according to Zenova *et al.,* (2003), suggest that habitat MI 5.1 shares related actinobacterial diversity with MI

6.3. These habitats most likely contained anaerobic and acidic soils due to poor aeration. Habitat MI 1.2 contains soils that are hypersaline compared to other two habitats. In addition, habitat MI 1.2 occurs on the shore zone and on surface made up of fibrous peat and volcanic ash. The soils of habitat MI 1.2 contain higher biotic indices due to manuring by marine animals. The presence of birds (mostly penguins) and marine mammals (mostly fur seals) that frequent the habitat may explain the wealth of *Micrococcineae* that seem to be endemic to habitat MI 1.2. The pH is higher compared to the other two habitats, ranging between 4.5 and 6.2, with a median of 5.6 (Smith, *et al.,* 2001). The *Micrococcineae* were abundant in habitat MI 1.2 compared to other groups. This is possibly because they may have been able to resist the desiccation caused by high salinity levels. The ability of *Micrococcineae* to resist desiccation has been attributed to the presence of thick lipopolysaccharides in their cell walls (Kochkina *et al.,* 2001).

Habitat MI 5.1 is richer in nutrients (phosphate and all forms of nitrogen). The habitat occurs on compact peat dominated by the plants *C. plumosa* and *P. Cookii* and pH ranges between 4.0 and 5.9, with a median of 4.5 (Smith, *et al.,* 2001). The soils of habitats MI 5.1 and MI 6.3 have lower pH (ranging between 4.0-5.9 and 4.3-5.5 respectively) compared to MI 1.2 (pH range, 4.5-6.2). Since habitat MI 5.1 occurs on compact peat and habitat MI 6.3 is a waterlogged bog, the soils should be anaerobic. The influence of manuring is thought to be much less in habitat MI 5.1 compared to most habitats influenced by animals. Instead,

more trampling occurs in habitat MI 5.1 rather than manuring (Smith & Steenkamp, 2001; Smith, *et al.,* 2001). Trampling results in compacted and less aerated soils (Mcnearney *et al.,* 2002). This should have resulted in high proportions of *Frankineae* and *Acidimicrobiales* in habitat MI 5.1. Analysis of 16S rRNA gene sequences showed that actinobacterial communities from habitats MI 5.1 and MI 6.3 were dominated by *Acidimicrobiales* and *Frankineae.* Habitat MI 1.2 was quite different, supporting more *Micrococcineae* and *Propionibacterineae.*

The high proportions of *Frankineae* in habitats MI 5.1 and MI 6.3 were most likely involved in anaerobic anabolic processes. The members of *Frankineae* form actinorhizal symbiotic associations with plants and contribute to the functioning of the nitrogen cycle (Verghese & Misra, 2002). Nitrogen fixation by the *Frankia* involves the nitrogenase enzyme, which converts dinitrogen to ammonia under anaerobic conditions (Verghese & Misra, 2002).

Similarly, the *Acidimicrobiales* are chemoautotrophs that fix CO_2 from the atmosphere under anaerobic conditions. These microorganisms obtain their energy through the biomineralization of metal ores (Cleaver *et al.,* 2007; Rawlings *et al.,* 2003). The processes are acidic and involve oxidation using ferrous iron, reduced inorganic sulfur compounds, or both as electron donors under anaerobic conditions (Rawlings *et al.,* 2003).

Bacteria are important agents for decomposition of plant litter. Their abundance varies directly with the amount and type of litter, and the stage of carbon recycling (decomposition of plant matter) (Carney & Matson, 2005). The microbial mineralization of carbon and nitrogen occurs at temperatures as low as -5ºC (Krivtsov *et al.,* 2005). The soil physiochemical parameters, vegetation, and mineralization activities also affect the microbiological diversity (Nüsslein & Tiedje, 1999). The carbon and nitrogen mineralization rates can be useful in predicting the abundance of specific groups of microorganisms (Fierer *et al.,* 2007). The high moisture content, such as that in habitat MI 6.3, is believed to be a major factor that retards the decomposition of nutrients on Marion Island (Smith, 1988).

The rates of mineralization of plant matter by actinomycetes may have been reduced in habitat MI 6.3 due to anaerobic soil conditions. This may also explain the high proportions of acidic actinobacteria (*Acidimicrobiales*) in MI 6.3. These may have been involved in chemoautotrophic production of energy from the oxidation of inorganic ferric and sulfate compounds. The anaerobic soil conditions may similarly have contributed to the proliferation of some *Acidimicrobiales* and *Frankineae* in habitat MI 5.1. This habitat is characterised by more trampling activities than manuring from marine mammals and birds (Smith & Steenkamp, 2001; Smith, *et al.,* 2001). The trampling of by animals was found to significantly increase the soil denitrification activities (Menneer *et al.,*

2005), which is coupled to denitrification (An *et al.,* 2001; Kuai & Verstraete, 1998; Menneer *et al.,* 2005).

Actinomycetes formed the majority of actinobacterial phylotypes identified in this study. Their community sizes and diversity depends on the soil characteristics, primarily soil type, pH, moisture and organic content. They proliferate under conditions of high available organic carbon and nitrogen contents (that is rich in organic matter) compared to oligotrophic conditions. In addition, actinomycetes utilize a wide variety of organic compounds than other bacteria (Paul & Clark, 1996). The data on the available carbon content was not provided (Smith, *et al.,* 2001). However, since both habitats MI 1.2 and MI 5.1 were frequently influenced by manuring activities, they probably had higher concentrations of organic carbon than MI 6.3. This may possibly explain the higher diversity indices and evenness in habitats MI 5.1 and MI 1.2 compared to MI 6.3.

Peats, tundra and waterlogged soils are unfavourable for the proliferation of actinomycetes. The optimum pH for actinomycetes is between 5.0 and 9.0 (Goodfellow & Williams, 1983). Actinomycetes are generally not acid tolerant and show low activity at low pH. Consequently, they proliferate in alkaline compared to acidic soils with pH lower than 5.0 (Paul & Clark, 1996). The higher pH may explain the higher diversity index in habitat MI 1.2 compared the other habitats. In addition, actinomycetes rarely occur in habitats with high moisture content, especially under water-logging conditions. High moisture content reduces the

availability of oxygen and limits aerobic metabolism, which is common to all soil actinomycetes (Paul & Clark, 1996). The data on the soil aeration in the Marion Island habitats studied was not available. The Acidobacteria and were found to be most abundant in soils with low rates of carbon mineralization of plant litter. They were also found to diminish in diversity and numbers as the amount of organic carbon content increases (Fierer *et al.,* 2007).

This study has demonstrated that the spatial distribution of microbial taxa is influenced by their genetic traits and adaptation to environmental factors in support of previous studies (Green *et al.,* 2008; Kassen and Rainey, 2004). They also support the claims that some microorganisms are endemic to particular environments (Martiny *et al.,* 2006).

4.7 Summary

This chapter reports culture-independent identification of the specific groups of actinobacteria occurring in different Marion Island terrestrial habitats. In most cases, clones from the same habitats phylogenetically clustered together, and few major groups dominated each habitat. These results were inconsistent with the findings of DGGE microbiological community profiling (Chapter 3). Habitats MI 1.2 and MI 5.1 consistently clustered together based on DGGE profiles. However, the community structures of habitats MI 5.1 and MI 6.3 closely resembled each other compared to habitat MI 1.2 based on sequence analysis. This was most likely because of poor soil aeration, which resulted in anaerobic

soil conditions compared to habitat MI 1.2. Consequently, habitat MI 6.3 had lower species evenness and richness, and diversity indices compared to MI 1.2. Less than 1% of the genotypes from the metagenomic libraries were cultured.

CHAPTER 5

Isolation of actinomycetes from Marion Island terrestrial habitats

5.1 Aims

This chapter was aimed at isolating novel Actinomycetes from Marion Island terrestrial habitats. The screening and isolation involved classical techniques of plating onto a variety of selective media. Identification of actinomycetes was based on the PCR amplification of the 16S rRNA genes and DNA sequencing. The gene sequences were used to determine their phylogenetic placement.

5.2 Background & literature review

The discovery of new actinomycete taxa is important, especially as a source of novel drugs. Culture-dependant techniques are severely limited in capacity to completely define microbial diversity because they cannot reveal the presence of most groups that are not culturable (Forney *et al.,* 2004; Rheims *et al.,* 1996). It is estimated that more than 99% of the total microorganisms unculturable or are uncultured using the standard laboratory techniques (Amann *et al.,* 1995). Some microorganisms are known to enter the non-culturable state under unsuitable conditions. Most unknown microorganisms are still uncultured because of lack of appropriate isolation techniques (Amann *et al.,* 1995; Keller & Zengler, 2004). Previously uncultured microorganisms have been isolated through the application

of new approaches such as use of oligotrophic media. Such microorganisms are thought to be resistant to high concentrations of nutrients or high cell densities, and were isolated on low nutrient media diluted to almost extinction (Button *et al.,* 1993; Connon & Giovannoni, 2002).

Several approaches have successfully led to the isolation of novel microorganisms. This includes simulating their natural environments in artificial chambers using membrane diffusion (Kaeberlein *et al.,* 2002; Keller & Zengler, 2004). An example is the isolation of deep-sea actinomycetes (Bull *et al.,* 2000). In addition, the use of filter traps is a modification that improves the simulation of natural environments (Gavrish *et al.,* 2008). The application of suitable selective pressure enhances the success of isolation. This includes the use of antibiotics such as cycloheximide and nystatin that allow the proliferation of the actinomycetes by inhibiting the growth of fungi. These antibiotics reduce the numbers of fast growing and unwanted microorganisms (Porter *et al.,* 1960).

In some cases, the prolongation of incubation periods to months improves isolation of slow growing actinomycetes. Such actinomycetes usually have slow doubling times (Goodfellow & Williams, 1983; Paul & Clark, 1996). Thermophilic actinomycetes have also been isolated using temperature selection. Actinomycetes that form sporangia are selected based on their ability to withstand desiccation (Goodfellow & Williams, 1983). Selective recovery has also been employed to preferentially isolate actinomycetes using methods such as the

rehydration and centrifugation (RC), developed for zoospore-forming actinomycetes (Hayakawa *et al.,* 2000). Membrane filtration (Hirsch & Christensen, 1983) and differential centrifugation (Hopkins *et al.,* 1991; Maldonado *et al.,* 2005b; Yamamura *et al.,* 2003) are useful techniques for concentrating actinomycetes from environmental samples. A modern and sophisticated isolation approach involves micro-encapsulation of microorganisms and selection of viable cells by fluorescence-activated cell sorting (FACS) (Toledo *et al.,* 2006).

In addition to use of new selective isolation procedures, many microorganism, have been isolated from previously unsampled habitats (sources other than just terrestrial soils). These include plant leaf litter, marine and lake sediments. This has contributed to the isolation of new groups of actinomycetes (Lazzarini *et al.,* 2000).

In this study, actinomycetes were isolated using standard actinomycete selective media most suited to the environmental properties of the habitat sources. The pH and sodium chloride contents of media were adjusted according to the characteristics of the respective habitats described by Smith *et al.* (2001).

5.3 Isolation and propagation of cultures

The suspensions of soils from habitats MI 5.1, MI 1.2, MI 6.3, MI 5.2 and MI 3.3 were prepared separately. The soils from habitats MI 1.1, MI 6.5 and MI 6.2 were

mixed together and designated Sample A whilst habitats MI 6.4, MI 5.3 and MI 4.3 were designated Sample B. The samples were mixed because they were identified as consisting of similar phylotypes based on cluster analysis of DGGE banding patterns of DNA fragments on DGGE (Chapter 3, Sections 3.8.4 and 3.8.5). The soil suspensions were diluted in sterile water and plated on 7H9, MC, CZ and HA agar media adjusted to pH 5.0 or 5.5.

Incubation at 16-20 ºC resulted in the growth of different microorganisms. Growth was inspected periodically and microorganisms with morphology typical of actinomycetes were preferentially selected. The selection was based on the appearance, size, shape, texture and pigmentation of colonies or mycelia. Most of the microorganisms suspected to be actinomycetes exhibited filamentous hyphae, or were powdery, consisted of leathery surfaces or were brightly coloured. The different representatives of all other microorganisms that were not easily identifiable were also selected for verification. However, colonies with shiny surfaces were not selected because they were thought to be Gram-negative bacteria. All the isolates were purified, sub-cultured and stored. Some of the actinomycetes isolated in this study are shown in Appendix A11.

The selection and purification of cultures was carried out over a period of approximately three months until a collection of pure cultures was established. Most actinomycetes were isolated on 7H9 and HA rather than CZ and MC agars. All Streptomycetes from habitat MI 3.3 were isolated on MC agar. The media

adjusted with 10% sodium chloride did not yield any actinomycetes. Some of the actinomycetes, especially those isolated on HA agar, were subsequently lost because they were difficult to propagate in liquid media. Isolates grown on HA medium were difficult to amplify by PCR. This was due to inhibition by humic acids (Hugenholtz & Goebel, 2001).

5.4 Isolation of DNA from cultures

High molecular weight genomic DNA was isolated from all cultures (Fig. 5.1). The yields and quality of DNA obtained were poor as indicated by low concentrations of DNA and high RNA and protein contamination. The nucleotide concentrations varied between 300 and 6200ng/µl after RNAse treatment showing that there was still high RNA than DNA content. However, additional RNAse treatment would require further protein purification, which would result in DNA losses. Agarose gel electrophoresis also showed smears of RNA contamination (Fig. 5.1). For most of the isolates, the 260/280nm ratio varied between 1.06 and 2.03 suggesting high levels of protein contamination in some samples (Sambrook *et al.,* 1989). Most actinomycetes form mycelial structures that consist of abundant cellular material (Romano & Sohler, 1956). Furthermore, unlike Gram-negative bacteria, actinomycetes have complex cell wall structures, which are difficult to disrupt. They contain several polysaccharides that interfere with the extraction of DNA (Sambrook *et al.,* 1989).

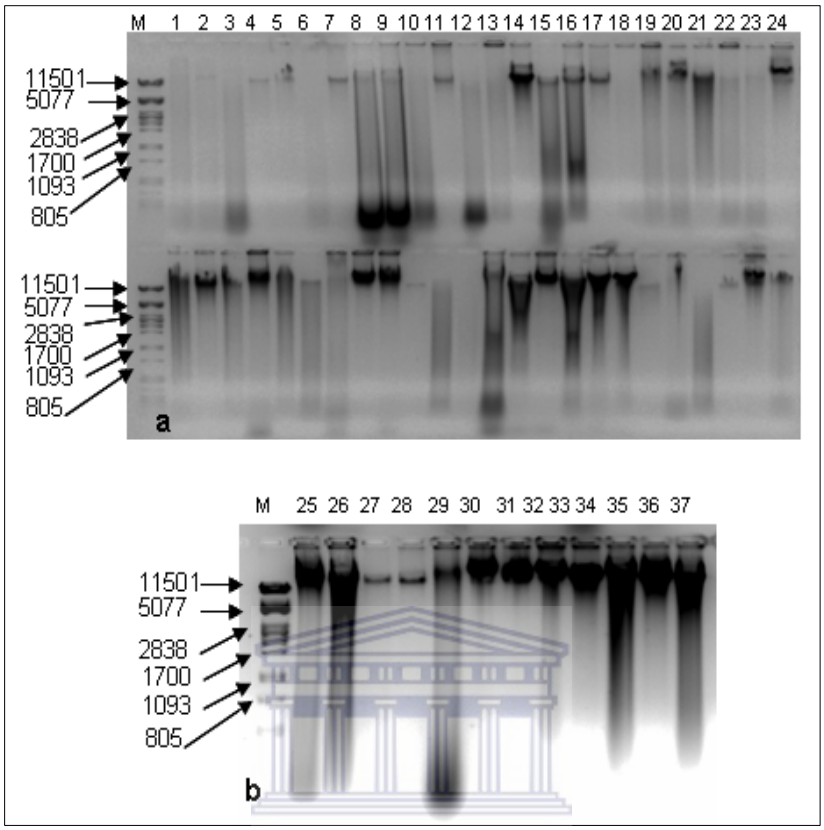


Fig. 5.1: Representative agarose gel (1%) showing the genomic DNA isolated from different microorganisms isolated from Marion Island terrestrial habitat soils. M, molecular weight marker of λ DNA digested using PstI; Lanes 1-37, genomic DNA of individual cultures.

5.5 PCR amplification of the 16S rRNA genes

The cultures were screened for actinomycete phylotypic signals using the PCR primer set S-C-Act-235-a-S-20 and S-C-Act-878-a-S-19 (Stach *et al.,* 2003b).

This primer set is specific for the actinobacterial 16S rRNA gene. The actinobacteria were identified by the presence of a 648bp fragment (Fig. 5.2(a)).

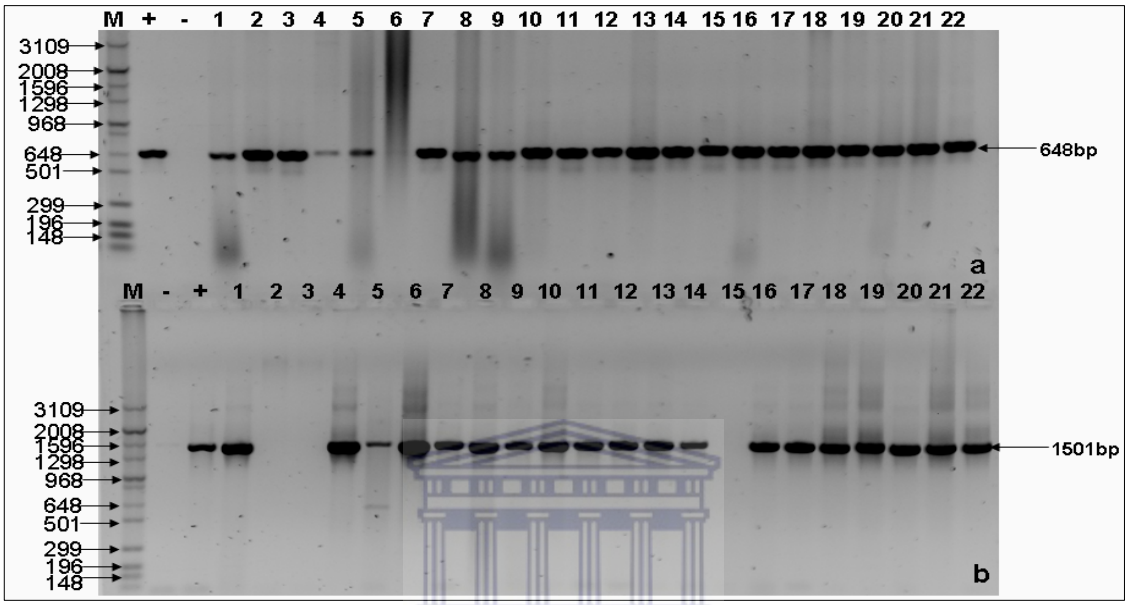


Fig. 5.2: Representative agarose gel (1%) showing the PCR amplified 16S rRNA genes from different microorganisms isolated from Marion Island terrestrial habitats soils. (a) using the actinobacterial-specific primers, S-C-Act-235-a-S-20 and S-C-Act-878-a-S-19 and (b) using the bacterial-specific primers E9f and U1510R. M, λ Pst molecular weight marker; +, Positive control (*Streptomyces albus*); -, negative control; Lanes 1-22, PCR products of individual isolates.

The bacterial full-length 16S rRNA gene contain more reliable sequence information for identification compared to the 648bp actinobacterial-specific gene. A corresponding PCR was therefore, conducted using the bacterial-specific primers E9F (Farrelly *et al.,* 1995) and U1510R (Reysenbach & Pace, 1995b).

Fragments of the expected size, approximately 1501bp, were obtained from the PCR amplification. This PCR step also verified that the cultures positively identified as actinobacteria, were also true bacteria (Fig. 5.2(b)).

Some of the cultures were identified as bacteria, but not actinobacteria as expected (for example in lane 6). Unexpectedly, the bacterial-specific 16S rRNA primers did not amplify the genes from some cultures that were thought to be actinobacteria (such as those in lanes 2, 3 and 15). According to Forney *et al.* (2004), "universal primers are not universal" because they are limited to targeting certain regions in the rRNA gene that differ in specificity for eubacteria (Forney *et al.,* 2004). Universal primers are designed from known sequences, which make them biased towards targeting unknown groups (Amann *et al.,* 1995). Since primer design is a compromise on a number of factors including specificity, this results in the under-representation of some genotypes from environmental samples (Baker *et al.,* 2003). In culture dependent studies, primer bias results in failure to identify microorganisms that belong to selected taxa.

A different primer set, 16S-F1 (Weisberg *et al.,* 1991) and 16S-R5 (Weisberg *et al.,* 1991), was therefore used to amplify those cultures. This primer set is specific for the bacterial 16S rRNA genes. In some cases, only the actinobacterial-specific primers were able to amplify the 16S rRNA genes from the isolates (Table 5.1). It is important to note that the primers E9F and 16S-F1 target similar regions of the 16S rRNA genes but have different specificities

(sequence composition and length) (Chapter 2, Section 2.6, Table 2.3). Likewise, the primers U1510R and 16S-R5 target similar regions, but have different lengths and similarities. The use of more than one primer set in this study set facilitated the identification of unknown microorganisms, which would have been overlooked by simply using one set of primer. Sequence analysis shows that primers U1510R and 16S-R5 were designed for the same region but differ in length, and are 84% similar in sequence identity.

5.6 Identification and phylogenetic placement of isolates

The approximately 1500bp 16S rRNA gene products (almost full-length) amplified from each bacterium using PCR were gel-band purified. The products from primer set E9F and U1510R corresponded to the *E.* coli positions 9 to 1510. The products of primers 16S-F1 and 16S-R5 corresponded to the *E.* coli positions 17F and 33R (U1510R). The gel-band purified PCR products were directly subjected to DNA sequencing. Alternatively, they were first cloned into a plasmid vector before sequencing. The sequences were identified by querying the NCBI database using BLAST. Table 5.1 shows the description of the 42 actinomycete isolates that were identified in this study, including a few non-actinomycetes that were of interest.

Table 5.1: Identification of actinomycete cultures isolated from Marion Island terrestrial habitats using BLAST. The E-value is zero in all cases.

Query/ Relative	Accession Number	Description, notes, source	Identity (%)
MI 1.2 V66			
Terrabacter terrae	AY944176.1	*Micrococcineae; Intrasporangiaceae; Terrabacter.* Isolated from soil in Spain (2005).	95
Uncultured bacterium, clone BS148.	AB240275.1	Bacteria; environmental samples. From rhizosphere community of Phragmites	95
Uncultured *Intrasporangiaceae* bacterium	EU016432.1	*Micrococcineae; Intrasporangiaceae;* environmental samples.	95
MI 6.3 U19			
Mycobacterium vanbaalenii, PYR-1	CP000511.1	*Corynebacterineae; Mycobacteriaceae; Mycobacterium.*	97
Mycobacterium austroafricanum	A16S-F190800.1	*Corynebacterineae; Mycobacteriaceae; Mycobacterium.*	97
[a]**MI 5.1 P301**			
Rahnella aquatilis, PTB2102	DQ862542.1	*Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Rahnella.*	96
MI 5.1 P18			
Streptomyces scabrisporus	AB249946.1	*Streptomycineae; Streptomycetaceae; Streptomyces.*	99
MI 5.1 P60			
Kitasatosporia mediocidica	U93324.1	*Streptomycineae; Streptomycetaceae; Kitasatospora.*	99
Streptomyces atroaurantiacus, NRRL B-24282	DQ026645.1	*Streptomycineae; Streptomycetaceae; Streptomyces.*	98
MI 1.2 V7			
Uncultured bacterium, Blai11	AJ318104.1	Bacteria; environmental samples.	97
Humicoccus flavidus strain DS-52	DQ321750.1	*Frankineae; Nakamurellaceae; Humicoccus.*	96
MI 5.1 P51			
Streptomyces scabrisporus	AB249946.1	*Streptomycineae; Streptomycetaceae; Streptomyces.*	99

[a] 640 bp fragment amplified using the actinobacterial specific primers Sc act 235/878; [b] Full 16S rRNA gene amplified using the primers 16S-F1 and 16S-R5; The rest of the sequences amplified using the primers E9F and U1510R.

Table continued

Query/ Relative	Accession Number	Description, notes, source	Identity (%)
MI 5.1 P25			
Mycobacterium sp. Ellin113	AF408955.1	*Corynebacterineae; Mycobacteriaceae; Mycobacterium.*	99
Mycobacterium hodleri	X93184.1	*Corynebacterineae; Mycobacteriaceae; Mycobacterium.*	98
[a]**MI 1.2 V46**			
Chryseobacterium sp. FRGDSA 4034/97	AY468464.1	*Flavobacteriales; Flavobacteriaceae; Chryseobacterium.* Isolated from diseased aquatic animals. Isolated from skin ulcer (rainbow trout, Landes, France, 1997).	98
Unidentified bacterium, W4-B50	AY345495.1	Bacteria; environmental samples. Identified a from Hawaiian Archipelago.	98
MI 5.1 P43			
Williamsia maris	AB010909.2	*Corynebacterineae; Williamsiaceae; Williamsia.* Isolated from deep-sea.	99
MI 5.1 P7b			
Microbacterium sp. PHD-5	DQ227343.1	*Micrococcineae; Microbacteriaceae; Microbacterium.* A phenol-degrading strain	99
Microbacterium paraoxydans	AJ491806.1	*Micrococcineae; Microbacteriaceae; Microbacterium.* Causes bacteremia in patients with leukemia.	99
MI 5.1 P20			
Curtobacterium sp. VKM Ac-1811	AB042089.1	*Micrococcineae; Microbacteriaceae; Curtobacterium.* Associated with plant-nematodes.	98
Curtobacterium sp. 2340	AY688358.1	*Micrococcineae; Microbacteriaceae; Curtobacterium.* Isolated from Human Clinical Specimens	98
Curtobacterium citreum	AM411064.1	*Micrococcineae; Microbacteriaceae; Curtobacterium.* A deep sea bacterium.	98
MI 1.2 V62			
Terrabacter terrae	AY944176.1	*Micrococcineae; Intrasporangiaceae; Terrabacter.*	95
Uncultured *Intrasporangiaceae*	EU016432.1	*Micrococcineae; Intrasporangiaceae; environmental samples.* Iron-reducing microorganisms involved in anaerobic benzene degradation	95

[a] 640 bp fragment amplified using the actinobacterial specific primers Sc act 235/878; [b] Full 16S rRNA gene amplified using the primers 16S-F1 and 16S-R5; The rest of the sequences amplified using the primers E9F and U1510R.

Table continued

Query/ Relative	Accession Number	Description, notes, source	Identity (%)
MI 5.1 P61			
Kitasatosporia mediocidica	U93324.1	*Streptomycineae; Streptomycetaceae; Kitasatospora.*	99
MI 5.1 P62			
Streptomyces scabrisporus	AB249946.1	*Streptomycineae; Streptomycetaceae; Streptomyces.*	99
MI 1.2 V47			
Arthrobacter sp. Nj-4	AM419018.1	*Micrococcineae; Micrococcaceae; Arthrobacter.* Bacteria species isolated from Antarctica	99
Arthrobacter stackebrandtii, CCM 2783	AJ640198.1	*Micrococcineae; Micrococcaceae; Arthrobacter.*	98
[a]**MI 5.1 P202a**			
Streptomyces scabrisporus	AB249946.1	*Streptomycineae; Streptomycetaceae; Streptomyces.*	100
[a]**MI 5.1 P207**			
Rhodococcus erythropolis, K22-19	EU333891.1	*Corynebacterineae; Nocardiaceae; Rhodococcus.* Isolated from a cold desert of the Indian Himalayas.	100
[a]**MI 1.2 V104**			
Streptomyces scabrisporus	AB249946.1	*Streptomycineae; Streptomycetaceae; Streptomyces.*	100
[a]**MI 5.1 P202b**			
Streptomyces scabrisporus	AB249946.1	*Streptomycineae; Streptomycetaceae; Streptomyces.*	99
[a]**MI 5.1 P101**			
Streptomyces scabrisporus	AB249946.1	*Streptomycineae; Streptomycetaceae; Streptomyces.*	99
BOB			
Streptomyces drozdowiczii, PhyCEm-1349	AM921646.1	*Streptomycineae; Streptomycetaceae; Streptomyces* .From rhizosphere bacterial communities associated with the specific host *Lolium perenne.*	99

[a] 640 bp fragment amplified using the actinobacterial specific primers Sc act 235/878.

[b] Full 16S rRNA gene amplified using the primers 16S-F1 and 16S-R5.

The rest of the sequences amplified using the primers E9F and U1510R.

Table continued

Query/ Relative	Accession Number	Description, notes, source	Identity (%)
BOA			
Arthrobacter sp. Nj-30	AM491454.1	*Micrococcineae; Micrococcaceae; Arthrobacter.*From Antarctica:China great wall station.	97
Uncultured soil bacterium TE9	DQ248287.1	Bacteria; environmental samples. Identified from from Carbon Tetrachloride Contaminated Soil.	97
Arthrobacter sp. An16	AJ551154.1	*Micrococcineae; Micrococcaceae; Arthrobacter.*	97
MI 5.2 K1			
Catellatospora citrea subsp. Citrea	A16S-F152106.1	*Micromonosporineae; Micromonosporaceae; Catellatospora.*	99
C.citrea (DSM 44097)	X93197.1	*Micromonosporineae; Micromonosporaceae; Catellatospora.*	99
MI 3.3 Y5			
Amycolatopsis minnesotaensis, 32U-4	DQ076483.1	*Pseudonocardineae; Pseudonocardiaceae; Amycolatopsis.*	97
Amycolatopsis minnesotaensis, 32U-2	DQ076482.1	*Pseudonocardineae; Pseudonocardiaceae; Amycolatopsis.* Isolated from a prairie soil.	97
MI 3.3 Y6			
Kitasatosporia mediocidica	U93324.1	*Streptomycineae; Streptomycetaceae; Kitasatospora.*	99
MI 6.3 U3b			
Streptomyces monomycini, NRRL B-24309	DQ445790.1	*Streptomycineae; Streptomycetaceae; Streptomyces.*	99
B1B			
Swingsiella fulva	AB100608.1	*Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Rhodanobacter.* A beta-galactosidase-producing gammaproteobacterium isolated from soil mixed with rotten rice straw.	97
Uncultured bacterium, LS4-200	AB234278.1	Bacteria; environmental samples. Lake Suwa, and Osaka sediment and soil, Japan: Nagano,	97

[a] 640 bp fragment amplified using the actinobacterial specific primers Sc act 235/878.

[b] Full 16S rRNA gene amplified using the primers 16S-F1 and 16S-R5.

The rest of the sequences amplified using the primers E9F and U1510R.

Table continued

Query/ Relative	Accession Number	Description, notes, source	Identity (%)
MI 3.3 Y9			
Streptomyces sp. NBRC 104276	AB441718.1	*Streptomycineae; Streptomycetaceae; Streptomyces.* Isolated from Bali, Indonesia (2008).	100
MI 3.3 Y7			
Streptomyces drozdowiczii PhyCEm-1349	emb|AM921646.1|	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces. Isolated from rhizosphere bacterial community associated with *Lolium perenne.*	99
MI 1.2 V13			
Cellulomonas sp. KAR4	EF451634.1	*Micrococcineae; Cellulomonadaceae; Cellulomonas.* Isolated from a high Arctic permafrost soil, Spitsbergen, Northern Norway.	98
Uncultured *Cellulomonadaceae,* HT06Ba17	EU016443.1	*Micrococcineae; Cellulomonadaceae;* environmental samples.	97
Cellulomonas sp. KAR1	EF451631.1	*Micrococcineae; Cellulomonadaceae; Cellulomonas.* Isolated from a high Arctic permafrost soil from Spitsbergen, Northern Norway.	98
MI 1.2 V3			
Dietzia sp. YIM 65001	EU375845.1	*Corynebacterineae; Dietziaceae; Dietzia.* A novel *sp.* isolated from plant tissues.	98
Dietzia sp. CNJ898 PL04	DQ448696.1	*Corynebacterineae; Dietziaceae; Dietzia.* From marine sediments (2007).	98
Dietzia sp. P27-19	DQ060380.1	*Corynebacterineae; Dietziaceae; Dietzia.* Isolated from Arctic Ocean Marine Sediments (2005).	98
[b]**A1A**			
Kitasatospora mediocidica, NBRC 14789.	AB184621.1	*Streptomycineae; Streptomycetaceae; Kitasatospora.*	99
Kitasatospora mediocidica	U93324.1	*Streptomycineae; Streptomycetaceae; Kitasatospora*	99
[b]**MI 3.3 Y2**			
Streptomyces niveoruber 173622	gi|183186415|gb|EU570497.1|	Streptomycineae; Streptomycetaceae; Streptomyces	100

[a] 640 bp fragment amplified using the actinobacterial specific primers Sc act 235/878.

[b] Full 16S rRNA gene amplified using the primers 16S-F1 and 16S-R5.

The rest of the sequences amplified using the primers E9F and U1510R.

Table continued

Query/ Relative	Accession Number	Description, notes, source	Identity (%)
B1A			
Rhodococcus sp. MOP100	AY927229.1	*Corynebacterineae; Nocardiaceae; Rhodococcus.* Alkyl ether-utilizing rhodococci isolated in 2007.	94
Rhodococcus *sp.* gf-6	EU287449.1	*Corynebacterineae; Nocardiaceae; Rhodococcus.* A cyhalothrin biodegradation bacterium isolated from a soil sample from XinZheng pesticide company (China, 2007).	94
Rhodococcus sp. L4	EF527237.1	*Corynebacterineae; Nocardiaceae; Rhodococcus.* Trichloroethylene (TCE) degrading bacteria (2007).	93
[b]**MI 1.2 V18**			
Humibacillus xanthopallidus, YM21-029.	AB286022.1	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Intrasporangiaceae; Humibacillus.	97
Terrabacter tumescens	AF005023.1	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Intrasporangiaceae; Terrabacter.	96
Humibacillus xanthopallidus KV-663	AB282888.1	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Intrasporangiaceae; Humibacillus.	96
[b]**MI 6.3 U2**			
Streptomyces niveoruber 173622	gb|EU570497.1	Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.	100
Streptomyces niveoruber strain HBUM173783	EU841654.1	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces. Isolated from Baoding.	99
[b]**MI 5.1 P16**			
Subtercola pratensis, DSM 14226T	AJ310412.1	*Micrococcineae; Microbacteriaceae; Agreia.* Isolated from grass-associated phyllosphere and litter layer after mulching the sward.	99
Agreia bicolorata, DSM 14575	AM410672.1	*Micrococcineae; Microbacteriaceae; Agreia.*	99
MI 3.3 Y4			
Kitasatospora mediocidica, NBRC 14789	AB184621.1	*Streptomycineae; Streptomycetaceae; Kitasatospora.*	99

[a] 640 bp fragment amplified using the actinobacterial specific primers Sc act 235/878.

[b] Full 16S rRNA gene amplified using the primers 16S-F1 and 16S-R5.

The rest of the sequences amplified using the primers E9F and U1510R.

Table continued

Query/ Relative	Accession Number	Description, notes, source	Identity (%)
[b]MI 5.1 P205			
Kitasatospora mediocidica, NBRC 14789.	AB184621.1	*Streptomycineae; Streptomycetaceae; Kitasatospora.*	99
Kitasatosporia mediocidica	U93324.1	*Streptomycineae; Streptomycetaceae; Kitasatospora.*	99
[b]MI 6.3 U12			
Mycobacterium aichiense, JS618	AF498656.1	*Corynebacterineae; Mycobacteriaceae; Mycobacterium.* Aerobic, vinyl chloride-assimilating bacteria from contaminated sites.	99
Mycobacterium sp. T104	U62890.1	*Corynebacterineae; Mycobacteriaceae; Mycobacterium.* Associated with toluene degradation, from a contaminated stream.	99
[b]MI 6.3 U306			
Mycobacterium aichiense, JS618	AF498656.1	*Corynebacterineae; Mycobacteriaceae; Mycobacterium.* Aerobic, vinyl chloride-assimilating bacteria from contaminated sites.	98
Mycobacterium sp. T104	U62890.1	*Corynebacterineae; Mycobacteriaceae; Mycobacterium.* Toluene degradation, from a contaminated stream.	98
Mycobacterium sp. T103	U62889.1	*Corynebacterineae; Mycobacteriaceae; Mycobacterium.* Toluene degradation, from a contaminated stream.	98
[b]MI 3.3 Y1			
Streptomyces sp. Soll14	AJ308572.1	*Streptomycineae; Streptomycetaceae; Streptomyces.* From an Agricultural soil (1999), degrades CM-cellulose only.	99
Uncultured actinomycete, Z851024	EU423876.1	environmental samples.	99
Streptomyces sp. GW25-12	EF471902.1	*Streptomycineae; Streptomycetaceae; Streptomyces.* Isolated from Antarctica.	99
[a]MI 6.3 U3			
Mycobacterium sp. H2-5	AB250800.1	*Corynebacterineae; Mycobacteriaceae; Mycobacterium.*	99
Mycobacterium aichiense, JS618	AF498656.1	*Corynebacterineae; Mycobacteriaceae; Mycobacterium.* Vinyl chloride assimilating bacteria from contaminated sites.	99

[a] 640 bp fragment amplified using the actinobacterial specific primers Sc act 235/878. [b] Full 16S rRNA gene amplified using the primers 16S-F1 and 16S-R5. The rest of the sequences amplified using the primers E9F and U1510R.

5.6.1 Comparing the actinomycetes isolated from different habitats

The actinomycetes that were culturable under the conditions used in this study belonged to the suborders *Corynebacterineae, Micromonosporineae, Frankineae, Pseudonocardineae, Streptomycineae* and *Micrococcineae* (Table 5.1). Only a *Streptomyces* (isolate A1A) and no other actinomycete, was culturable from Sample A, consisting of soils from habitats MI 6.4, MI 1.1 and MI 4.3. A few actinomycetes (MI BOA, MI B1A and MI BOB) were isolated from the Sample B, consisting of mixed soils from habitats MI 5.3, MI 6.2 and MI 6.5. However, other non-actinomycete bacteria were isolated from these samples suggesting that most actinomycetes were unculturable using the techniques employed in this study. The low numbers of culturables indicate that each habitat requires different media and techniques optimized for isolation of actinomycetes. Each habitat contributed soil in mixed samples that was equivalent to third of the total quantity used. However, the final quantities of soil used for the isolation procedures were equal to those used for unmixed samples. The quantities were therefore, not the limiting factor to the yields of culturable actinomycetes obtained.

Most members of the suborder *Micrococcineae* were isolated from habitat MI 1.2. Most members of *Streptomycineae* were isolated from habitats MI 5.1 and MI 3.3. The only *Frankineae,* MI 1.2 V7, was isolated from habitat MI 1.2. The only *Pseudonocardineae,* MI 3.3 Y5, occurred in habitat MI 3.3. The *Corynebacterineae* occurred in habitats MI 5.1 and MI 6.3. The only exception

was a *Dietziaceae,* MI 1.2 V3 from habitat MI 1.2. There were no other actinomycetes isolated from habitat MI 5.2 except for a single *Micromonosporineae*, MI 5.2 K1 that related to *Catellatospora citrea*.

5.6.2 Phylogenetic placement of the actinomycete Isolates

Most of the *Corynebacterineae* belonged to the genera *Mycobacterium*. The isolates MI 5.1 P25, MI U19 and MI U306 belonged to an independent cluster defined by a deep branch (100% bootstraps) compared to the other *Mycobacteria.* Isolate MI 5.1 P25 showed 98% similarity to *M. holderi* and belonged to an independent sub-cluster (100% bootstraps) (Fig. 5.3). The isolate MI B1A showed 94% sequence identity to a *Rhodococcus sp.* MOP100 and was divergent, characterised a long evolutionary distance. The isolate belonged to an independent sub-cluster (96% bootstraps). The isolate MI 1.2 V3, was related to a *Dietzia* species by 98% sequence identity. One of the closest relatives, a *Dietzia sp.* CNJ898 PL04, was isolated from Arctic Ocean Marine Sediments.

Amongst the *Frankineae,* isolate MI 1.2 V7 related to *Humicoccus Flavidus* with 96% sequence identity, and was also related to a neighbouring clade consisting of an uncultured bacterium. The isolate MI 3.3 Y5 was related to *Amycolatopsis minnesotaensis* (*Pseudonocardineae*) and other members of *Amycolatopsis* with 97% sequence identity.

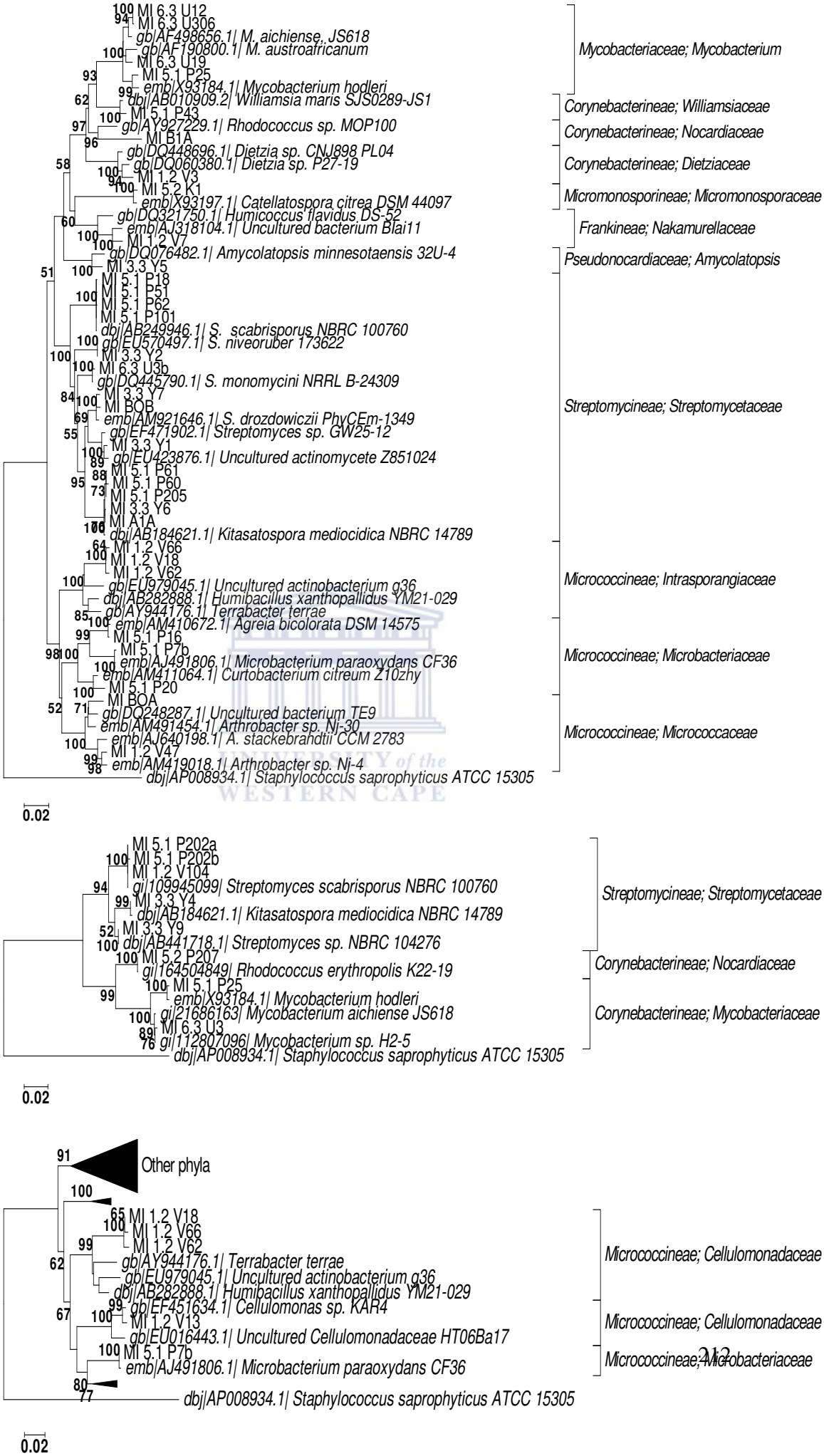


Fig. 5.3: Phylogenetic relationships amongst the actinomycetes isolated from Marion Island terrestrial habitats to known isolates in culture collections. Trees were drawn based on 1350bp (a); 630bp (b) and 750bp (c) alignments using *MEGA 4* (Tamura *et al.,* 2007): Neighbor-Joining method, 1000 bootstrap replicates, Maximum Composite Likelihood substitution model with pairwise deletion of gaps, substitutions included transitions and transversions, and pattern among lineages assumed homogeneous (Felsenstein, 1985; Saitou & Nei, 1987; Tamura *et al.,* 2004). Scale shows units of the number of base substitutions per site.

Most isolates of the genera *Streptomyces* showed 99% sequence identity to other cultured relatives. One of the major clusters consisted of much less divergent isolates of *Streptomyces.* These isolates related to *Streptomyces scabrisporus* and were phylogenetically separated by very short evolutionary distances. This micro-heterogeneity could indicate that these isolates are sub-species. Alternatively, the sequence variation may have arisen from cloning and DNA sequencing artifacts. Sequence alignment shows that these members of *Streptomyces* belonging to that clade relate to each other by 99% sequence identity and may represent only two or three different species. These slight differences were most likely due to high 16S rRNA gene copy numbers. These are known to result in variable genes that are characterised by slight differences in nucleotide composition (Acinas *et al.,* 2004).

Similarly, the other *Streptomyces* isolates, related to *Kitasatospora mediocidica* and were separated by short evolutionary distances. However, they belonged to

clearly distinct branches and to a clade supported by high bootstrap values (100%). The isolate MI 3.3 Y1 belonged to an independent clade (100% bootstraps) of uncultured bacteria. These were related to *Streptomyces drozdowiczii* PhyCEm-1349. Isolates MI 3.3 Y7 and MI BOB belonged to a neighbouring clade (69% bootstrap). MI 3.3 Y1 showed 99% sequence identity to these closest relatives even though it may possibly be a new *Streptomyces* species.

The isolates MI 1.2 V66, MI 1.2 V18 and MI 1.2 V62 belonged to a clade consisting of uncultured bacteria. This clade separated independently from a neighbouring clade (100% bootstraps) consisting of *Micrococcineae.* The *Micrococcineae* related to *Terrabacter terrae*, which was isolated from soil in Spain. The three isolates showed sequence identities of 95% to *T. terrae,* 97% to *T. xanthopallidus* and 97% to *T. terrae* respectively. They are therefore, likely to be new *Terrabacter* species or new genera of actinomycetes.

The isolate MI 5.1 P20 was related to *Curtobacterium citreum* with 98% sequence identity, but diverged from neighbouring clades as a deep branch, supported by a bootstrap value of 100%. The isolate MI BOA belonged to a clade of uncultured bacteria, consisting of a sister *Athrobacter* clade that was related to *Arthrobacter stackebrandtii.* The isolate MI 1.2 V47 was also related to *A. stackebrandtii* by 98% sequence identity. The same isolate was also related to an *Arthrobacter sp.* Nj-4 (97% sequence identity) that was isolated from

Antarctica. Isolate MI 1.2 V13 was related to *Cellulomonas* species with sequence identities between 97 and 98%. One of the *Cellulomonas,* species KAR4 was isolated from a high Arctic permafrost soil in Spitsbergen, Northern Norway, perhaps indicating that MI 1.2 V13 is a psychrophile and probably endemic to Antarctic environments.

5.6.2 1 Recovery of culturable actinomycetes

A comparison was made between the phylotypes identified from three metagenomic clone libraries (habitats MI 1.2, MI 5.1 and MI 6.3) (Chapter 4, Section 4.5) and the actinomycetes subsequently isolated in this study. The comparison was aimed at establishing the extent of culturable actinomycetes that were recovered.

A total of 80 actinomycete phylotypes were identified from the three metagenomic libraries. Subsequently, 27 different actinomycetes were isolated using culture dependent studies. These constituted 33% (27 isolates out of 80 phylotypes) of the phylotypes predicted using culture independent studies. Most of the isolates belonged to the suborders identified using culture-independent techniques (Fig. 5.3).

Only isolate MI 1.2 V18 was identified amongst the phylotypes from metagenomic clone libraries. This constituted a proportion of only 4% of the isolates. This isolate had 100% 16S rRNA gene sequence identity with a

phylotype represented by clones B3-MIV or A3-MIV. These phylotypes related to *Terrabacter terrae* and *Humicoccus xanthopallidus* amongst the other cultured representatives. Thus, only 1 out of 80 phylotypes (1.3% of the clones) identified using culture independent studies were isolated in this study. The slight differences in sequence could have been caused by errors that may have arisen during PCR amplification and sequencing. The remaining 96% of the isolates were not identified using culture independent studies.

Even though the remaining 26 isolates belonged to the taxa predicted using culture independent studies, they were not related to the phylotypes identified from metagenomic clone libraries (Fig. 5.4). There was not enough evidence to show that the gene sequences identified from the remaining 98.7% of phylotypes identified using culture independent studies were identical to the other 96% of the isolates. In most cases, isolates did not belong to the same phylogenetic branches with the clone phylotypes even though they belonged to related neighbouring clades.

The phylotypes from the metagenomic clone libraries phylogenetically separated from the isolates by divergent evolutionary branches. Notable evolutionary distances and high bootstraps characterised the separation of these clades or branches. This showed that culture dependent studies resulted in the identification of additional actinobacteria to those identified using culture independent techniques.

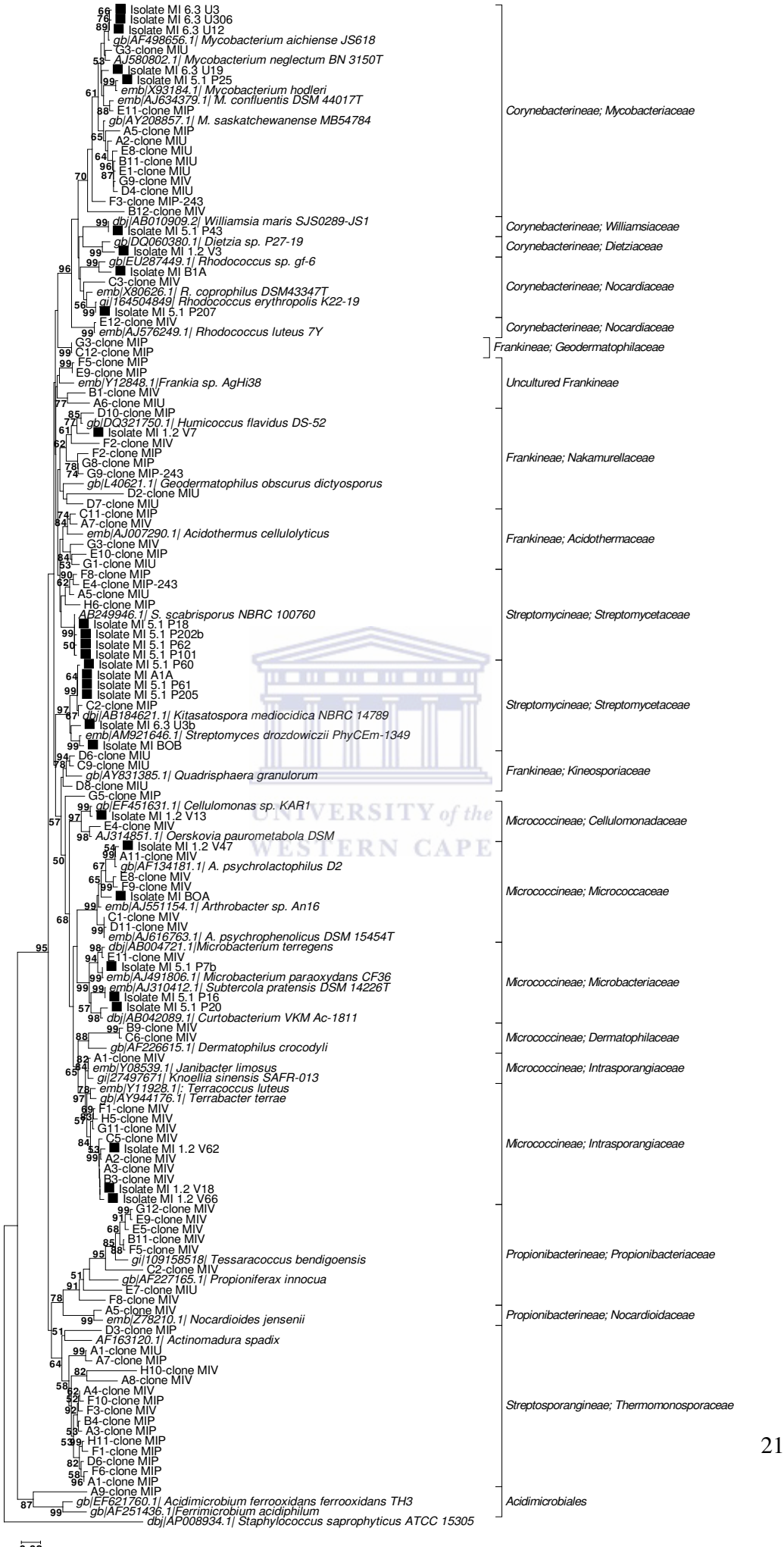


Fig. 5.4: Comparison of actinomycetes isolated from Marion Island terrestrial habitats MI 5.1, MI 1.2 and MI 6.3 to the clones identified using culture-independent studies. The tree was drawn based on 570bp alignment using *MEGA 4* (Tamura *et al.,* 2007): Neighbor-Joining method, 1000 bootstrap replicates, Maximum Composite Likelihood substitution model with pairwise deletion of gaps, substitutions included transitions and transversions, and pattern among lineages assumed homogeneous (Felsenstein, 1985; Saitou & Nei, 1987; Tamura *et al.,* 2004). Scale shows units of the number of base substitutions per site.

The nine isolates of the suborder *Corynebacterineae* belonged to the families *Mycobacteriaceae* (5)*, Williamsiaceae* (1)*, Dietziaceae*a (1) and *Nocardiaceae* (2). Only a few of the *Mycobacteriaceae* isolates were related to the phylotypes from metagenomic clone libraries.

Most phylotypes identified from culture independent studies were represented by clones that belonged to a neighbouring independent clade (61% bootstraps). This suggests that there were still many uncultured *Mycobacteriaceae.* The *Dietzia* isolate MI 1.2 V3 was not detected using culture independent studies. Only one *Frankineae* was isolated from habitat MI 1.2 and was related to *Humicoccus flavidus.* The isolate was phylogenetically distant from clones representing the phylotypes identified from the same habitat.

The *Streptomyces* clones (4 different genotypes) that were related to *S. scabrisporus* belonged to a clade that was independent (99% bootstraps). The other *Streptomyces* isolates (4 different genotypes) were related to *Kitasatospora mediocidica* and clone C2-MIP. The use of culture independent studies did not result in the identification of *Streptomyces* phylotypes from habitat MI 6.3. However, isolate MI 6.3 U3b was isolated from this habitat.

The *Micrococcineae,* isolate MI 1.2 V13 related to a phylotype represented by clone E4-MIV, but belonged to a phylogenetically separate clade (97% bootstraps). The case was similar with the *Micrococcineae,* isolate MI 5.1 P7b and clone E11-MIV. The *Micrococcineae* isolates MI 5.1 P16 and MI 5.1 P20 were not detected in metagenomic clone libraries from habitat MI 5.1. None of the members of suborders *Streptosporangineae* and *Propionibacterineae* was isolated in this study. However, these were identified in metagenomic libraries and may require special isolation techniques.

5.7 Discussion

Culture dependent studies were consistent with the findings of Chapter 4 that specific groups of actinomycetes occur in specific habitats. Most of the isolates identified using culture-dependent techniques were not of the same genotypes identified using culture-independent techniques. However, they were related. In addition, the members of the genus *Streptomyces* that were not commonly

identified using culture-independent studies were frequently isolated from habitat MI 5.1.

Some of the isolates were related to other cultured relatives by less than 97% 16S rRNA gene sequence identities. They may represent new families or genera according to the general rules of classifying bacteria (Wayne *et al.,* 1987). However, actinobacteria with less than 99% 16S rRNA sequence identities belong to different species because they typically show less than 70% DNA-DNA homology (Stach *et al.,* 2003a; Stackebrandt & Goebel, 1994). Most actinomycetes isolated in this study showed less than 99% 16S rRNA gene sequence identities to other cultured relatives. They are therefore, most likely to be new species. It is possible that the isolates showing 99% similarity will have different morphological and biochemical properties, especially the members of the family *Streptomycetaceae*. In a number of studies, apparently identical actinomycete isolates were reclassified as different species using morphological, biochemical and taxonomic characteristics (Stackebrandt, *et al.,* 2004; Stackebrandt & Schumann, 2000; Yi *et al.*, 2007).

Kochkina *et al.* (2001) identified actinobacteria as the most dominant group, contributing 50-90% of the total viable counts of bacteria isolated from ancient Arctic and Antarctic permafrost sediments samples. Actinomycetes of genera *Arthrobacter, Micrococcus, Kocuria, Rhodococcus, Gordonia, Microbacterium, Brevibacterium, Nocardioides* and *Mycobacterium* particularly constituted the

majority of the actinobacteria. Members of the family *Micrococcaceae* and the suborder *Corynebacterineae*, which possess a thick lipopolysaccharide in cell walls formed the major groups amongst the actinobacteria isolated (Kochkina *et al.,* 2001). In our study, similar groups of actinomycetes were isolated except for members of the genera *Kocuria, Gordonia* and *Brevibacterium.* Similarly, the *Micrococcineae* were in high proportions in this study, suggesting that they are adapted to lower temperature environments.

A comparison of clone libraries to the isolated cultures showed that culture-independent studies alone were not enough to provide information about the composition of microbiological communities. For example, culture-dependent studies showed that the *Micrococcineae* were also present in habitat 5.1, which were not identified using culture-independent studies. In addition, other groups of actinobacteria such as *Dietzia* were isolated, but not detected using culture-independent methods. There are many actinomycetes yet to be cultured because most of those cultures did not share related phylogenetic branches with the clones identified using culture-independent studies.

5.8 Summary

Actinomycetes were successfully isolated from Marion Island terrestrial habitats using well-established selective media and classical microbiological techniques. A total of 42 different actinomycetes were obtained and identified based on their 16S rRNA gene sequences. Amongst these, 27 were from habitats studied using

culture independent techniques. Only one isolate was identified amongst the phylotypes from metagenomic clone libraries. Amongst all the isolates, 13 were related to other cultured relatives by sequence identities ranging between 94 and 98%. These constituted 30% of the total number of actinomycetes isolated. Amongst these 13 isolates, five isolates (MI 6.3 U19, MI BOA, MI 3.3 Y5, MI B1B and MI 1.2 V18) showed 97% sequence identities to the cultured relatives. Another four isolates showed less than 97% sequence identities. These were MI 1.2 V66 (95%), MI 1.2 V7 (96%), MI 1.2 V62 (95%) and MI 1.2 B1A (93-94%). Most of these may need to be reclassified into new genera because they showed low sequence identities to other cultured relatives. A number of actinomycetes isolated from habitat MI 1.2 were related to actinomycetes from psychrotrophic environments.

CHAPTER 6

6.0 General Discussion, Conclusions and Recommendations

6.1 Summary of discussions

In this Chapter, results are interpreted in reference to other studies. The limitations associated with metagenomic community analysis are also discussed.

The choice of sampling strategy, particularly the effective dissociation of cells from soil particles and efficient cell lysis is crucial for the recovery of representative community DNA (Heuer *et al.,* 2001). DNA extraction methods do not equally lyse all cells. Most microorganisms that form vegetative cells, spores and hyphae, especially actinomycetes, are difficult to lyse in soil. This was established by extracting metagenomic DNA from soils inoculated with known microorganisms (Frostegård *et al.,* 1999). Harsh mechanical soil disruption methods were developed for efficient in-situ lysis of actinomycete cells. The most efficient methods are soil grinding (Frostegård *et al.,* 1999) and bead-beating (Tsai & Rochelle, 2001).

In this study, high molecular weight DNA was obtained using bead-beating and chemical cell-disruption methods. The DNA yields varied between 78 to 190µg per gram of soil. The DNA purity was high as indicated by the $A_{260/280nm}$ ratios (Tsai & Rochelle, 2001), which ranged between 1.6 and 1.8 after removal of humic acids. DNA extraction was conducted in triplicate and the reliability of the

method verified using DGGE analysis. The analysis showed that the community microbiological diversity obtained was reproducible based on the techniques used.

Humic substances inhibit PCR reactions (Hugenholtz & Goebel, 2001). In this study, the humic acids were removed to ensure that PCR reactions would not be inhibited. All DNA samples used were of $A_{260/280nm}$ ratios greater than 1.9 after PVPP purification. The limitations of PCR-based methods include the bias and distortion of PCR due to differential amplification of target rRNA genes (Baker *et al.,* 2003; Forney *et al.,* 2004). This is caused by problems that include primer specificity, primer reannealing, variable denaturation of templates, gene copy number and genome size. This subsequently distorts the interpretation of microbiological community data (Hugenholtz & Goebel, 2001).

In this study, two precautions were taken to minimize the limitations of PCR. The primer S-C-Act-235-a-S-20 is claimed to have 99.7% coverage for actinomycetes whilst S-C-Act-878-a-A-19 has 87% (Stach *et al.,* 2003b). However, these claims are based on sequences from cultured actinomycetes and may not necessarily be true for the unknown groups. Even though these primers have a higher coverage, the primer F243 was also used in combination with the bacterial specific primer U1510R (Heuer *et al.,* 1997). Primer F243 was however, not very specific for actinomycetes and amplified mostly *Verrucomicrobia.* Actinomycetes

constituted only 14% of the clones in the metagenomic library from habitat MI 5.1 (Cotula Herbfield Habitat).

The products obtained from the PCR amplification of metagenomic DNA were evaluated for suitability as templates for nested PCR-DGGE. The products from the primer set E9F and U1510R were unsuitable for nested PCR-DGGE using primers 341F-GC and 534R. These formed PCR-DGGE products that were believed to be chimeras, most likely caused by primer 3'-end complementarities with incompletely transcribed products. The products were difficult to identify as false positives because they were of almost the same size as the fragments expected. Such products could have provided a false representation of the bacterial communities if not compared to products arising from other primer sets. Other PCR products used for DGGE were found to be suitable.

The DGGE denaturing gradients were optimized for maximum electrophoretic separation of gene fragments in order to minimize the problem of co-migration. Such fragments exhibit the same electrophoretic mobility because they share related nucleotide compositions or may be closely phylogenetically related (Heuer *et al.,* 1997). The co-migration of DNA fragments on DGGE results in the underestimation of community diversity.

The dissimilarities between habitat clusters identified using soil characteristics were due to soil solution sodium (32-55%) and moisture content (55%). Total

plant cover was the most important determinant of similarities (67-79%) amongst plant characteristics. The first principal component explained 42% of the variability in soil physiochemical properties and separated habitats mainly based on salinity and nutrient availability. In a related study, salinity was identified as the most important factor determining the composition of microbiological communities (Lozupone & Knight, 2007). PCA analysis also showed that different combinations of nutrients were important in different habitats. Habitat MI 5.1, MI 1.1 and MI 1.2 were most separated from the other habitats using MDS ordination. These three habitats are influenced by sea salt-spray and manuring from marine birds and mammals (Smith e*t al.*, 2001; Smith & Steenkamp, 2001).

Three habitat clusters were consistently identified using hierarchical clustering of both bacterial and actinobacteria diversities. MDS analysis showed that most of the habitats were scattered and thus, not as closely related. However, the similarities in habitat characteristics did not predict similarities in microbiological diversity of habitat clusters.

The soil pH positively correlated the most (35%) with actinobacteria. A combination of soil pH, organic carbon, total potassium and exchangeable magnesium accounted for 26.4% of the positive correlation. The tussock graminoids, *Poa Annua*, mire bryophytes, *Bryum/Breutelia* plants, and in some cases, cushion bryophytes, lichens and tussock graminoids positively correlated with actinobacteria (32%) whilst the *Bryum/Breutelia* plants negatively correlated.

Total calcium, exchangeable calcium, soil solution calcium, magnesium, and potassium, total sodium and exchangeable sodium positively correlated (35-36%) with bacterial diversity. Amongst the plants, *P. annua* positively correlated (21%) with bacterial distribution. In addition, *P. annua* positively correlated with bacterial distribution (21%) in combination with lichens, epiphytic graminoids or brachythecium mosses.

The habitat clusters obtained from the analysis of environmental data in this study were not congruent to those obtained using microbiological diversity. MDS ordination showed that most of the habitats were not closely clustered based on microbiological diversity. Different habitat characteristics, therefore explained the microbiological diversities observed. However, an analysis of more soil characteristics is recommended in order to clearly identify the factors that resulted in similar habitat clusters using both bacterial and actinobacterial diversities.

Hierarchical clustering showed that some habitats consisted of unique groups of microorganisms, especially actinobacteria. This demonstrates the importance of DGGE in directing microbiological community research efforts towards the unique habitats. Most unique phylotypes were identified in habitats MI 1.2 (Coastal Fellfield Habitat), MI 5.3 (Biotic Lawn Habitat), MI 5.1 (Cotula Herbfield Habitat), MI 5.2 (Biotic Mud Habitat) and MI 1.1 (Coastal Herbfield Habitat). These habitats were identified using PCA as having high salinity and nutrient availability, typically from the impact of animals. This suggested that the high

salinity levels were major determinants of unique microbiological diversity. The identification of unique phylotypes and subsequent culturing of unique actinomycetes from habitat MI 1.2 confirmed this to be true. A few of these unique actinobacterial genospecies were also identified in habitats MI 3.3 (Mesic Fernbrake Habitat), MI 6.2 (Mesic Mire Habitat) and MI 6.5 (Biotic Mire Habitat) which contained low nutrient concentrations except for MI 6.5.

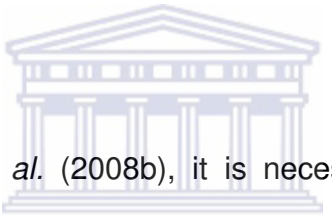
Actinomycetes were estimated to constitute between 50 and 52% of the total bacterial diversity, based on the diversity patterns of DNA fragments on DGGE. Unexpectedly, lower numbers of unique bacteria were obtained based on bacterial diversity. This suggests that DGGE could have failed to separate the increased numbers of DNA fragments even though optimized for the most possible separation. This could also have been due to different treatments of the samples during amplification of metagenomic DNA. The products were amplified from the metagenome using different primer sets, which could have led to the anomaly. The primer set S-C-act-235-a-S-20 and S-c-act-878-a-S-19 was used to amplify actinobacteria from metagenomic DNA. The PCR-DGGE primers, 341-F GC and 534R, are universally specific for bacteria and were used to amplify metagenomic DNA. Since the attempt to initially amplify the metagenomic DNA using the primers E9F and U1510R had resulted in false products, direct PCR-DGGE of metagenomic DNA was used for bacterial diversity. This strategy was also used for DGGE characterization of actinomycete communities (Heuer *et al.,* 1997).

DGGE showed almost similar species richness to that identified from screening metagenomic clones libraries. DGGE analysis showed the presence of 38, 36 and 41 phylotypes whilst clone libraries consisted of 34, 38 and 39 phylotypes in habitats MI 5.1, MI 6.3 and MI 1.2, respectively. This result demonstrated the excellent reproducibility of the methods used in this study. Coverage of the metagenomic clone libraries was estimated at 88%, 84% and 85% for habitats MI 5.1, MI 6.3 and MI 1.2, respectively.

In most previous studies, DGGE and statistical analysis were only used to determine how microbial communities related according to similarities of their habitat conditions. Other studies focused on identifying the important environmental factors that cause variability amongst the environmental factors. These studies and their references are summarized in Table 3.1 (Chapter 3, Section 3.2). In this study, statistical analysis was used to associate bacterial and actinobacterial phylotypes to specific habitats. Hierarchical clustering was used to identify the phylotypes that were similarly distributed across the habitats and to determine how habitats related each other.

The statistical analysis and classification described here could be improved by including the relative abundance of the phylotypes. This could enable to cluster the habitats, not only based on the presence of phylotypes, but also according to their proportions. However, the limitations of PCR and resolution on DGGE may distort the community data. This makes DGGE an unreliable quantitative method

for studying the distribution (or abundance) of genospecies. Suitable quantitative methods include real time quantitative PCR (qPCR) (Malik *et al.,* 2008), FISH (Keller & Zengler, 2004; Malik *et al.,* 2008; Steele & Streit, 2005) and microarrays (Malik *et al.,* 2008; Zhou, 2003). These could be used to provide greater insight into the distribution and functions of specific phylotypes. Since the methods used in this study detect DNA, both dead and living genospecies are also detected, which complicates the study. Since RNA has a more rapid turnover and therefore, more accurately represents active genospecies, the use of total cellular RNA as the template for reverse transcription PCR-DGGE is recommended.



According to Macdonald *et al.* (2008b), it is necessary to develop molecular markers for characterizing specific habitats (Macdonald *et al.,* 2008b). The identification and characterization of the biotypes into specific clusters according to their occurrence in habitats may enable the association of specific groups of microorganisms with specific habitats and functions. The screening of metagenomic libraries by DGGE demonstrates one way in which environmental microbiological markers could be developed.

Even though PCA identified the most important soil variables (Chapter 3, Section 3.7.4), these may not account completely for the actinobacterial distribution observed. Some of the habitat characteristics such as oxygen availability were not analyzed because the data was not available. Such missing data may have

been equally important. In addition, the impact of important variables such as pH is difficult to identify using PCA. The habitat characteristics such as soil structure and vegetation type are rather qualitative. Their impacts can only be assessed indirectly by measuring associated factors such as oxygen consumption, mineralization activities and fixation rates of inorganic nutrients.

Culture-independent studies and phylogenetic analysis suggested that oxygen availability and pH were also important factors that determined the distribution of actinobacteria. The availability of oxygen has implications for the composition of microbiological diversity and community functions. This is particularly true for the microbiological mineralization of plant litter. The abundance of bacteria varies directly with the amount and type of litter, and the stage of carbon recycling (decomposition of plant matter) (Carney & Matson, 2005). The microbial mineralization of carbon and nitrogen was shown to occur at temperatures as low as -5ºC (Krivtsov *et al.,* 2005). Temperature was therefore not expected to be a major limiting factor in mineralization activities on Marion Island (mean temperature, 5-6.5ºC). The high moisture content, such as water-logging in habitat MI 6.3, is thought to be a major factor that retards the decomposition of nutrients on Marion Island (Smith, 1988). The mineralization activities of plant litter by actinomycetes may have been lowered in habitat MI 6.3 due to the anaerobic conditions. This should have resulted in the proliferation of *Acidimicrobiales* and *Frankineae*, which are involved in generating energy under anaerobic conditions (Rawlings *et al.,* 2003; Verghese & Misra, 2002).

The physical properties of soil and the vegetation coverage affect the diversity of microbiological communities (Nüsslein & Tiedje, 1999). This includes how microorganisms compete with vascular plants for nutrients (Smith, 1988). The high proportions of *Frankineae* in habitats MI 5.1 and MI 6.3 may have been due to the type of vegetation and anaerobic conditions. *Frankineae* frequently form symbiotic relationships with plants, including vascular plants (Verghese & Misra, 2002), and are typically involved in nitrogen-fixation under anaerobic conditions. The *Acidimicrobiales* are iron-oxidizing and sulphate reducing chemoautotrophic bacteria. They are involved in the biomineralization of metal ores (Rawlings *et al.,* 2003) and mineral sulfide oxidation (Cleaver *et al.,* 2007). The *Acidimicrobiales* were found to be dominant in soils with very low carbon availability due to low carbon mineralization rates of plant litter (Fierer *et al.,* 2007).

The actinobacterial community structure of habitat MI 5.1 also comprised high proportions of *Frankineae* and *Acidimicrobiales.* This habitat is influenced by trampling from marine birds and mammals, which has been shown to compact the soils causing anaerobic conditions (Smith & Steenkamp, 2001; Smith, *et al.,* 2001). The trampling of soil was shown to significantly increase the soil denitrification activities (Menneer *et al.,* 2005). The denitrification activities are coupled to nitrogen fixation and involve energy production from nitrates and nitrites under anaerobic conditions (An *et al.,* 2001; Menneer *et al.,* 2005). Thus, culture-independent studies suggested that the *Acidimicrobiales* were involved in anaerobic production of energy from inorganic compounds in habitats MI 6.3.

The *Frankineae* were most likely involved in nitrogen fixation activities in habitat MI 5.1. Habitats MI 1.2 and MI 5.1 were characterised by higher diversity indices, compared to MI 6.3. However, MI 1.2 had the highest species richness due to the presence of unique groups (*Micrococcineae* and *Propionibacterineae*), which did not occur in MI 5.1 or MI 6.3.

Most of the *Micrococcineae, Corynebacterineae* and *Propionibacterineae* phylotypes identified from habitat MI 1.2 were previously uncultured and related to isolates from psychrophilic environments, suggesting that they were endemic to cold habitats. Members of the *Micrococcineae* were subsequently isolated using culture-dependent studies. The *Streptosporangineae* and some of the *Frankineae* identified from habitat MI 5.1 were related to phylotypes only identified from Antarctic environments.

This study supported the claims that genetic traits and adaptation to environmental factors influence the spatial distribution of microbial taxa (Green *et al.,* 2008; Kassen and Rainey, 2004) and that some microorganisms are endemic (Martiny *et al.,* 2006) to specific habitats. Our findings contradicted recent studies where it has been suggested that marine environments were better sources of novel actinomycetes than terrestrial environments (Bull *et al.,* 2005; Bull *et al.,* 2000; Maldonado *et al.,* 2005a). Members of a bacterial species are at least 97% identical in 16S rRNA gene sequence (Wayne *et al.,* 1987). However, actinomycetes of the same species show at least 99% identity (Stach *et al.,*

2003a; Stackebrandt & Goebel, 1994). In this study, 13 isolates showed 16S rRNA gene sequence identities of 98% or less with known actinomycetes, suggesting that they may be new species. In addition, four of the isolates showed sequence identities of less than 97% to known actinomycetes. These may belong to new genera.

A total of 111 actinobacterial phylotypes identified from metagenomic clone libraries. Most of these related to database sequences by identities ranging between 89 and 99%. Only one isolate, representing 0.9% of the diversity, showed 100% sequence identity to a cultured representative. This finding supported the claim that more than 99% of the environmental microorganisms are yet to be cultured (Amann *et al.,* 1995).

The actinomycetes comprised of 80 phylotypes that were identified from metagenomic clone libraries. A comparison with culture independent studies showed that most of the isolates clustered independently from the related phylotypes. A total of 27 actinomycetes were isolated from the habitats that were studied using culture independent techniques. Only one isolate had a sequence that was 100% identical to that of a phylotype identified using culture independent studies. This isolate constituted only 4% of the actinomycetes isolated from these habitats. There was no sufficient evidence to show that the remaining 96% of the isolates identified using culture dependent studies were amongst those phylotypes identified from metagenomic clone libraries. Thus,

only 1.3% (1 out of 80) of the actinomycete phylotypes identified using culture independent techniques was cultured in this study. The remaining 98.7% phylotypes were not cultured. Some of the actinomycetes may have been in the form of dormant structures such as spores that were resistant to lysis. This could explain the isolation of more members of the genus *Streptomyces* than those identified using culture-independent techniques.

New actinomycete isolates are a potential source of novel therapeutic agents such as antibiotics (Baltz, 2007; Donadio *et al.,* 2002). Their biosyntheses are mostly mediated by the action of PKS and NRPS enzymes (Amoutzias *et al.,* 2008; Baltz, 2007; Busti *et al.,* 2006; Caffrey *et al.,* 1992; Demain, 1999; Donadio *et al.,* 2007). Members of *Arthrobacter* and *Rhodococcus* may be of industrial importance because they are associated with the degradation of complex synthetic substances (Linos *et al.,* 2000) and production of biodegrading enzymes (McCarthy & Williams, 1992). *Rhodococcus* is important for producing nitrile-hydrolyzing enzymes (Brandão & Bull, 2003; Brandão *et al.,* 2002). These new actinomycete isolates derived from this study should be explored for these potential applications.

6.2 Overall conclusions

This study has provided additional insight to the microbiology of a psychrotrophic environment and resulted in the identification of previously uncultured actinobacterial phylotypes in several Marion Island habitats.

The actinobacterial diversity in Marion Island terrestrial habitats is determined by environmental factors that distinguish the habitats. Salinity and nutrient availability are the most important environmental factors, each resulting in different actinobacterial community structures. The microorganisms in habitat MI 6.3 were associated with autotrophic production of energy generated by the *Acidimicrobiales* and the nitrogen fixation from *Frankineae.* These processes occur under anaerobic conditions that are commonly found in soils associated with the two habitats. The Marion Island terrestrial habitats were classified according to actinobacterial diversity. Conversely, general bacterial diversity was not very useful for classifying the habitats based on the methods used in this study. A number of novel *Micrococcineae* isolated from habitat MI 1.2 were unique to that habitat. However, most of the actinomycetes identified using culture-independent techniques are yet to be isolated.

6.3 Recommendations and future perspectives

This work is far from being exhaustive, and should be extended to the phylogenetic characterization of the remaining 18 habitats. The studies should encompass further classification of the habitats based on microbiological diversity. Furthermore, the study of microbiological involvement in biogeochemical cycles would be worth investigating. Such studies should include measuring the fixation of inorganic compounds and mineralization activities by specific groups of microorganisms under anaerobic conditions. In addition,

isolation of actinomycetes using a variety of techniques is recommended. These should comprise the use of differential centrifugation, membrane filters, simulation of natural environment, application of selective pressure, prolonged incubation periods, taxon-specific media and isolation on oligotrophic media (Chapter 5, Section 5.2). Different types of media and culture conditions should be explored for the isolation of uncultured actinomycetes. In addition, the *Acidimicrobiales* identified as the dominant group in habitat MI 6.3 should be cultured using recommended media (Johnson, 1995). These are important for industrial applications, such as bioleaching, biomineralization of metal ores and sulfate reduction. The newly isolated actinomycetes must be evaluated for their applications in industrial biosyntheses, such as in the production of therapeutic compounds. This could be determined by conducting plate-inhibition assays and identifying novel PKS and NRPS genes and enzymes. Isolates showing less than 99% identities in 16S rRNA gene sequences may belong to new species. Therefore, chemotaxonomic and biochemical tests are recommended to discriminate amongst these isolates.

The use of more than one set of taxon-specific primers is imperative when conducting microbiological community studies. This approach has the potential to increase the coverage of diversity. The findings from this study clearly showed that both culture-dependent and culture-independent methods complement each other. The use of both approaches is therefore, recommended when studying the diversity of microbiological communities.

Appendices

Appendix A1: Average values of soil physiochemical properties in Marion Island terrestrial habitats (Smith, *et al.,* 2001).

	MI_1.1	MI_1.2	MI 2.1	MI 2.2	MI 3.1	MI 3.2	MI_3.3	MI 3.4	MI 3.5	MI 3.6	MI 4.1
Bulk density	252.0	237.0	467.0	225.0	143.0	152.0	124.0	136.0	148.0	77.0	164.0
pH	5.8	5.5	5.8	5.4	4.8	4.6	5.2	4.5	5.7	6.2	4.0
Moisture	400.0	571.0	147.0	329.0	526.0	614.0	704.0	658.0	707.0	1218.0	525.0
Organic C	20.9	28.4	6.3	13.0	19.4	25.6	24.2	24.1	18.9	16.0	36.2
Total N	1.5	1.7	0.4	0.8	1.5	2.0	1.8	2.1	1.7	1.8	3.7
Ammonium N	8.4	11.0	0.5	0.5	5.2	3.7	5.0	0.5	4.0	6.5	20.8
Nitrate N	2.8	0.4	0.1	0.0	2.0	1.7	2.1	0.2	3.2	5.6	15.1
Nitrite N	0.4	0.4	0.1	0.0	0.3	0.0	0.3	0.0	0.1	0.3	0.5
Total P	1.2	0.9	1.2	1.3	1.4	1.9	1.8	2.5	2.0	2.6	2.1
Phosphate P	66.5	55.5	3.9	6.0	19.4	34.9	6.5	8.2	13.7	13.7	233.5
Total Ca	49.3	50.0	24.9	16.1	12.3	10.4	10.5	11.2	16.0	15.5	3.6
Total Mg	15.0	13.6	15.0	11.2	3.5	3.2	4.4	1.9	6.9	6.2	0.7
Total K	1.8	1.8	0.7	0.6	0.8	0.7	0.8	0.7	0.7	0.7	0.4
Total Na	15.0	14.6	2.2	1.5	1.6	0.9	1.1	1.0	1.5	1.5	1.0
[a]C.E.C	79.6	78.9	38.1	73.0	93.3	121.0	98.4	109.9	85.9	85.1	74.5
[b]Exch. Ca	22.0	14.5	20.0	10.3	6.3	6.0	4.0	9.5	16.3	11.7	9.3
[b]Exch. Mg	25.9	30.3	3.1	4.1	8.3	8.4	6.4	4.8	10.5	7.6	2.3
[b]Exch. K	1.0	1.2	0.1	0.6	0.7	0.9	0.6	0.3	0.5	0.4	0.0
[b]Exch. Na	22.1	20.1	1.8	1.1	2.0	1.2	0.6	0.6	2.2	1.9	1.3
[c]Sln Ca	154.0	202.0	72.0	67.0	74.0	96.0	128.0	119.0	117.0	103.0	104.0
[c]Sln Mg	90.0	114.0	14.0	24.0	38.0	51.0	45.0	62.0	55.0	67.0	53.0
[c]Sln K	661.0	687.0	78.0	182.0	360.0	223.0	479.0	304.0	196.0	227.0	221.0
[c]Sln Na	9627.0	6675.0	97.0	182.0	273.0	308.0	334.0	377.0	215.0	272.0	534.0

[a]C.E.C = cation exchange capacity, [b]Exch. = exchangeable, [c]Sln = solution

Appendix A continued

Bulk density	MI_6.4	MI_6.3	MI_4.3	MI_4.3b	MI_5.1	MI_5.2	MI_5.3	MI 6.1	MI_6.2	MI_6.5	MI 6.6
pH	10.0	55.0	170.0	157.0	143.0	81.0	144.0	112.0	71.0	63.0	63.0
Moisture	5.8	4.8	4.2	4.7	4.8	5.0	5.3	5.0	4.7	5.1	4.9
Organic C	995.0	1839.0	483.0	552.0	658.0	1018.0	679.0	898.0	1296.0	1573.0	1686.0
Total N	17.0	38.8	34.9	21.1	32.2	36.7	27.3	23.4	36.8	39.9	43.5
Ammonium N	1.1	1.9	3.7	2.1	2.9	5.9	2.9	1.8	2.3	2.6	2.1
Nitrate N	8.8	36.4	29.4	53.4	59.9	326.3	147.1	1.7	10.0	55.0	41.9
Nitrite N	0.6	1.1	5.5	2.7	10.9	65.4	8.7	0.1	0.9	3.0	0.3
Total P	0.3	0.4	0.5	0.1	0.5	1.0	0.7	0.1	0.3	1.7	0.0
Phosphate P	1.5	1.1	2.1	1.5	2.2	1.3	1.0	1.6	1.2	1.6	1.0
Total Ca	5.2	96.0	218.8	120.8	258.5	132.1	268.4	28.2	70.3	222.1	116.5
Total Mg	7.6	5.2	3.0	15.6	19.9	4.9	11.6	10.4	4.0	5.1	5.7
Total K	2.6	1.9	0.8	5.6	5.6	1.4	4.3	3.4	1.3	2.1	3.1
Total Na	0.5	0.7	0.6	0.3	1.2	0.3	0.7	0.8	0.9	0.9	0.9
C.E.C	1.4	1.2	1.4	2.3	5.1	1.6	2.4	1.4	1.3	1.9	4.6
Exch. Ca	81.0	85.8	75.2	48.3	83.8	74.4	64.8	84.3	81.7	72.1	66.8
Exch. Mg	8.8	7.0	9.0	16.2	12.1	9.1	15.4	10.0	6.6	5.5	9.2
Exch. K	5.5	10.4	2.8	12.9	12.0	8.8	8.9	7.2	7.7	10.8	28.6
Exch. Na	0.3	0.6	0.1	0.1	0.6	0.0	0.3	0.7	1.1	0.6	0.8
Sln Ca	0.9	2.3	1.8	2.5	5.3	1.9	2.6	1.2	2.2	3.0	9.8
Sln Mg	98.0	119.0	96.0	99.0	108.0	75.0	86.0	110.0	80.0	85.0	140.0
Sln K	33.0	82.0	60.0	120.0	87.0	88.0	65.0	533.0	71.0	88.0	154.0
Sln Na	234.0	447.0	341.0	184.0	502.0	175.0	321.0	353.0	687.0	578.0	575.0
	260.0	701.0	1020.0	1187.0	3222.0	881.0	886.0	457.0	667.0	984.0	4057.0

[a]C.E.C = cation exchange capacity
[b]Exch. = exchangeable
[c]Sln = solution

Appendix A2: PCA analysis

log (x+1) transformed data of Marion Island soil physiochemical properties

	MI_5.3	MI_5.1	MI_1.2	MI_6.5	MI_6.2	MI_5.2	MI_3.3	MI_1.1	MI_6.4	MI_6.3	MI_4.3	MI_4.3b
Bulk density	2.161	2.158	2.377	1.806	1.857	1.914	2.097	2.403	1.041	1.748	2.233	2.199
pH	0.799	0.763	0.813	0.785	0.756	0.778	0.792	0.833	0.833	0.763	0.716	0.756
Moisture	2.833	2.819	2.757	3.197	3.113	3.008	2.848	2.603	2.998	3.265	2.685	2.743
Organic C	1.452	1.521	1.468	1.612	1.577	1.576	1.401	1.34	1.255	1.6	1.555	1.344
Total N	0.591	0.591	0.431	0.556	0.519	0.839	0.447	0.398	0.322	0.462	0.672	0.491
Ammonium N	2.171	1.785	1.079	1.748	1.041	2.515	0.778	0.973	0.991	1.573	1.483	1.736
Nitrate N	0.987	1.076	0.146	0.602	0.279	1.822	0.491	0.58	0.204	0.322	0.813	0.568
Nitrite N	0.23	0.176	0.146	0.431	0.114	0.301	0.114	0.146	0.114	0.146	0.176	0.041
Total P	0.301	0.505	0.279	0.415	0.342	0.362	0.447	0.342	0.398	0.322	0.491	0.398
Phosphate P	2.43	2.414	1.752	2.348	1.853	2.124	0.875	1.829	0.792	1.987	2.342	2.086
Total Ca	1.1	1.32	1.708	0.785	0.699	0.771	1.061	1.702	0.934	0.792	0.602	1.22
Total Mg	0.724	0.82	1.164	0.491	0.362	0.38	0.732	1.204	0.556	0.462	0.255	0.82
Total K	0.23	0.342	0.447	0.279	0.279	0.114	0.255	0.447	0.176	0.23	0.204	0.114
Total Na	0.531	0.785	1.193	0.462	0.362	0.415	0.322	1.204	0.38	0.342	0.38	0.519
C.E.C	1.818	1.928	1.903	1.864	1.918	1.877	1.997	1.906	1.914	1.939	1.882	1.693
Exch. Ca	1.215	1.117	1.19	0.813	0.881	1.004	0.699	1.362	0.991	0.903	1	1.236
Exch. Mg	0.996	1.114	1.496	1.072	0.94	0.991	0.869	1.43	0.813	1.057	0.58	1.143
Exch. K	0.114	0.204	0.342	0.204	0.322	0	0.204	0.301	0.114	0.204	0.041	0.041
Exch. Na	0.556	0.799	1.324	0.602	0.505	0.462	0.204	1.364	0.279	0.519	0.447	0.544
Sln Ca	1.94	2.037	2.307	1.934	1.908	1.881	2.111	2.19	1.996	2.079	1.987	2
Sln Mg	1.82	1.944	2.061	1.949	1.857	1.949	1.663	1.959	1.531	1.919	1.785	2.083
Sln K	2.508	2.702	2.838	2.763	2.838	2.246	2.681	2.821	2.371	2.651	2.534	2.267
Sln Na	2.948	3.508	3.825	2.993	2.825	2.945	2.525	3.984	2.417	2.846	3.009	3.075

log (x+1) transformed and normalized data of Marion Island soil physiochemical properties

	MI_5.3	MI_5.1	MI_1.2	MI_6.5	MI_6.2	MI_5.2	MI_3.3	MI_1.1	MI_6.4	MI_6.3	MI_4.3	MI_4.3b
Bulk density	0.437	0.429	1.019	-0.523	-0.384	-0.232	0.263	1.091	-2.589	-0.679	0.631	0.538
pH	0.497	-0.551	0.894	0.088	-0.772	-0.122	0.294	1.466	1.466	-0.551	-1.936	-0.772
Moisture	-0.35	-0.415	-0.709	1.393	0.991	0.49	-0.275	-1.447	0.442	1.717	-1.056	-0.78
Organic C	-0.199	0.389	-0.059	1.157	0.867	0.857	-0.626	-1.143	-1.865	1.056	0.677	-1.11
Total N	0.469	0.469	-0.695	0.216	-0.06	2.276	-0.58	-0.939	-1.491	-0.469	1.06	-0.258
Ammonium N	1.278	0.554	-0.769	0.485	-0.84	1.923	-1.334	-0.968	-0.934	0.157	-0.012	0.462
Nitrate N	0.7	0.889	-1.088	-0.118	-0.806	2.477	-0.353	-0.165	-0.964	-0.713	0.331	-0.19
Nitrite N	0.51	-0.019	-0.311	2.466	-0.624	1.197	-0.624	-0.311	-0.624	-0.311	-0.019	-1.33
Total P	-1.144	1.685	-1.453	0.435	-0.571	-0.303	0.881	-0.571	0.199	-0.851	1.494	0.199
Phosphate P	0.957	0.928	-0.274	0.809	-0.09	0.402	-1.865	-0.133	-2.015	0.152	0.797	0.332
Total Ca	0.115	0.707	1.752	-0.735	-0.968	-0.774	0.008	1.736	-0.333	-0.716	-1.229	0.438
Total Mg	0.197	0.51	1.642	-0.568	-0.994	-0.933	0.224	1.773	-0.355	-0.663	-1.343	0.51
Total K	-0.268	0.753	1.709	0.172	0.172	-1.331	-0.042	1.709	-0.764	-0.268	-0.509	-1.331
Total Na	-0.136	0.665	1.951	-0.354	-0.672	-0.504	-0.797	1.986	-0.614	-0.733	-0.614	-0.177
C.E.C	-0.911	0.558	0.213	-0.302	0.412	-0.123	1.477	0.264	0.363	0.693	-0.062	-2.583
Exch. Ca	0.927	0.426	0.801	-1.136	-0.787	-0.154	-1.721	1.681	-0.221	-0.673	-0.176	1.033
Exch. Mg	-0.184	0.29	1.821	0.121	-0.409	-0.202	-0.691	1.557	-0.917	0.061	-1.852	0.407
Exch. K	-0.531	0.261	1.475	0.261	1.298	-1.531	0.261	1.112	-0.531	0.261	-1.168	-1.168
Exch. Na	-0.213	0.455	1.897	-0.087	-0.354	-0.471	-1.181	2.006	-0.976	-0.317	-0.513	-0.247
Sln Ca	-0.735	0.053	2.225	-0.776	-0.985	-1.207	0.641	1.283	-0.284	0.388	-0.355	-0.249
Sln Mg	-0.361	0.427	1.159	0.458	-0.123	0.458	-1.349	0.518	-2.176	0.267	-0.576	1.298
Sln K	-0.435	0.465	1.097	0.749	1.097	-1.655	0.37	1.019	-1.071	0.231	-0.314	-1.554
Sln Na	-0.268	0.914	1.582	-0.172	-0.528	-0.273	-1.161	1.917	-1.389	-0.483	-0.139	0

Contribution of principal components

```
> summary(x1)
Importance of components:
                          PC1    PC2    PC3    PC4    PC5    PC6    PC7    PC8
Standard deviation     3.096 2.270 1.770 1.348 1.1570 0.7875 0.7068 0.6044
Proportion of Variance 0.417 0.224 0.136 0.079 0.0582 0.0270 0.0217 0.0159
Cumulative Proportion  0.417 0.641 0.777 0.856 0.9142 0.9412 0.9629 0.9788
                          PC9    PC10    PC11      PC12
Standard deviation     0.5164 0.42513 0.20278 2.11e-16
Proportion of Variance 0.0116 0.00786 0.00179 0.00e+00
Cumulative Proportion  0.9903 0.99821 1.00000 1.00e+00
> x1
Standard deviations:
 [1] 3.095895e+00 2.269930e+00 1.769957e+00 1.348303e+00 1.157004e+00
 [6] 7.874565e-01 7.068010e-01 6.044031e-01 5.164458e-01 4.251282e-01
[11] 2.027758e-01 2.108030e-16
```

Eigenvalue loadings used to construct biplots

```
Rotation:
                    PC1         PC2         PC3          PC4          PC5
Bulk.density  0.17521206  0.25034984 -0.02868266  0.343129630  0.061728233
pH            0.17319201 -0.16898458  0.11854719 -0.409723927 -0.389371553
Moisture     -0.16951769 -0.09831303 -0.30676833 -0.402630019  0.147694945
Organic.C    -0.11327273  0.19334007 -0.45409409  0.023845260  0.095349988
Total.N      -0.16555585  0.33213742 -0.08583329  0.087753617 -0.204196528
Ammonium.N   -0.14027432  0.35600225  0.04148869 -0.221863584 -0.103456198
Nitrate.N    -0.12171791  0.31494855  0.06293700  0.015443098 -0.442335886
Nitrite.N    -0.08725646  0.18295336 -0.27672302 -0.258274988 -0.396029446
Total.P      -0.12525063 -0.00790758  0.03378030  0.523941409 -0.220611218
Phosphate.P  -0.02002876  0.39237883 -0.14669479  0.037142110  0.147843578
Total.Ca      0.30251031  0.02970468  0.14227082 -0.014629641 -0.106188973
Total.Mg      0.30049889  0.00772607  0.14170901 -0.032554457 -0.088303022
Total.K       0.27816994 -0.03768400 -0.24384689  0.089957387 -0.136434932
Total.Na      0.30560748  0.10162408  0.01138354 -0.004296897 -0.134552469
C.E.C         0.02153445 -0.24419798 -0.31177223  0.151918192 -0.400514317
Exch..Ca      0.20053996  0.20825805  0.28511625 -0.060219229  0.045652265
Exch..Mg      0.27893592  0.10184306 -0.03977973 -0.262058965  0.054719456
Exch..K       0.22534190 -0.15545683 -0.32104049 -0.028726305  0.098556965
Exch..Na      0.29487812  0.13677653 -0.07777364 -0.044453180 -0.026043808
Sln.Ca        0.27569989 -0.10576602 -0.00984860  0.115240785  0.002191507
Sln.Mg        0.14027941  0.32095048 -0.09213328 -0.085036255  0.321538394
Sln.K         0.18470232 -0.10058107 -0.41407548  0.130421757  0.030508313
Sln.Na        0.27115923  0.21149093 -0.05814703  0.082815202 -0.047439481
```

```
                      PC6          PC7          PC8          PC9         PC10
Bulk.density -0.277811929 -0.015682708  0.539323392 -0.07544338 -0.07792784
pH            0.053877042 -0.036315096  0.158625615 -0.05546143  0.03019613
Moisture      0.002428033  0.228162178 -0.241561470 -0.11593644 -0.14628467
Organic.C    -0.157235648  0.002445363 -0.085996519  0.13475576 -0.06806763
Total.N      -0.279803002 -0.087662828  0.011543693 -0.01706164  0.29382393
Ammonium.N   -0.053053337  0.053350026 -0.078775612 -0.07054983 -0.47145453
Nitrate.N    -0.245604321  0.016325322 -0.106035051 -0.31480765  0.08259520
Nitrite.N     0.372475509  0.118800935  0.388510773  0.35660597  0.08065750
Total.P       0.481472446  0.479517387 -0.224019500 -0.13092806 -0.01164623
Phosphate.P   0.286258503 -0.195298129 -0.036824728 -0.02867360 -0.42401429
Total.Ca     -0.036522141  0.190772273  0.014615617 -0.17545579 -0.20285222
Total.Mg     -0.016318819  0.230209788  0.166653792 -0.19729634 -0.25271466
Total.K       0.133411507 -0.149755835 -0.008840204 -0.03646226 -0.08599929
Total.Na      0.073844574 -0.047745455 -0.165340748  0.12936332  0.15712921
C.E.C        -0.349190390 -0.057293525 -0.337453334 -0.01906782 -0.17572191
Exch..Ca      0.141906806 -0.400751805 -0.289386596 -0.02086485 -0.12136627
Exch..Mg     -0.083683191  0.319177431 -0.056554325 -0.17801037  0.09353488
Exch..K       0.024107540 -0.102935323  0.024710733 -0.39053703  0.10632439
Exch..Na      0.081826897 -0.116293466 -0.202921762  0.20060479  0.22967329
Sln.Ca       -0.280834789  0.247733968 -0.049688348  0.58491762 -0.31231064
Sln.Mg       -0.081522250  0.406122353 -0.066226192 -0.03999773  0.27090969
Sln.K         0.161415607 -0.148319919  0.182309468 -0.20366279 -0.06892314
Sln.Na        0.092159122 -0.033164429 -0.245065580  0.10907600  0.18422483

                     PC11         PC12
Bulk.density -0.321652614  0.11619392
pH           -0.117375578  0.55721006
Moisture     -0.176138873  0.20389528
Organic.C     0.192329845  0.18872814
Total.N       0.437752910  0.09731410
Ammonium.N    0.233497644 -0.31637954
Nitrate.N    -0.262251357  0.10269735
Nitrite.N    -0.057004141 -0.29270181
Total.P       0.004904854  0.12442190
Phosphate.P  -0.120812650  0.36270031
Total.Ca      0.338737819  0.11858254
Total.Mg      0.073884992 -0.21986967
Total.K       0.169142140  0.13927997
Total.Na      0.292589609  0.06335681
C.E.C        -0.249790813 -0.21226963
Exch..Ca     -0.150664666 -0.15287089
Exch..Mg     -0.118775254 -0.18554915
Exch..K       0.260903989 -0.02727677
Exch..Na     -0.113744068 -0.03819117
Sln.Ca        0.028985220  0.13639861
Sln.Mg       -0.128594288  0.03136276
Sln.K        -0.096495429 -0.18420236
Sln.Na       -0.201577860 -0.04012094
> 
```

Appendix A3: The plant cover characteristics in Marion Island terrestrial habitats

	MI 5.3	MI 5.1	MI 1.2	MI 6.5	MI 6.2	MI 5.2	MI 3.3	MI 1.1	MI 6.4	MI 6.3	MI 4.3
Cushion Dicot	0	3	39	0	0.3	0	6	6	2	0	0
Cushion Bryophyte	0	0	1	0	0	0	1	0	0	0	0
Lichen	0	0	0	0	0	0	0	0	0.3	0	0
Epiphytic Graminoid	0	0	0	0	0	0	0	0	0	0	0
Tussock Graminoid	3	15	1	7	0.3	1	0	1	0	0	42
Poa Annua	23	0	0	0	0	0	0	0	0	0	14
Pteridophyte	0	0	0	0	0.3	0	73	0	0.3	0.3	0
Rosette Dicot	4	81	21	0	0	0	0	7	0	0	6
Erect Dicot	0	0	37	0	0	0	0	86	0	0	0
Mat Dicot	70	2	0	10	1	98	0	0	0.3	1	39
Brachythecium Moss	0	0	0	1	0	0	0	0	0	0	0
Deciduous Shrub	0	0.3	0	0	0	0	0	0	1	0.3	0
Mire Graminoid	1	0.3	1	23	42	0.3	18	0	40	14	0
Mire Bryophyte	0	0	0	59	55	0	2	0	10	84	0
Bryum/Breutelia	0	0	0	0	0	0	0	0	46	0	0
Total Cover	123	103	93	106	137	102	104	85	169	112	52
Rock Cover	0	1	9	0	0.3	0	1	15	1	0	0
Shore	139	153	41	470	541	134	1200	20	2130	521	54
Altitude	14	20	12	40	47	17	105	16	179	45	9
Biotic	8	8	3	5	1	9	1	4	1	0	10
Salt	6	5	7	2	0	4	0	8	0.3	0	5

Values given as 0.3 are arbitrarily assigned values. These were not given by Simth and Steenkamp (2001), but were mentioned to be between 0 and 1.

Appendix A4: SIMPER analysis of hierarchical clusters of habitats generated from analysis of soil physiochemical properties using PRIMER5

PRIMER 2/14/2009

SIMPER analysis of soil physiochemical characteristics

Similarity Percentages - species contributions

Worksheet

File: C:\Documents and Settings\FungaiWalter Sanyika\My Documents\Walter\Walter phd 1st September to date\Walter PhD thesis\Stats analysis_Gibbons\soilbiochem.pri
Sample selection: All
Variable selection: All

Parameters

Standardise data: No
Transform: None
Cut off for low contributions: 90.00%
Factor name: Distance = 800

Factor groups
Cluster hab B3
Cluster hab B1
Cluster hab A
Cluster hab B2

Group Cluster hab B3

Average similarity: 72.35

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Moisture	775.80	28.01	3.50	38.71	38.71
Sln Na	676.20	19.49	1.99	26.94	65.66
Sln K	310.00	10.58	3.04	14.62	80.27
Sln Ca	96.60	3.76	4.34	5.20	85.47
C.E.C	78.76	3.15	5.24	4.35	89.82
Phosphate P	126.20	2.04	0.67	2.82	92.64

Group Cluster hab B1

Less than 2 samples in group

Group Cluster hab A

Average similarity: 83.56

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Sln Na	8151.00	67.26	#######	80.49	80.49
Sln K	674.00	6.66	#######	7.97	88.46
Moisture	485.50	4.03	#######	4.82	93.28

Group Cluster hab B2

Average similarity: 86.48

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%

Moisture	1569.33	40.52	14.87	46.86	46.86
Sln Na	784.00	19.85	36.15	22.96	69.82
Sln K	570.67	14.38	5.96	16.63	86.45
Sln Ca	94.67	2.39	42.34	2.76	89.22
Phosphate P	129.47	2.30	7.28	2.66	91.87

Groups Cluster hab B3 & Cluster hab B1

Average dissimilarity = 44.63

Species	Group Cluster hab B3 Av.Abund	Group Cluster hab B1 Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Sln Na	676.20	3222.00	34.72	4.97	77.81	77.81
Sln K	310.00	502.00	2.56	1.68	5.74	83.56
Moisture	775.80	658.00	2.54	1.19	5.68	89.24
Phosphate P	126.20	258.50	1.91	1.14	4.28	93.52

Groups Cluster hab B3 & Cluster hab A

Average dissimilarity = 70.17

Species	Group Cluster hab B3 Av.Abund	Group Cluster hab A Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Sln Na	676.20	8151.00	60.66	8.08	86.46	86.46
Sln K	310.00	674.00	3.01	3.06	4.29	90.75

Groups Cluster hab B1 & Cluster hab A

Average dissimilarity = 37.47

Species	Group Cluster hab B1 Av.Abund	Group Cluster hab A Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Sln Na	3222.00	8151.00	32.20	3.28	85.92	85.92
Phosphate P	258.50	61.00	1.33	6.03	3.55	89.47
Sln K	502.00	674.00	1.16	4.31	3.10	92.57

Groups Cluster hab B3 & Cluster hab B2

Average dissimilarity = 29.33

Species	Group Cluster hab B3 Av.Abund	Group Cluster hab B2 Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Moisture	775.80	1569.33	13.82	2.63	47.14	47.14
Sln Na	676.20	784.00	5.61	1.52	19.14	66.28
Sln K	310.00	570.67	4.66	1.83	15.89	82.17
Phosphate P	126.20	129.47	1.92	1.59	6.54	88.71
Ammonium N	103.32	33.80	1.57			

0.90 5.34 94.05

Groups Cluster hab B1 & Cluster hab B2

Average dissimilarity = 43.49

Species	Group Cluster hab B1 Av.Abund	Group Cluster hab B2 Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Sln Na	3222.00	784.00	28.72	9.25	66.03	66.03
Moisture	658.00	1569.33	10.66	3.59	24.50	90.53

Groups Cluster hab A & Cluster hab B2

Average dissimilarity = 66.26

Species	Group Cluster hab A Av.Abund	Group Cluster hab B2 Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Sln Na	8151.00	784.00	54.69	8.39	82.54	82.54
Moisture	485.50	1569.33	8.12	4.62	12.25	94.80

Appendix A5: SIMPER analysis of hierarchical clusters of habitats generated from analysis of plant cover characteristics using PRIMER5

PRIMER 2/15/2009

SIMPER

Similarity Percentages - species contributions

Worksheet

File: C:\Documents and Settings\FungaiWalter Sanyika\My Documents\Walter\Walter phd 1st September to date\Walter PhD thesis\Stats analysis_Gibbons\Plant COVER data.pri
Sample selection: All
Variable selection: All

Parameters

Standardise data: No
Transform: None
Cut off for low contributions: 90.00%
Factor name: merged clusters

Factor groups
Plant cluster CD
Plant cluster AB

Group Plant cluster CD

Average similarity: 53.42

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Total cover	93.00	42.33	4.54	79.24	79.24
Mat Dicot	34.83	5.25	0.49	9.82	89.06
Rosette Dicot	19.83	2.58	0.86	4.83	93.89

Group Plant cluster AB

Average similarity: 67.34

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Total cover	125.60	49.00	15.80	72.77	72.77
Mire Bryophyte	42.00	9.39	0.81	13.94	86.71
Mire Graminoid	27.40	8.67	2.93	12.87	99.58

Groups Plant cluster CD & Plant cluster AB

Average dissimilarity = 54.19

Species	Group Plant cluster CD Av.Abund	Group Plant cluster AB Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Mire Bryophyte	0.00	42.00	10.45	1.29	19.28	19.28
Total cover	93.00	125.60	8.69	1.21	16.04	35.32
Mat Dicot	34.83	2.46	8.22	0.95	15.18	50.49
Mire Graminoid	0.43	27.40	6.50	2.55	12.00	62.49

```
Erect Dicot                          20.50                    0.00
5.21     0.63      9.62  72.11
Rosette Dicot                        19.83                    0.00
4.82     0.71      8.90  81.01
Pteridophyte                          0.00                   14.78
3.87     0.50      7.14  88.15
Tussock Graminoid                    10.50                    1.46
2.70     0.70      4.97  93.12
```

SIMPER 2

Similarity Percentages - species contributions

Worksheet

File: C:\Documents and Settings\FungaiWalter Sanyika\My Documents\Walter\Walter phd 1st September to date\Walter PhD thesis\Stats analysis_Gibbons\Plant COVER data.pri
Sample selection: All
Variable selection: All

Parameters

Standardise data: No
Transform: None
Cut off for low contributions: 90.00%
Factor name: Plant cover

Factor groups
Plant Cluster C
Plant Cluster D
Plant Cluster B
Plant Cluster A

Group Plant Cluster C

Average similarity: 64.28

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Total cover	92.33	34.97	3.10	54.40	54.40
Mat Dicot	69.00	25.21	3.76	39.21	93.61

Group Plant Cluster D

Average similarity: 63.93

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Total cover	93.67	49.34	12.04	77.18	77.18
Erect Dicot	41.00	7.41	0.58	11.59	88.76
Rosette Dicot	36.33	6.56	1.44	10.27	99.03

Group Plant Cluster B

Average similarity: 73.74

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Total cover	131.00	49.17	14.46	66.68	66.68
Mire Bryophyte	52.00	15.03	1.25	20.38	87.06
Mire Graminoid	29.75	9.15	2.39	12.40	99.46

Group Plant Cluster A

Less than 2 samples in group

Groups Plant Cluster C & Plant Cluster D

Average dissimilarity = 53.70

Species	Group Plant Cluster C Av.Abund	Group Plant Cluster D Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Mat Dicot	69.00	0.67	18.21	2.90	33.92	33.92
Erect Dicot	0.00	41.00	11.35	1.11	21.13	55.04
Rosette Dicot	3.33	36.33	8.61	1.00	16.04	71.08
Total cover	92.33	93.67	7.44	1.54	13.86	84.95
Tussock Graminoid	15.33	5.67	4.52	0.85	8.41	93.35

Groups Plant Cluster C & Plant Cluster B

Average dissimilarity = 55.82

Species	Group Plant Cluster C Av.Abund	Group Plant Cluster B Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Mat Dicot	69.00	3.08	15.39	2.80	27.57	27.57
Mire Bryophyte	0.00	52.00	12.71	1.78	22.78	50.34
Total cover	92.33	131.00	10.49	1.21	18.79	69.13
Mire Graminoid	0.43	29.75	6.87	2.56	12.31	81.44
Tussock Graminoid	15.33	1.83	3.91	0.80	7.00	88.44
Poa Annua	12.33	0.00	2.92	1.30	5.22	93.67

Groups Plant Cluster D & Plant Cluster B

Average dissimilarity = 53.11

Species	Group Plant Cluster D Av.Abund	Group Plant Cluster B Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Mire Bryophyte	0.00	52.00	13.14	1.79	24.75	24.75
Erect Dicot	41.00	0.00	10.26	1.13	19.31	44.06
Total cover	93.67	131.00	8.89	1.48	16.74	60.80
Rosette Dicot	36.33	0.00	8.68	1.13	16.34	77.13
Mire Graminoid	0.43	29.75	7.08	2.61	13.34	90.47

Groups Plant Cluster C & Plant Cluster A

Average dissimilarity = 56.46

Species	Group Plant Cluster C Av.Abund	Group Plant Cluster A Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Pteridophyte	0.00	73.00	18.79	10.41	33.29	33.29
Mat Dicot	69.00	0.00	17.41	2.58	30.85	64.13
Total cover	92.33	104.00	6.61	0.89	11.70	75.83
Mire Graminoid	0.43	18.00	4.53	8.14	8.02	83.86
Tussock Graminoid	15.33	0.00	4.31	0.65	7.63	91.49

Groups Plant Cluster D & Plant Cluster A

Average dissimilarity = 49.74

Species	Group Plant Cluster D Av.Abund	Group Plant Cluster A Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Pteridophyte	0.00	73.00	19.46	15.72	39.11	39.11
Erect Dicot	41.00	0.00	11.11	0.97	22.33	61.45
Rosette Dicot	36.33	0.00	9.36	0.97	18.83	80.27
Mire Graminoid	0.43	18.00	4.68	21.57	9.40	89.67
Total cover	93.67	104.00	2.81	1.16	5.64	95.31

Groups Plant Cluster B & Plant Cluster A

Average dissimilarity = 42.27

Species	Group Plant Cluster B Av.Abund	Group Plant Cluster A Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Pteridophyte	0.23	73.00	17.06	15.56	40.35	40.35
Mire Bryophyte	52.00	2.00	12.02	1.58	28.43	68.79
Total cover	131.00	104.00	6.01	0.98	14.22	83.01
Mire Graminoid	29.75	18.00	3.12	1.33	7.38	90.39

Appendix A6: SIMPER, and BIOENV comparison of actinobacaterial diversity-based hierarchical clusters of habitats to the soil physiochemical characteristics using PRIMER5

PRIMER 2/13/2009

BIOENV
Biota and/or Environment matching

Worksheet

File: C:\Documents and Settings\FungaiWalter Sanyika\Desktop\soilbiochem.pri
Sample selection: All
Variable selection: All

Similarity Matrix

File: C:\Documents and Settings\FungaiWalter Sanyika\Desktop\Actinobacterial Bray Curtis similarity.sid
Data type: Similarities
Sample selection: All

Parameters

Rank correlation method: Spearman
Maximum number of variables: 5

Similarity Matrix Parameters for sample data worksheet:
Analyse between: Samples
Similarity measure: Bray Curtis
Standardise: No
Transform: Square root

Variables

1 pH
2 Moisture
3 Organic C
4 Total N
5 Ammonium N
6 Nitrate N
7 Nitrite N
8 Total P
9 Phosphate P
10 Total Ca
11 Total Mg
12 Total K
13 Total Na
14 C.E.C
15 Exch. Ca
16 Exch. Mg
17 Exch. K
18 Exch. Na
19 Sln Ca
20 Sln Mg
21 Sln K
22 Sln Na

Best results

Var	Corr.	Selections
1	0.353	1
4	0.277	1,3,15,16
3	0.265	3,15,16
3	0.264	1,15,16
5	0.264	1,3,12,15,16
5	0.263	1,3,11,15,16
5	0.259	1,3,8,15,16
5	0.256	1,3,7,15,16
4	0.253	3,11,15,16
4	0.253	1,11,15,20

SIMPER analysis of actinobacterial diversity (informative results only)
PRIMER 2/14/2009

SIMPER
Similarity Percentages - species contributions

Worksheet

File: C:\Documents and Settings\FungaiWalter Sanyika\My Documents\Walter\Walter phd 1st September to date\Walter PhD thesis\Stats analysis_Gibbons\Actinobacterial diversity.pri
Sample selection: All
Variable selection: All

Parameters

Standardise data: No
Transform: None
Cut off for low contributions: 90.00%
Factor name: Distance = 35

Factor groups
Cluster Act 2
Cluster Act 3
Cluster Act 1

Group Cluster Act 2

Average similarity: 42.02

Group Cluster Act 3

Average similarity: 40.98

Group Cluster Act 1

Average similarity: 45.79

Groups Cluster Act 2 & Cluster Act 3

Average dissimilarity = 67.46

Groups Cluster Act 2 & Cluster Act 1

Average dissimilarity = 68.25

Groups Cluster Act 3 & Cluster Act 1

Average dissimilarity = 75.04

PRIMER 2/15/2009

SIMPER
Similarity Percentages - species contributions

Worksheet

File: C:\Documents and Settings\FungaiWalter Sanyika\My Documents\Walter\Walter phd 1st September to date\Walter PhD thesis\Stats analysis_Gibbons\Actinobacterial diversity.pri
Sample selection: All
Variable selection: All

Parameters

Standardise data: No
Transform: None
Cut off for low contributions: 90.00%
Factor name: Distance = 30

Factor groups
Cluster Act 2
Cluster Act 1

Group Cluster Act 2

Average similarity: 36.57

Group Cluster Act 1

Average similarity: 45.79

Groups Cluster Act 2 & Cluster Act 1

Average dissimilarity = 72.49

SIMPER
Similarity Percentages - species contributions

Worksheet

File: C:\Documents and Settings\FungaiWalter Sanyika\My Documents\Walter\Walter phd 1st September to date\Walter PhD thesis\Stats analysis_Gibbons\Actinobacterial diversity.pri
Sample selection: All
Variable selection: All

Parameters

Standardise data: No
Transform: None
Cut off for low contributions: 90.00%
Factor name: Distance = 35

Factor groups
Cluster Act 2a
Cluster Act 2b
Cluster Act 1

Group Cluster Act 2a

Average similarity: 42.02

Group Cluster Act 2b

Average similarity: 40.98

Group Cluster Act 1

Average similarity: 45.79

Groups Cluster Act 2a & Cluster Act 2b

Average dissimilarity = 67.46

Groups Cluster Act 2a & Cluster Act 1

Average dissimilarity = 68.25

Groups Cluster Act 2b & Cluster Act 1

Average dissimilarity = 75.04

Appendix A7: BIOENV comparison of hierarchical clusters of habitats generated from the analysis of actinobacterial diversity to the plant cover characteristics using PRIMER5

BIOENV

Biota and/or Environment matching

Worksheet

File: C:\Documents and Settings\FungaiWalter Sanyika\My Documents\Walter\Walter phd 1st September to date\Walter PhD thesis\Stats analysis_Gibbons\Plant COVER data.pri
Sample selection: All
Variable selection: All

Similarity Matrix

File: C:\Documents and Settings\FungaiWalter Sanyika\My Documents\Walter\Walter phd 1st September to date\Walter PhD thesis\Stats analysis_Gibbons\Actinobacterial Bray Curtis similarity.sid
Data type: Similarities
Sample selection: All

Parameters

Rank correlation method: Spearman
Maximum number of variables: 5

Similarity Matrix Parameters for sample data worksheet:
Analyse between: Samples
Similarity measure: Bray Curtis
Standardise: No
Transform: None

Variables

1 Cushion Bryophyte
2 Lichen
3 Epiphytic Graminoid
4 Tussock Graminoid
5 Poa Annua
6 Pteridophyte
7 Rosette Dicot
8 Erect Dicot
9 Mat Dicot
10 Brachythecium Moss
11 Deciduous Shrub
12 Mire Graminoid
13 Mire Bryophyte
14 Total cover
15 Bryum/Breutelia

Best results

Var	Corr.	Selections
5	0.319	4,5,13-15
5	0.313	1,5,13-15
4	0.312	5,13-15
5	0.312	2,5,13-15

```
5     0.312 3,5,13-15
5     0.312 5,10,13-15
5     0.311 5,11,13-15
4     0.304 4,13-15
5     0.304 2,4,13-15
5     0.304 3,4,13-15
```

Appendix A8: SIMPER, and BIOENV comparison of bacterial diversity-based hierarchical clusters of habitats to the soil physiochemical characteristics using PRIMER5

BIOENV

Biota and/or Environment matching

Worksheet

File: C:\Documents and Settings\FungaiWalter Sanyika\Desktop\soilbiochem.pri
Sample selection: All
Variable selection: All

Similarity Matrix

File: C:\Documents and Settings\FungaiWalter Sanyika\Desktop\Bray curtis bacterial similarity matrix.sid
Data type: Similarities
Sample selection: All

Parameters

Rank correlation method: Spearman
Maximum number of variables: 5

Similarity Matrix Parameters for sample data worksheet:
Analyse between: Samples
Similarity measure: Bray Curtis
Standardise: No
Transform: Square root

Variables

1 pH
2 Moisture
3 Organic C
4 Total N
5 Ammonium N
6 Nitrate N
7 Nitrite N
8 Total P
9 Phosphate P
10 Total Ca
11 Total Mg
12 Total K
13 Total Na
14 C.E.C
15 Exch. Ca
16 Exch. Mg
17 Exch. K
18 Exch. Na
19 Sln Ca
20 Sln Mg
21 Sln K
22 Sln Na

Best results

Var	Corr.	Selections
5	0.364	10,13,15,19,21
5	0.362	11,13,15,19,21
5	0.358	10,15,18,19,21
5	0.354	8,10,15,19,21
5	0.353	13,15,18,19,21
5	0.352	1,10,15,19,21
5	0.351	10,11,18,19,21
5	0.351	10,11,15,19,21
5	0.351	10,12,13,19,21
5	0.348	8,10,13,19,21

PRIMER 2/14/2009

SIMPER

Similarity Percentages - species contributions

Worksheet

File: C:\Documents and Settings\FungaiWalter Sanyika\My Documents\Walter\Walter phd 1st September to date\Walter PhD thesis\Stats analysis_Gibbons\bacterial diversity.pri
Sample selection: All
Variable selection: All

Parameters

Standardise data: No
Transform: None
Cut off for low contributions: 90.00%
Factor name: Clusters1

Factor groups
Cluster bact A
Cluster bact B
Cluster bact C

Group Cluster bact A

Average similarity: 47.97

Group Cluster bact B

Average similarity: 43.90

Group Cluster bact C

Average similarity: 41.43

Groups Cluster bact A & Cluster bact B

Average dissimilarity = 61.81

Groups Cluster bact A & Cluster bact C

Average dissimilarity = 63.22

Groups Cluster bact B & Cluster bact C

Average dissimilarity = 64.49

Appendix A9: BIOENV comparison of hierarchical clusters of habitats generated from the analysis of bacterial diversity to the plant cover characteristics using PRIMER5

BIOENV
Biota and/or Environment matching

Worksheet

File: C:\Documents and Settings\FungaiWalter Sanyika\My Documents\Walter\Walter phd 1st September to date\Walter PhD thesis\Stats analysis_Gibbons\Plant COVER data.pri
Sample selection: All
Variable selection: All

Similarity Matrix

File: C:\Documents and Settings\FungaiWalter Sanyika\My Documents\Walter\Walter phd 1st September to date\Walter PhD thesis\Stats analysis_Gibbons\Bray curtis bacterial similarity matrix.sid
Data type: Similarities
Sample selection: All

Parameters

Rank correlation method: Spearman
Maximum number of variables: 5

Similarity Matrix Parameters for sample data worksheet:
Analyse between: Samples
Similarity measure: Bray Curtis
Standardise: No
Transform: None

Variables

1 Cushion Bryophyte
2 Lichen
3 Epiphytic Graminoid
4 Tussock Graminoid
5 Poa Annua
6 Pteridophyte
7 Rosette Dicot
8 Erect Dicot
9 Mat Dicot
10 Brachythecium Moss
11 Deciduous Shrub
12 Mire Graminoid
13 Mire Bryophyte
14 Total cover
15 Bryum/Breutelia

Best results

Var	Corr.	Selections
1	0.206	5
2	0.206	2,5
2	0.206	3,5

```
2     0.206 5,10
2     0.206 5,15
3     0.206 2,3,5
3     0.206 2,5,10
3     0.206 2,5,15
3     0.206 3,5,10
3     0.206 3,5,15
```

Appendix A10: Summary of the BLAST results used to identify the actinobacteria in metagenomic clone libraries from Marion Island terrestrial habitats MI 1.2, MI 5.1, and MI 6.3.

The E-value is zero for all sequences and query coverage at least 98%.

Query/ Relative	Accession number	Description	% Identity
F8-MI 6.3			
Uncultured bacterium pLW-82	gi|67550303|gb|DQ067025.1|	Uncultured, Bacteria; environmental samples, Sediment of Lake Washington	97
C8-MI 6.3			
Uncultured actinobacterium CrystalBog1D10	gi|60326373:1-1514	Uncultured, Bacteria; Actinobacteria; environmental samples, Humic Lake, USA: Northern Wisconsin	99
Acidimicrobium ferrooxidans strain TH3	gb|EF621760.1|:1-1325	Bacteria; Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium, Heterotrophic iron-oxidizing and extremely acidophilic.	90
C3-MI 6.3			
Uncultured actinobacteriumAmb_16S_1709	gi|134020521:1-1362	Uncultured Bacteria; Actinobacteria; environmental samples. Trembling aspen rhizosphere under ambient CO_2 conditions	99
Acid streamer iron-oxidizing bacterium CS11	gi|78214562:1-1404	Bacteria; Actinobacteria, Streamer in acidic, iron-rich water	91
Acidimicrobium ferrooxidans TH3	gb|EF621760.1|:1-1325	Bacteria; Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium, Actinobacteria, heterotrophic iron-oxidizing, extremely acidophilic Actinobacteria	91
E7-MI 6.3			
Actinobacterium MH3-4	gi|123192448:1-1393	Bacteria; Actinobacteria, Permafrost soil	97
Aestuariimicrobium kwangyangensis R47	gb|DQ830985.1|:1-1472	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; propionibacteriaceae; Aestuariimicrobium, Actinobacteria, isolated from the enrichment with degradation activity of diesel oil	94
F9-MI 6.3			
Uncultured actinobacterium GASP-MA1S2_H06	Gi|151349688:1-832	Uncultured Bacteria; Actinobacteria; environmental samples, Cropland on GASP KBS-LTER sampling site, USA: Michigan	99
Acidimicrobium ferrooxidans TH3	gb|EF621760.1|:1-1325	Bacteria; Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium,	92
Ferrimicrobium acidiphilum	gb|AF251436.1|:1-1449	Actinobacteria; Ferrimicrobium, Heterotrophic acidophiles and their roles in the bioleaching of sulfide minerals, iron-oxidizing acidophile isolated from acid mine waters	91
A9-MI 6.3			
Frankia sp.	gb|U60287.1|FSU60287:1-1450	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Frankiaceae; Frankia. Specific host: Alnus nepalensis nodule.	98

Appendix A10 continued

Query/ Relative	Accession number	Description	% Identity
E11-MI 6.3			
Uncultured actinobacterium CK-48	gi|120971672:1-638	Bacteria; Actinobacteria; environmental samples. Soil used for rice-wheat cultivation for centuries, China: SuZhou, Jiangsu Province	**98**
Acidimicrobium ferrooxidans TH3	gb|EF621760.1|:1-1325	Bacteria; Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium,	**91**
G4-MI 6.3			
Uncultured actinobacterium CK-11	gi|120971693:1-639	Uncultured Bacteria; Actinobacteria; environmental samples. Soil used for rice-wheat cultivation for centuries, China: SuZhou, Jiangsu Province	**98**
Ferrimicrobium acidiphilum	gb|AF251436.1|:1-1449	Bacteria; Actinobacteria; Ferrimicrobium, Plays a role in the bioleaching of sulfide minerals, heterotrophic,iron-oxidizing acidophile isolated from acid mine waters	**91**
A3-MI 6.3			
Uncultured actinobacterium Elev_16S_853	gi|134021116:1-1362	Uncultured Bacteria; Actinobacteria; environmental samples. Trembling aspen rhizosphere under elevated CO_2 conditions	**100**
Acidimicrobium ferrooxidans TH3	gb|EF621760.1|:1-1325	Bacteria; Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium, Heterotrophic iron-oxidizing, extremely acidophilic Actinobacteria	**90**
Ferrimicrobium acidiphilum	gb|AF251436.1|:1-1449	Bacteria; Actinobacteria; Ferrimicrobium,	**90**
B11-MI 6.3			
Uncultured actinobacterium TH3-101	emb|AM690900.1|:1-883	uncultured Bacteria; Actinobacteria; environmental samples. Large, shallow, freshwater, subtropical Taihu Lake, China	**99**
Mycobacterium sp. JS621	gi|22711853:1-1517	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium. Bodegrades the groundwater pollutant vinyl chloride	**98**
Mycobacterium anthracenicum	emb|Y15709.1|:1-1450	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium. PAH-degrading	**98**
E8-MI 6.3			
Uncultured actinobacterium TH3-101	emb|AM690900.1|:1-883	uncultured Bacteria; Actinobacteria; environmental samples. Large, shallow, freshwater, subtropical Taihu Lake, China Freshwater lake, China:Lake Taihu	**98**
Mycobacterium anthracenicum	emb|Y15709.1|:1-1450	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium. PAH-degrading PAH-degrading	**98**
F1-MI 6.3			
Uncultured bacterium Amb_16S_1075	gi|134019874|gb|EF018450.1|	Uncultured Bacteria; Actinobacteria; environmental samples. Trembling aspen rhizosphere under ambient CO_2 conditions	**99**
Acidimicrobium ferrooxidans TH3	gb|EF621760.1|:1-1325	Bacteria; Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium, Heterotrophic iron-oxidizing, extremely acidophilic Actinobacteria	**90**

Appendix A10 continued

Query/ Relative	Accession number	Description	% Identity
Acidimicrobium ferrooxidans	gb|U75647.1|AFU75647:1-1465	Bacteria; Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium. Heterotrophic iron-oxidizing, extremely acidophilic Actinobacteria. A moderately thermophilic mineral-sulphide oxidising bacterium	89
Candidatus Microthrix calida TNO2-4	gi|73532959:1-1461	Bacteria; Actinobacteria; Actinobacteria (class); unclassified Actinobacteria; Candidatus Microthrix. Isolated from industrial activated sludge wastewater treatment plants	89
B5-MI 6.3			
Uncultured actinobacterium Elev_16S_853	gi|134021116:1-1362	Bacteria; Actinobacteria; environmental samples. Trembling aspen rhizosphere under elevated CO_2 conditions	100
Acidimicrobium ferrooxidans TH3	gb|EF621760.1|:1-1325	Bacteria; Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium, Heterotrophic iron-oxidizing, extremely acidophilic Actinobacteria	90
Ferrimicrobium acidiphilum	gb|AF251436.1|:1-1449	Bacteria; Actinobacteria; Ferrimicrobium. Heterotrophic iron-oxidizing acidophile isolated from acid mine waters	90
Actinocorallia caverna N3-7	gb|AY966427.1|:1-1407	Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinocorallia, type of Actinocorallia caverna, South Korea: Jeju	89
E1-MI 6.3			
Uncultured actinobacterium TH3-101	emb|AM690900.1|:1-883	uncultured Bacteria; Actinobacteria; environmental samples. Large, shallow, freshwater, subtropical Taihu Lake, China Large, shallow, freshwater lake, subtropical Taihu Lake, China	99
Mycobacterium anthracenicum	emb|Y15709.1|:1-1450	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium. PAH-degrading PAH-degrading	98
Mycobacterium saskatchewanense MB54784	gb|AY208857.1|:1-1511	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium. Slowly growing scotochromogenic species from human clinical isolates	98
E6-MI 6.3			
Uncultured bacterium Amb_16S_1075	gi|134019874:1-1368	Uncultured Bacteria; Actinobacteria; environmental samples. Trembling aspen rhizosphere under ambient CO_2 conditions	99
Ferrimicrobium acidiphilum	gb|AF251436.1|:1-1449	Bacteria; Actinobacteria; Ferrimicrobium. Heterotrophic acidophiles, roles in the bioleaching of sulfide minerals	91
Acidimicrobium ferrooxidans TH3	gb|EF621760.1|:1-1325	Bacteria; Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium, Actinobacteria, Heterotrophic iron-oxidizing, extremely acidophilic	91
G2-MI 6.3			
Uncultured actinobacterium Elev_16S_1837	gi|134021719:1-1364	Bacteria; Actinobacteria; environmental samples. Trembling aspen rhizosphere under elevated CO_2 conditions	99

Appendix A10 continued

Query/ Relative	Accession number	Description	% Identity
Acidimicrobium ferrooxidans	gi|1649046|gb|U75647.1|AFU75647	Bacteria; Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium, Heterotrophic iron-oxidizing, extremely acidophilic Actinobacteria	90
Actinocorallia caverna N3-7 A10-MI 6.3	gb|AY966427.1|:1-1407	Streptosporangineae; Thermomonosporaceae; Actinocorallia, isolated from a natural cave in Jeju, Korea	89
Uncultured actinobacterium GASP-WDOW3_H01	gi|118042893:1-864	Uncultured Bacteria; Actinobacteria; environmental samples. Soil bacterial community affected by animal manure application in pasture and cropping systems of the Southern Piedmont USA, Georgia, GASP Watkinsville sampling site	96
Acidimicrobium ferrooxidans TH3	gb|EF621760.1|:1-1325	Bacteria; Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium,	92
Ferrimicrobium acidiphilum	gb|AF251436.1|:1-1449	Bacteria; Actinobacteria; Ferrimicrobium. Plays role in the bioleaching of sulfide minerals	91
Acidimicrobium ferrooxidans	gb|U75647.1|AFU75647:1-1465	Bacteria; Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium, Heterotrophic iron-oxidizing, extremely acidophilic Actinobacteria	91
B7-MI 6.3			
Uncultured actinobacterium Elev_16S_853	gi|134021116:1-1362	Bacteria; Actinobacteria; environmental samples. Trembling aspen rhizosphere under elevated CO_2 conditions	100
Actinocorallia caverna N3-7	gb|AY966427.1|:1-1407	Actinomycetales, type strain, isolated South Korea: Jeju	89
D8-MI 6.3			
Bacterium Ellin5024	gi|37961598:1-1343	Actinobacteria; Actinomycetales; Frankineae; Kineosporiaceae	99
Quadrisphaera granulorum AG019	gb|AY831385.1|:1-1483	Actinobacteria; Actinomycetales; Frankineae; Quadrisphaera. Type of Quadrisphaera granulorum	97
Kineosporia rhamnosa: I-132(=JCM9954)	dbj|AB003935.1|:1-1474	Actinobacteria; Actinomycetales; Frankineae; Kineosporiaceae; Kineosporia, isolated from plant samples	97
B10-MI 6.3			
Uncultured Actinomycetales (TM208)	emb|X92703.1|:1-1349	Environmental samples; Actinobacteria; Actinomycetales. Peat bog	99
Ferrimicrobium acidiphilum	gb|AF251436.1|:1-1449	Bacteria; Actinobacteria; Ferrimicrobium. Heterotrophic iron-oxidizing acidophile isolated from acid mine waters	92
Acidimicrobium ferrooxidans TH3	gb|EF621760.1|:1-1325	Bacteria; Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium, Heterotrophic iron-oxidizing, extremely acidophilic Actinobacteria	92
Streptomyces aurantiogriseus: NBRC 12842	dbj|AB184188.2|:1-1480	Actinobacteria; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces	90

Appendix A10 continued

Query/ Relative	Accession number	Description	% Identity
A2-MI 6.3			
Uncultured bacterium HTDD3	gi|18141096:1-1483	Uncultured bacteria; Environmental samples. Microbial diversity associated with metal-rich particles from a freshwater reservoir, USA: Colorado, Fort collins, Horsetooth Reservoir	99
Mycobacterium saskatchewanense MB54784	gb|AY208857.1|:1-1511	Actinobacteria; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium	99
G3-MI 6.3			
Mycobacterium neglectum type BN 3150T	emb|AJ580802.1|:1-1470	Actinobacteria; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium, drinking water biofilm, Germany: Duisburg	99
B9-MI 6.3			
Uncultured bacterium pLW-82	gi|67550303:1-1382	Uncultured Bacteria; environmental samples. Sediment of Lake Washington	97
Gemmatimonas aurantiaca	dbj|AB072735.1|:1-1446	Bacteria; Gemmatimonadetes; Gemmatimonadales; Gemmatimonadaceae; Gemmatimonas	90
A1-MI 6.3			
Uncultured bacterium FW95	gi|21952404:1-1246	Uncultured Bacteria; environmental samples. Forested wetland	98
Actinomadura cremea subsp. Cremea	gi|7159024:1-1432	Actinobacteria; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura	95
Thermomonospora formosensis	gb|AF002263.1|AF002263:1-1456	Actinobacteria; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura	95
D2-MI 6.3			
Uncultured actinobacterium CrystalBog022D6	gi|60326367:1-1510	Uncultured Bacteria; environmental samples.Humic Lake, USA: Northern Wisconsin	99
Nostocoida limicola II (Ben17)	emb|X85211.1|:1-1509	Actinobacteria; Actinomycetales; Micrococcineae; Intrasporangiaceae; Nostocoida type II, a filamentous bacterium from activated sludge	92
C1-MI 6.3			
Uncultured bacterium FCPT456	gi|145285356:1-1440	Uncultured bacteria; Environmentalsamples. Grassland soil, USA: northern California, Angelo Coast Range Reserve	96
Geodermatophilus obscurus dictyosporus	gb|L40621.1|GEDRG16SB:1-1411	Actinobacteria; Actinomycetales; Frankineae; Geodermatophilaceae; Geodermatophilus	90
D11-MI 6.3			
Uncultured bacterium Amb_16S_1075	gi|134019874:1-1368	Uncultured Bacteria; Actinobacteria; environmental samples. trembling aspen rhizosphere under ambient CO_2 conditions	100

Appendix A10continued

Query/ Relative	Accession number	Description	% Identity
Acidimicrobium ferrooxidans TH3	gb|EF621760.1|:1-1325	Bacteria; Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium, Heterotrophic iron-oxidizing, extremely acidophilic	91
Bacterium TH3	gi|173823:1-1365	Actinobacteria; Acidimicrobidae	91
Acidimicrobium ferrooxidans	gb|U75647.1|AFU75647:1-1465	Bacteria; Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium, Heterotrophic iron-oxidizing, extremely acidophilic Actinobacteria. A moderately thermophilic mineral-sulphide oxidising bacterium	90
Candidatus Microthrix calida TNO1-2	gi|73532963:1-1463	Bacteria; Actinobacteria; Actinobacteria (class); unclassified Actinobacteria; Candidatus Microthrix. Microthrix species isolated from industrial activated sludge wastewater treatment plants	89
Actinoplanes minutisporangius	dbj|AB037007.1|:1-1470	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Kineosporiaceae; Cryptosporangium.	89
D4-MI 6.3			
Uncultured actinobacterium TH3-101	emb|AM690900.1|:1-883	uncultured Bacteria; Actinobacteria; environmental samples. Large, shallow, freshwater, subtropical Taihu Lake, China	98
Mycobacterium anthracenicum	emb|Y15709.1|:1-1450	Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium. PAH-degrading PAH-degrading	98
C9-MI 6.3			
Bacterium Ellin5024	gi|37961598:1-1343	Actinobacteria; Actinomycetales; Frankineae; Kineosporiaceae	99
Frankiaceae (isolate 10;Namibia)	emb|X92365.1|:1-1432	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Frankiaceae, isolated from dry soil, rocks, and monument surfaces, G10, dolomite marbel, Namibia	97
Quadrisphaera granulorum AG019	gb|AY831385.1|:1-1483	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Quadrisphaera, Gram-positive polyphosphate-accumulating coccus in tetrads	97
G1-MI 6.3			
Actinomycetales Gsoil 1632	dbj|AB245397.1|:1-1470	Actinobacteria; Actinobacteridae; Actinomycetales, ginseng field soil, South Korea:Daejeon	98
Acidothermus cellulolyticus	emb|AJ007290.1|:1-1470	Actinobacteridae; Actinomycetales; Frankineae; Acidothermaceae; Acidothermus	96
A5-MI 6.3			
Uncultured bacterium ARFS-35	emb|AJ277701.1|:1-1110	Uncultured bacteria, Actinobacteria; environmental samples, Italian rice paddy soil	98
Streptomyces scabrisporus: NBRC 100760	dbj|AB249946.1|:1-1449	Actinobacteria; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces	97
A6-MI 6.3			
Uncultured actinobacteria 403T3	gi|71041253:	Actinobacteria; environmental samples	99

Appendix A10 continued

Query/ Relative	Accession number	Description	% Identity
Sporichthya polymorpha	emb|X72377.1|:1-1445	Actinobacteria; Actinomycetales; Frankineae; Sporichthyaceae; Sporichthya	96
D6-MI 6.3			
Bacterium Ellin5024	gi|37961598:1-1343	Actinobacteridae; Actinomycetales; Frankineae; Kineosporiaceae	98
Quadrisphaera granulorum AG019	gb|AY831385.1|:1-1483	Actinomycetales; Frankineae; Quadrisphaera, Gram-positive polyphosphate-accumulating coccus in tetrads	97
Kineosporia aurantiaca	emb|X87110.1|:1-1501	Actinomycetales; Frankineae; Kineosporiaceae; Kineosporia	97
G5-MI 6.3			
Unidentified bacterium LWSR-4	gi|33392072:1-1486	Lake Wai'ele'ele water	97
Ferrimicrobium acidiphilum	gb|AF251436.1|:1-1449	Bacteria; Actinobacteria; Ferrimicrobium	89
Kibdelosporangium albatum DSM 44149T	emb|AJ512462.1|:1-1489	Actinobacteria; Actinomycetales; Pseudonocardineae; Pseudonocardiaceae; Kibdelosporangium	88
A8-MI 6.3			
Uncultured bacterium FCPT456	gi|145285356:1-1440	Uncultured bacteria; Environmental samples. Grassland soil, USA: northern California, Angelo Coast Range Reserve	96
Blastococcus sp. BC448	emb|AJ316571.1|:1-1483	Actinobacteria; Actinomycetales; Frankineae; Geodermatophilaceae; Blastococcus	90
Geodermatophilus obscurus dictyosporus	gb|L40621.1|GEDRG16SB:1-1411	Actinomycetales; Frankineae; Geodermatophilaceae; Geodermatophilus	90
D1-MI 6.3			
Uncultured Actinomycetales (TM208)	emb|X92703.1|:1-1349	Actinobacteria; Actinomycetales; environmental samples, peat bog, Germany	99
Ferrimicrobium acidiphilum	gb|AF251436.1|:1-1449	Bacteria; Actinobacteria; Ferrimicrobium	93
Streptomyces mashuensis NRRL B-8164T	gb|DQ442526.1|:1-1489	Actinobacteria; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces	90
D7-MI 6.3			
Actinomycetales str. Ellin143	gi|33309385:1-1338	Actinobacteria; Actinomycetales	96
Blastococcus jejuensis KST3-10	gb|DQ200983.1|:1-1404	Actinomycetales; Frankineae; Geodermatophilaceae; Blastococcus, marine sediment	95
Geodermatophilus obscurus dictyosporus	gb|L40621.1|GEDRG16SB:1-1411	Actinomycetales; Frankineae; Geodermatophilaceae; Geodermatophilus	95

Appendix A10 continued

Query/ Relative	Accession number	Description	% Identity
A7-MI 6.3			
Uncultured Actinomycetales (TM208)	emb|X92703.1|:1-1349	Uncultured Actinobactreia; Actinobacteridae; Actinomycetales; environmental samples. Peat bog, Germany	99
Ferrimicrobium acidiphilum	gb|AF251436.1|:1-1449	Bacteria; Actinobacteria; Ferrimicrobium. Actinobacteria; Ferrimicrobium, heterotrophic iron-oxidizing acidophile isolated from acid mine waters	93
Streptomyces aurantiogriseus: NBRC 12842	dbj|AB184188.2|:1-1480	Actinobactria; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces	90
A4-MI 6.3			
Uncultured actinobacterium Amb_16S_1709	gi|134020521:1-1362	Uncultured Bacteria; Actinobacteria; environmental samples, trembling aspen rhizosphere under ambient CO_2 conditions	99
Ferrimicrobium acidiphilum	gb|AF251436.1|:1-1449	Bacteria; Actinobacteria; Ferrimicrobium. Heterotrophic acidophiles and their roles in the bioleaching of sulfide minerals	91
Actinoplanes minutisporangius	dbj|AB037007.1|:1-1470	Actinobactria; Actinomycetales; Frankineae; Kineosporiaceae; Cryptosporangium	89
D9-MI 6.3			
Uncultured actinobacterium GASP-38KA-9-A02	gi|154869504:1-1385	Actinobacteria; environmental samples, subtropical Altamaha and Ohopee River Valley inland dune fields, USA: Georgia	98
Acidimicrobium ferrooxidans TH3	gb|EF621760.1|:1-1325	Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium, heterotrophic iron-oxidizing, extremely acidophilic Actinobacteria	89
G9-MI 6.3			
Uncultured actinobacterium TH3-101	emb|AM690900.1|:1-883	uncultured Bacteria; Actinobacteria; environmental samples. Large, shallow, freshwater, subtropical Taihu Lake, China. Bacterioplankton community composition in the large, shallow, subtropical Taihu Lake, freshwater lake, China	99
Mycobacterium sp.JS621	gi|22711853:1-1517	Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium, biodegrades the groundwater pollutant vinyl chloride	98
Mycobacterium anthracenicum	emb|Y15709.1|:1-1450	Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium, PAH-degrading , isolated by Saowanit Tongpim and M.A Pickard, Univ. of Alberta, Canada	98
B1-MI 1.2			
Uncultured bacterium ARFS-24	emb|AJ277695.1|:1-1113	Actinobacteria; environmental samples, flooded anoxic rice paddy soil	97
Acidothermus cellulolyticus	emb|AJ007290.1|:1-1470	Actinobacteridae; Actinomycetales; Frankineae; Acidothermaceae; Acidothermus	95

Appendix A10 continued

Query/ Relative	Accession number	Description	% Identity
A1-MI 1.2			
Janibacter sp. G5	gi|110226603:1-1034	Actinobactria; Actinobacteridae; Actinomycetales; Micrococcineae; Intrasporangiaceae; Janibacter, Bacterium-bacterium inhibitory interactions among psychrotrophic bacteria isolated from Antarctic seawater (Terra Nova Bay, Ross Sea)	92
Janibacter limosus	emb|Y08539.1|:1-1473	Actinobactria; Actinobacteridae; Actinomycetales; Micrococcineae; Intrasporangiaceae; Janibacter, actinomycete with meso-diaminopimelic acid in the cell wall	99
A8-MI 1.2			
Uncultured bacterium FAC78	gi|90992962:1-1471	Fushan, Forest Soils of Taiwan	94
Streptosporangium vulgare (DSM44112)	emb|X89957.1|:1-1450	Actinobacteridae; Actinomycetales; Streptosporangineae; Streptosporangiaceae; Streptosporangium, sub_species "antibioticum"	92
D11-MI 1.2			
Uncultured bacterium Par-w-17	gi|149350584:1-1489	Uncultured bacteria; Environmental samples. Aquatic environments of the high altitude Andean Altiplano (northern Chile), freshwater	99
Arthrobacter psychrophenolicus DSM 15454T	emb|AJ616763.1|:1-1501	Actinomycetales; Micrococcineae; Micrococcaceae; Arthrobacter	99
G9-MI 1.2			
Arthrobacter sp. TSBY-69	gi|89258116:1-1377	Actinobactria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae; Arthrobacter	98
Arthrobacter psychrolactophilus D2	gb|AF134181.1|:1-1372	Actinobacteridae;Actinomycetales; Micrococcineae; Micrococcaceae; Arthrobacter, psychrophilic Arthrobacter isolate related to Arthrobacter psychrolactophilus	98
G3-MI 1.2			
Uncultured actinobacterium NPK-58	gi|120971710:1-643	Actinobacteria; environmental samples, rice-wheat growing soils in China under long-term manure and chemical fertilizer treatments, China: SuZhou, Jiangsu Province	98
Humicoccus flavidus DS-52	gb|DQ321750.1|:1-1475	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Nakamurellaceae; Humicoccus	96
A4-MI 1.2			
Uncultured Thermomonosporaceae GASP-MA3W3_B05	gi|151351130:1-812	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; environmental samples, grassland at the GASP KBS-LTER sampling site, USA: Michigan	99
Actinomadura spadix	gi|6690564:1-1458	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura	96
A5-MI 1.2			
Uncultured actinobacterium CK-71	gi|120971651:1-642	Actinobacteria; environmental samples, rice-wheat growing soils in China under long-term manure and chemical fertilizer treatments, China: SuZhou, Jiangsu Province	98

Appendix A10 continued

Query/ Relative	Accession number	Description	% Identity
Nocardioides jensenii	emb|Z78210.1|:1-1467	Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Nocardioides, an LL-diaminopimelic acid-containing actinomycete from Antarctic sandstone	98
B4-MI 1.2			
Uncultured bacterium ANTLV2_H06	gi|102415972:1-1437	Bacteria; environmental samples, perennial ice cover of Lake Vida, Antarctica, Antarctica: Southern Victoria Land, Victoria Valley	99
Candidatus Microthrix calida TNO2-4	gi|73532959|gb|DQ147284.1|	Bacteria; Actinobacteria; Actinobacteria (class); unclassified Actinobacteria; Candidatus Microthrix. Industrial activated sludge wastewater treatment plants	90
Streptomyces mashuensis NRRL B-8164T	gb|DQ442526.1|:1-1489	Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces	88
C10-MI 1.2			
Uncultured Gemmatimonadete Skagenf3	gi|108947782:1-1400	Uncultured bacteria; Gemmatimonadetes; environmental samples, activated sludge, enhanced biological phosphorus removal plant	96
Gemmatimonas aurantiaca	dbj|AB072735.1|:1-1446	Gemmatimonadetes; Gemmatimonadales; Gemmatimonadaceae; Gemmatimonas	95
Corynebacterium flavescens NCDO 1320	emb|X84441.1|:1-1489	Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Corynebacteriaceae; Corynebacterium	83
B11-MI 1.2			
Uncultured bacterium ANTLV2_C06	gi|102415949:1-1444	Perennial ice cover of Lake Vida, Antarctica, McMurdo Dry Valleys, Antarctica	98
Tessaracoccus bendigoensis	gi|109158518:1-1442	Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Propionibacteriaceae; Tessaracoccus, also found in wastewater generated in the production of stainless steel in a SBR reactor	97
E11-MI 1.2			
Microbacterium sp. KV-490	dbj|AB248875.1|:1-1421	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Microbacterium	99
Microbacterium terregens	dbj|AB004721.1|:1-1437	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Microbacteriaceae; Microbacterium, "synonym:Aureobacterium terregens"	98
F5-MI 1.2			
Uncultured bacterium ANTLV2_C06	gi|102415949:1-1444	Perennial ice cover of Lake Vida, Antarctica, 4.8 m, Lake Vida ice cover, McMurdo Dry Valleys, Antarctica: Southern Victoria Land, Victoria Valley	98
Tessaracoccus bendigoensis	gi|109158518:1-1442	Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Propionibacteriaceae; Tessaracoccus, also found in wastewater generated in the production of stainless steel in a SBR reactor	97

Appendix A10 continued

Query/ Relative	Accession number	Description	% Identity
G12-MI 1.2			
Uncultured bacterium ANTLV2_C06 16S	gi|102415949:1-1444	Bacteria; environmental samples, perennial ice cover of Lake Vida, Antarctica, 4.8 m, Lake Vida ice cover, McMurdo Dry Valleys, Antarctica, Antarctica: Southern Victoria Land, Victoria Valley	99
Tessaracoccus bendigoensis	gi|109158518:1-1442	Actinobacteridae; Actinomycetales; Propionibacterineae; Propionibacteriaceae; Tessaracoccus, also found in wastewater generated in the production of stainless steel in a SBR reactor	97
B6-MI 1.2			
Uncultured Actinomycetales (TM208)	emb|X92703.1|:1-1349	Actinobacteria; Actinobacteridae; Actinomycetales; environmental samples, peat bog, Germany	99
Ferrimicrobium acidiphilum	gb|AF251436.1|:1-1449	Bacteria; Actinobacteria; Ferrimicrobium. Heterotrophic acidophiles and their roles in the bioleaching of sulfide minerals, iron-oxidizing acidophile isolated from acid mine waters	93
Acidimicrobium ferrooxidans TH3	gb|EF621760.1|:1-1325	Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium, heterotrophic iron-oxidizing, extremely acidophilic Actinobacteria	92
Acidimicrobium ferrooxidans	gb|U75647.1|AFU75647:1-1465	Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium, moderately thermophilic mineral-sulphide oxidizing bacterium	91
Streptomyces aurantiogriseus : NBRC 12842	dbj|AB184188.2|:1-1480	Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces	90
B9-MI 1.2			
Uncultured bacterium KD1-70	gi|29027911:1-1480	Bacteria in Penguin Droppings Sediments from Ardley Island, Antarctica	99
Dermatophilus crocodyli	gb|AF226615.1|AF226615:1-1263	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Dermatophilaceae; Dermatophilus, also isolated from crocodiles, Dermatophilus crocodyli sp. nov., isolated from Crocodylus porosus (saltwater crocodile) with 'brown spot' disease	95
E5-MI 1.2			
Uncultured bacterium ANTLV2_C06	gi|102415949:1-1444	Perennial ice cover of Lake Vida, Antarctica, 4.8 m, Lake Vida ice cover, McMurdo Dry Valleys, Antarctica, Antarctica: Southern Victoria Land, Victoria Valley	98
Tessaracoccus bendigoensis	gi|109158518:1-1442	Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Propionibacteriaceae; Tessaracoccus, also found in wastewater generated in the production of stainless steel in a SBR reactor	97
F9-MI 1.2			
Arthrobacter sp. KAR36	gi|133723273:1-884	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae; Arthrobacter, high Arctic permafrost soil from, Spitsbergen, permafrost.	99

Appendix A10 continued

Query/ Relative	Accession number	Description	% Identity
Arthrobacter stackebrandtii CCM 2783	emb|AJ640198.1|:1-1517	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae; Arthrobacter	98
H10-MI 1.2			
Uncultured Thermomonosporaceae GASP-MA3W2_B02	gi|151351037:1-812	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; environmental samples, grassland at the GASP KBS-LTER sampling site, USA: Michigan	93
Actinomadura libanotica	gb|U49007.1|ALU49007:1-1404	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinocorallia	90
A3-MI 1.2			
Uncultured actinobacterium AI-1M_H02	gi|146428905:1-894	Actinobacteria; environmental samples, soil environments under mosses (Sanionia uncinata) on Anchorage Island, Antarctica	99
Terracoccus luteus	emb|Y11928.1|:1-1479	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Intrasporangiaceae; Terracoccus, an LL-diaminopimelic acid-containing coccoid actinomycete from soil	97
C6-MI 1.2			
Uncultured bacterium KD1-70	gi|29027911:1-1480	Bacteria; environmental samples, Penguin Droppings Sediments from Ardley Island, Antarctica	98
Dermatophilus crocodyli	gb|AF226615.1|AF226615:1-1263	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Dermatophilaceae; Dermatophilus, Dermatophilus crocodyli sp. nov., isolated from Crocodylus porosus (saltwater crocodile) with 'brown spot'disease	95
E4-MI 1.2			
Glacial ice bacterium G200-C18	gi|19568769:1-1432	Bacteria; unclassified Bacteria; unclassified Bacteria (miscellaneous). Glacial and subglacial environments, 200 year-old glacial ice from Guliya	99
Oerskovia paurometabola DSM 14281	emb|AJ314851.1|:1-1486	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Cellulomonadaceae; Oerskovia	98
F8-MI 1.2			
Uncultured soil bacterium F41_Pitesti	gi|87243057:1-1478	Uncultured Bacteria; environmental samples. Oil-polluted soil, Romania	99
Propionicicella superfundia BL-10T	gb|DQ176646.1|:1-1469	Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Nocardioidaceae; Propionicicella, chlorosolvent-tolerant propionate-forming, facultative anaerobic bacterium isolated from contaminated groundwater, type of Propionicicella superfundia	96
Propioniferax innocua	gb|AF227165.1|AF227165:1-1465	Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Propionibacteriaceae; Propioniferax	94

Appendix A10 continued

Query/ Relative	Accession number	Description	% Identity
H5-MI 1.2			
Uncultured actinobacterium NPK-33	gi|120971719:1-643	Actinobacteria; environmental samples, rice-wheat growing soils in China under long-term manure and chemical fertilizer treatments, soil used for rice-wheat cultivation for centuries, China: SuZhou, Jiangsu Province	98
Knoellia sinensis SAFR-013	gi|27497671:1-1324	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Intrasporangiaceae; Knoellia, Microbial diversity of spacecraft assembly facilities	98
B3-MI 1.2			
Uncultured actinobacterium AI-1M_H02	gi|146428905:1-894	Actinobacteria; environmental samples, Antarctic terrestrial habitats, soil environments under mosses (Sanionia uncinata) on Anchorage Island, Antarctica	99
Terracoccus luteus	emb|Y11928.1|:1-1479	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Intrasporangiaceae; Terracoccus, an LL-diaminopimelic acid-containing coccoid actinomycete from soil	97
Janibacter limosus	emb|Y08539.1|:1-1473	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Intrasporangiaceae; Janibacter, actinomycete with meso-diaminopimelic acid in the cell wall	97
C1-MI 1.2			
Uncultured bacterium Par-w-17	gi|149350584:1-1489	Uncultured bacterium, aquatic environments of the high altitude Andean Altiplano (northern Chile), Chile: Altiplano, Bofedal de Parinacota	99
Arthrobacter psychrophenolicus DSM 15454T	emb|AJ616763.1|:1-1501	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae; Arthrobacter, isolated from an alpine ice cave	99
E8-MI 1.2			
Arthrobacter sp. KAR36	gi|133723273:1-884	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae; Arthrobacter, high Arctic permafrost soil from, Spitsbergen, permafrost, isolated from pure culture	99
Arthrobacter psychrolactophilus D2	gb|AF134181.1|:1-1372	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae; Arthrobacter, psychrophilic Arthrobacter isolate related to Arthrobacter psychrolactophilus	98
G11-MI 1.2			
Uncultured actinobacterium NPK-33	gi|120971719:1-643	Actinobacteria; environmental samples, rice-wheat growing soils in China under long-term manure and chemical fertilizer treatments, soil used for rice-wheat cultivation for centuries, China: SuZhou, Jiangsu Province	98
Knoellia sinensis SAFR-013	gi|27497671:1-1324	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Intrasporangiaceae; Knoellia, Microbial diversity of spacecraft assembly facilities	98

Appendix A10 continued

Query/ Relative	Accession number	Description	% Identity
Janibacter limosus	emb|Y08539.1|:1-1473	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Intrasporangiaceae; Janibacter, actinomycete with meso-diaminopimelic acid in the cell wall	98
A7-MI 1.2			
Uncultured actinobacterium SI-1M_F04	gi|146430665|gb|EF221481.1|	Actinobacteria; environmental samples, soil environments under mosses (Chorisodontium aciphyllum) on Signy Island, Antarctica	98
Acidothermus cellulolyticus	emb|AJ007290.1|:1-1470	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Acidothermaceae; Acidothermus	97
E9-MI 1.2			
Uncultured bacterium ANTLV2_C06	gi|102415949:1-1444	uncultured bacterium. Perennial ice cover of Lake Vida, Antarctica, 4.8 m, Lake Vida ice cover, McMurdo Dry Valleys, Antarctica, Antarctica: Southern Victoria Land, Victoria Valley	99
Tessaracoccus bendigoensis	gi|109158518:1-1442	Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Propionibacteriaceae; Tessaracoccus, isolated from wastewater generated in the production of Stainless steel in a SBR reactor	97
F1-MI 1.2			
Uncultured actinobacterium NPK-33	gi|120971719:1-643	Actinobacteria; environmental samples, rice-wheat growing soils in China under long-term manure and chemical fertilizer treatments, soil used for rice-wheat cultivation for centuries, China: SuZhou, Jiangsu Province	98
Knoellia sinensis SAFR-013	gi|27497671:1-1324	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Intrasporangiaceae; Knoellia, Microbial diversity of spacecraft assembly facilities	98
C2-MI 1.2			
Uncultured bacterium ANTLV2_C06	gi|102415949:1-1444	uncultured bacterium. Perennial ice cover of Lake Vida, Antarctica, 4.8 m, Lake Vida ice cover, McMurdo Dry Valleys, Antarctica, Antarctica: Southern Victoria Land, Victoria Valley	93
Tessaracoccus bendigoensis	gi|109158518:1-1442	Actinobacteria; Actinobacteridae; Actinomycetales; Propionibacterineae; Propionibacteriaceae; Tessaracoccus, Isolated from wastewater generated in the production of stainless steel in a SBR reactor	92
A2-MI 1.2			
Uncultured actinobacterium AI-1M_H02	gi|146428905:1-894	Actinobacteria; environmental samples, soil environments under mosses (Sanionia uncinata) on Anchorage Island, Antarctica	99
Terracoccus luteus	emb|Y11928.1|:1-1479	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Intrasporangiaceae; Terracoccus, an LL-diaminopimelic acid-containing coccoid actinomycete from soil	97

Appendix A10 continued

Query/ Relative	Accession number	Description	% Identity
C3-MI 1.2			
Rhodococcus sp. 5/3	gb|EU041714.1|:1-1396	Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Nocardiaceae; Rhodococcus, Hexadecane mineralization activity in ornithogenic soil from Seabee Hook, Cape Hallet, Antarctica, ornithogenic soil	98
Rhodococcus coprophilus DSM43347T	emb|X80626.1|:1-1481	Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Nocardiaceae; Rhodococcus	96
E6-MI 1.2			
Uncultured Actinomycetales (TM208)	emb|X92703.1|:1-1349	Actinobacteria; Actinomycetales; environmental samples, peat bog, Germany	99
Ferrimicrobium acidiphilum	gb|AF251436.1|:1-1449	Bacteria; Actinobacteria; Ferrimicrobium. Heterotrophic acidophiles and their roles in the bioleaching of sulfide minerals, heterotrophic iron-oxidizing acidophile isolated from acid mine waters	93
Streptomyces mashuensis NRRL B-8164T	gb|DQ442526.1|:1-1489	Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces	90
A11-MI 1.2			
Arthrobacter sp. TSBY-69	gi|89258116:1-1377	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae; Arthrobacter, alpine permafrost in the Tianshan Mountains, northwestern China, frozen soil, China: Tianshan Mountains	100
Arthrobacter psychrolactophilus D2	gb|AF134181.1|:1-1372	Actinobacteria; Actinobacteridae; Actinomycetales; Micrococcineae; Micrococcaceae; Arthrobacter, psychrophilic Arthrobacter isolate related to Arthrobacter psychrolactophilus	99
C5-MI 1.2			
Uncultured actinobacterium AI-1M_H02	gi|146428905|gb|EF219721.1|	uncultured bacterium. Antarctic terrestrial habitats, soil environments under mosses (Sanionia uncinata) on Anchorage Island, Antarctica	98
E12-MI 1.2			
Rhodococcus sp. 11/16a	gi|83701104|gb|DQ310479.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Nocardiaceae; Rhodococcus, Antarctic hydrocarbon-degrading bacteria	99
Rhodococcus luteus 7Y	gi|33111952|emb|AJ576249.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Nocardiaceae; Rhodococcus, Antarctica:Schirmacher Oasis, Lake Zub	99
F3-MI 1.2			
Uncultured Thermomonosporaceae GASP-MA3W3_B05	gi|151351130|gb|EF663839.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; environmental samples, grassland at the GASP KBS-LTER sampling site, USA: Michigan	99
Actinomadura spadix	gi|6690564|gb|AF163120.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura	95

Appendix A10 continued

Query/ Relative	Accession number	Description	% Identity
B12-MI 1.2			
Mycobacterium engbaekii	gi|19387304|gb|AF480577.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium	97
F2-MI 1.2			
Uncultured actinobacterium FBP218	gi|29893288|gb|AY250865.1|	uncultured bacterium. Cryptoendolithic Communities from the McMurdo Dry Valleys, Antarctica, lichen-dominated Antarctic cryptoendolithic community, Antarctica: Southern Victoria Land, McMurdo dry valleys	95
Humicoccus flavidus DS-52	gi|83977435|gb|DQ321750.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Nakamurellaceae; Humicoccus	95
D1-MI 5.1			
Uncultured actinobacterium Elev_16S_853	gi|134021116|gb|EF019692.1|	Uncultured actinobacterium. Trembling aspen rhizosphere under elevated CO_2 conditions	99
Acidimicrobium ferrooxidans	gb|U75647.1|AFU75647:1-1465	Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium, a moderatelythermophilic mineral-sulphide oxidising bacterium	89
Actinocorallia caverna N3-7	gi|66476209|gb|AY966427.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinocorallia, isolated from a natural cave in Jeju, Korea	89
E9-MI 5.1			
Uncultured actinobacterium A03_WMSP1	gi|91221246|gb|DQ450794.1|	uncultured bacterium. Saturated alpine tundra wet meadow soil, 0-20 cm	99
Frankia sp. AgHi38	gi|2765253|emb|Y12848.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Frankiaceae; Frankia, ineffective Frankia nodule from A.glutinosa host from the Netherlands	97
Sporichthya polymorpha	gi|535061|emb|X72377.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Sporichthyaceae; Sporichthya	96
F7-MI 5.1			
Uncultured actinobacterium GASP-38KA-9-A02	gi|154869504|gb|EU044043.1|	Actinobacteria; environmental samples, subtropical Altamaha and Ohopee River Valley inland dune fields, USA: Georgia	99
Ferrimicrobium acidiphilum	gi|7595962|gb|AF251436.1|	Bacteria; Actinobacteria; Ferrimicrobium. Heterotrophic acidophiles and their roles in the bioleaching of sulfide minerals, a heterotrophic iron-oxidizing acidophile isolated from acid mine waters	89
F10-MI 5.1			
Uncultured Thermomonosporaceae GASP-MA3W3_B05	gi|151351130|gb|EF663839.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; environmental samples, soil communities in Michigan, rassland at the GASP KBS-LTER sampling site	99
Actinomadura spadix	gi|6690564|gb|AF163120.1|AF163120	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura	95

Appendix A10 continued

Query/ Relative	Accession number	Description	% Identity
F5-MI 5.1			
Uncultured actinobacterium A03_WMSP1	gi|91221246|gb|DQ450794.1|	Actinobacteria; environmental samples, saturated alpine tundra wet meadow soil 0-20 cm, USA: Colorado, Rocky Mountain Front Range, Niwot Ridge, elev. 3461 m	99
Frankia sp. AgHi38	gi|2765253|emb|Y12848.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Frankiaceae; Frankia, ineffective Frankia nodule from A.glutinosa host from the Netherlands	96
A3-MI 5.1			
Uncultured Thermomonosporaceae GASP-MA3W3_B05	gi|151351130|gb|EF663839.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; environmental samples, agricultural land management practices on bacterial soil communities in Michigan, agricultural land management practices on bacterial soil communities in Michigan, grassland at the GASP KBS-LTER sampling site	99
Actinomadura spadix	gi|6690564|gb|AF163120.1|AF163120	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura	96
H6-MI 5.1			
Uncultured bacterium BF0001B033	gi|145582839|emb|AM697007.1|	uncultured bacterium. Bacteria; environmental samples, indoor dust, Finland	100
Frankia sp. FE37	gi|3201707|gb|AF063641.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Frankiaceae; Frankia, isolated from root nodules (FE37) of Coriaria arborea collected in New Zealand	96
Acidothermus cellulolyticus	gi|5102592|emb|AJ007290.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Acidothermaceae; Acidothermus, A recA gene phylogenetic analysis confirms the close proximity of Frankia to Acidothermus	96
F2-MI 5.1			
Unidentified actinomycete L43598	gi|1448913|gb|L43598.1|ARRRRDA	Actinobacteria; environmental samples, characterization of Frankia and of close phyletic neighbors from an Alnus viridis rhizosphere, Alnus rhizosphere soil amplicon	99
Frankiaceae bacterium KVD-unk-16	gi|94995748|gb|DQ490442.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Frankiaceae, heterotrophic isolates in respective libraries from recent Hawaiian volcanic deposits (<300 yr old), volcanic deposits, USA: Hawaii, Kilauea volcano	97
Humicoccus flavidus DS-52	gi|83977435|gb|DQ321750.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Nakamurellaceae; Humicoccus, isolated from soil	96
G8-MI 5.1			
Bacterium Ellin5091	gi|37961665|gb|AY234508.1|	Actinobacteria; Actinobacteridae, Cultivation of Widespread and Previously Uncultured Soil Bacteria	99
Frankiaceae bacterium KVD-unk-16	gi|94995748|gb|DQ490442.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Frankiaceae, libraries from recent Hawaiian volcanic deposits (<300 yr old), volcanic deposits, USA: Hawaii, Kilauea volcano	98

Appendix A10 continued

Query/ Relative	Accession number	Description	% Identity
Streptomyces serianimatus YIM 45720	gi|116294342|gb|DQ997046.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces, isolated from a rhizophere soil	96
E12-MI 5.1			
Uncultured actinobacterium FI-1M_F07	gi|146429798|gb|EF220614.1|	Actinobacteria; environmental samples, diversity across a range of Antarctic terrestrial habitats, soil environments under Empetrum rubrum at the Falkland Islands, United Kingdom: Falkland Islands	97
Acidimicrobium ferrooxidans TH3	gi|148767452|gb|EF621760.1|	Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium, heterotrophic iron-oxidizing, extremely acidophilic Actinobacteria	92
F6-MI 5.1			
Uncultured actinobacterium SI-1M_A08	gi|146430614|gb|EF221430.1|	Actinobacteria; environmental samples, diversity across a range of Antarctic terrestrial habitats, soil environments under mosses(Chorisodontium aciphyllum) on Signy Island, Antarctica	98
Actinomadura sp. AC104	gi|77998078|gb|DQ239428.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura, isolated from an Algerian Saharan soil	95
Actinomadura spadix	gi|6690564|gb|AF163120.1|AF163120	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura	95
B4-MI 5.1			
Uncultured Thermomonosporaceae GASP-MA3W3_B05	gi|151351130|gb|EF663839.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; environmental samples, Impact of agricultural land management practices on bacterial soil communities in Michigan, grassland at the GASP KBS-LTER sampling site	99
Actinomadura spadix	gi|6690564|gb|AF163120.1|AF163120	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura	96
E11-MI 5.1			
Mycobacterium sp. CNJ859 PL04	gi|92091034|gb|DQ448716.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium, gram-positive bacteria cultured from marine sediments	99
Mycobacterium confluentis DSM 44017T	gi|45771901|emb|AJ634379.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium	99
H10-MI 5.1			
Uncultured actinobacterium FI-1M_F07	gi|146429798|gb|EF220614.1|	Actinobacteria; environmental samples, diversity across a range of Antarctic terrestrial habitats, soil environments under Empetrum rubrum at the Falkland Islands, United Kingdom	97
Acidimicrobium sp. Y0018	gi|23953857|gb|AY140240.1	Geothermal sites in Yellowstone National Park	92

Appendix A10 continued

Query/ Relative	Accession number	Description	% Identity
A1-MI 5.1			
Thermomonosporaceae GASP-MA3W3_B05	gi|151351130|gb|EF663839.1| Uncultured	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; environmental samples, Impact of agricultural land management practices on bacterial soil communities in Michigan, grassland at the GASP KBS-LTER sampling site, USA: Michigan	98
Actinomadura sp. AC104	gi|77998078|gb|DQ239428.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura, isolated from an Algerian Saharan soil	95
D6-MI 5.1			
Uncultured bacterium FAC78	gi|90992962|gb|DQ451517.1|	Fushan Forest Soils of Taiwan	98
Actinomadura spadix	gi|6690564|gb|AF163120.1|AF163120	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura	95
G9-MI 5.1			
Uncultured bacterium NR.1.120	gi|145203120|gb|EF494371.1|	Nunnock River granitic landscape, Australia	96
Ferrimicrobium acidiphilum	gi|7595962|gb|AF251436.1|	Bacteria; Actinobacteria; Ferrimicrobium	91
Actinomadura oligospora IMSNU 22174T	gi|38567360|emb|AJ293709.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura	91
G3-MI 5.1			
Uncultured actinobacterium AI-1F_A06	gi|146428677|gb|EF219493.1|	Actinobacteria; environmental samples, Antarctic terrestrial habitats, unvegetated soil environments on Anchorage Island, Antarctica	99
Modestobacter sp. CNJ793 PL04	gi|92091016|gb|DQ448698.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Geodermatophilaceae; Modestobacter, marine sediment, Palau	97
Blastococcus jejuensis KST3-10	gi|77997755|gb|DQ200983.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Geodermatophilaceae; Blastococcus, marine sediment	97
C11-MI 5.1			
Uncultured actinobacterium SI-1M_F04	gi|146430665|gb|EF221481.1|	Actinobacteria; environmental samples, range of Antarctic terrestrial habitats, soil environments under mosses (Chorisodontium aciphyllum) on Signy Island, Antarctica	99
Acidothermus cellulolyticus	gi|5102592|emb|AJ007290.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Acidothermaceae; Acidothermus	97
F8-MI 5.1			
Uncultured bacterium ARFS-35	gi|9944270|emb|AJ277701.1|	Actinobacteria; environmental samples, Italian rice paddy soil, environmental sample from flooded anoxic rice paddy soil	99
Sporichthya polymorpha	gi|535061|emb|X72377.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Sporichthyaceae; Sporichthya	96

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Query/ Relative	Accession number	Description	% Identity
D10-MI 5.1			
Uncultured actinobacterium lhac5	gi|109727756|gb|DQ648937.1|	Actinobacteria; environmental samples, PCB contaminated soil	99
Humicoccus flavidus DS-52	gi|83977435|gb|DQ321750.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Nakamurellaceae; Humicoccus, isolated from soil	98
A7-MI 5.1			
Uncultured bacterium FW95	gi|21952404|gb|AF524001.1|	Bacteria; environmental samples, forested wetland impacted by reject coal	98
Actinomadura sp. 171824	gi|146395020|gb|EF546421.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura	94
Thermomonospora formosensis	gi|2465535|gb|AF002263.1|AF002263	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura	94
A9-MI 5.1			
Uncultured actinobacterium Amb_16S_1709	gi|134020521|gb|EF019097.1|	Uncultured Bacteria; Actinobacteria; environmental samples. Trembling aspen rhizosphere under ambient CO_2 conditions	99
Acidimicrobium ferrooxidans TH3	gi|148767452|gb|EF621760.1|	Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium, heterotrophic iron-oxidizing, extremely acidophilic Actinobacteria	91
C12-MI 5.1			
Uncultured actinobacterium AI-1F_A06	gi|146428677|gb|EF219493.1|	Actinobacteria; environmental samples, Antarctic terrestrial habitats, unvegetated soil environments on Anchorage Island, Antarctica	99
Modestobacter sp. CNJ793 PL04	gi|92091016|gb|DQ448698.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Geodermatophilaceae; Modestobacter, marine sediment	97
Blastococcus jejuensis KST3-10	gi|77997755|gb|DQ200983.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Geodermatophilaceae; Blastococcus, marine sediment	97
C2-MI 5.1			
Kitasatospora mediocidica: NBRC 14789	gi|90960442|dbj|AB184621.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; Kitasatospora	99
Streptomyces spiroverticillatus	gi|94470230|gb|DQ487019.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces	99
F1-MI 5.1			
Uncultured actinobacterium SI-1M_A08	gi|146430614|gb|EF221430.1|	Actinobacteria; environmental samples, a range of Antarctic terrestrial habitats, soil environments under mosses (Chorisodontium aciphyllum) on Signy Island, Antarctica	98
Actinomadura spadix	gi|6690564|gb|AF163120.1|AF163120	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura	94

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Query/ Relative	Accession number	Description	% Identity
Streptoalloteichus hindustanus	gi|94470228|gb|DQ487017.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Pseudonocardineae; Pseudonocardiaceae; Streptoalloteichus	94
G5-MI 5.1			
Kineococcus-like bacterium AS2978	gi|6073809|gb|AF060676.1|AF060676	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Kineosporiaceae, endemic to the Mojave Desert	97
Kineosporia rhamnosa: I-132 (=JCM9954)	gi|3894223|dbj|AB003935.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Kineosporiaceae; Kineosporia, isolated from plant samples	97
E10-MI 5.1			
Uncultured bacterium Elev_16S_1485	gi|134021501|gb|EF020077.1|	Trembling aspen rhizosphere under elevated CO_2 conditions	96
Frankia sp. BCU110505	gi|84794997|gb|DQ336135.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Frankiaceae; Frankia, adaptation of frankia to different discaria (rhamnaceae) host species growing in Patagonia	95
Streptomyces serianimatus YIM 45720	gi|116294342|gb|DQ997046.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces	95
H11-MI 5.1			
Uncultured Thermomonosporaceae GASP-MA3W3_B05	gi|151351130|gb|EF663839.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; environmental samples. From grassland at the GASP KBS-LTER sampling site	98
Actinomadura spadix	gi|6690564|gb|AF163120.1|AF163120	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura	94
Streptoalloteichus hindustanus	gi|94470228|gb|DQ487017.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Pseudonocardineae; Pseudonocardiaceae; Streptoalloteichus	94
A5-MI 5.1			
Mycobacterium saskatchewanense MB54784	gi|37784496|gb|AY208857.1|	Slowly growing scotochromogenic species from human clinical isolates related to Mycobacterium interjectum and Accuprobe-positive for Mycobacterium avium complex	99
F1-MI 5.1			
Uncultured actinobacterium SI-1M_A08	gi|146430614|gb|EF221430.1|	Actinobacteria; environmental samples, Antarctic terrestrial habitats, soil environments under mosses (Chorisodontium aciphyllum) on Signy Island	98
Uncultured Thermomonosporaceae GASP-MA3W3_B05	gi|151351130|gb|EF663839.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; environmental samples, agricultural land under management practices in Michigan, grassland at the GASP KBS-LTER sampling site	98

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Query/ Relative	Accession number	Description	% Identity
Actinomadura spadix	gi|6690564|gb|AF163120.1|AF163120	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura	94
D3-MI 5.1			
Uncultured actinobacterium FI-1M_G05	gi|146429808|gb|EF220624.1|	Actinobacteria; environmental samples, soil environments under Empetrum rubrum at the Falkland Islands, Antarctic terrestrial habitats	99
Thermomonosporaceae str. IMP-7387	gi|7110081|gb|AF131647.1|AF131647	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae, tropical rainforests of Singapore	93
Frankia sp. (Dryas)	gi|710511|gb|L40616.1|FRARG16SE	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Frankiaceae; Frankia, organism, "Frankia sp."	94
E7-MI 5.1			
Uncultured actinobacterium Elev_16S_853	gi|134021116|gb|EF019692.1|	Actinobacteria; environmental samples, Elevated CO_2 Affects Soil Microbial Diversity Associated with Trembling Aspen, trembling aspen rhizosphere under elevated CO_2 conditions	99
Acidimicrobium ferrooxidans TH3	gi|148767452|gb|EF621760.1|	Actinobacteria; Acidimicrobidae; Acidimicrobiales; Acidimicrobineae; Acidimicrobiaceae; Acidimicrobium, heterotrophic iron-oxidizing, extremely acidophilic Actinobacteria	90
A8-MI 5.1			
Actinomycete (Ep_T1.148)	gi|1523828|emb|Z73370.1|	Actinobacteria; Actinobacteridae; Actinomycetales; environmental samples, temperate forest soil	99
Blastococcus jejuensis KST3-10	gi|77997755|gb|DQ200983.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Geodermatophilaceae; Blastococcus, an actinomycete from beach sediment, South Korea: Jeju coast	96
Frankia sp. BCU110505	gi|84794997|gb|DQ336135.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Frankiaceae; Frankia, adaptation of frankia to different discaria (rhamnaceae) host species growing in Patagonia	96
F3-MI 5.1			
Uncultured bacterium FAC78	gi|90992962|gb|DQ451517.1|	Environmental samples, Fushan Forest Soils of Taiwan	99
Uncultured Thermomonosporaceae GASP-MA3W3_B05	gi|151351130|gb|EF663839.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; environmental samples, agricultural land under management practices in Michigan, grassland at the GASP KBS-LTER sampling site	98
Actinomadura spadix	gi|6690564|gb|AF163120.1|AF163120	Actinobacteria; Actinobacteridae; Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura	95
A8-MI 5.1			
Actinomycete (Ep_T1.148)	gi|1523828|emb|Z73370.1|	Actinobacteria; Actinobacteridae; Actinomycetales; environmental samples, temperate forest soil	99

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Query/ Relative	Accession number	Description	% Identity
Blastococcus jejuensis KST3-10	gi|77997755|gb|DQ200983.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Geodermatophilaceae; Blastococcus, an actinomycete from beach sediment, South Korea: Jeju coast	96
Frankia sp. BCU110505	gi|84794997|gb|DQ336135.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Frankiaceae; Frankia, adaptation of frankia to different discaria (rhamnaceae) host species growing in Patagonia	96
F5-MI 5.1-F243			
Uncultured bacterium NR.1.120	gi|145203120|gb|EF494371.1|	Nunnock River granitic landscape, Australia	96
Uncultured bacterium FAC78	gi|90992962|gb|DQ451517.1|	FAC78Environmental samples, Fushan Forest Soils of Taiwan	99
Kineococcus-like bacterium AS2978	gi|6073809|gb|AF060676.1|AF060676	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Kineosporiaceae, endemic to the Mojave Desert	97
F3-MI 5.1-F243			
Streptomyces spiroverticillatus	gi|94470230|gb|DQ487019.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces	99
C4-MI 5.1-F243			
Uncultured bacterium AH73	gi|66394045:1-1426	Isolated from soil	97
Unknown Actinomycete (MC 26)	emb|X68468.1|:1-1067	Genomic DNA	98
Clostridia indicus (IndiB4)	emb|X75788.1|:1-1515	RDP Query	-
E4-MI 5.1-F243			
Uncultured bacterium ARFS-35	emb|AJ277701.1|:1-1110	Flooded anoxic rice paddy soil	99
Acidothermus cellulolyticus 1315	emb|AJ007290.1|:1-1470	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Acidothermaceae; Acidothermus	96
Sporichthya polymorpha	gi|6009629|dbj|AB025317.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Sporichthyaceae; Sporichthya	95
H4-MI 5.1-F243			
Mycobacterium sp. DSM 44605	emb|AJ431371.1|:1-1481	Strain DSM 44605, Mycobacterium pyrenivorans, Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium	99
Mycobacterium tokaiense	gb|AF480590.1|:1-1451	Mycobacterium tokaiense, Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium	99
G9-MI 5.1-F243			
Frankiaceae bacterium KVD-unk-16	Gi|94995748:1-1487	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Frankiaceae. Volcanic deposits, Hawaii, Kilauea volcano	97

Appendix A10 continued

Query/ Relative	Accession number	Description	% Identity
Frankia sp. FE37	Gi|3201707|gb|AF063641.1|	Actinobacteria; Actinobacteridae; Actinomycetales; Frankineae; Frankiaceae; Frankia, frankia strains in root nodules of plants from the families elaeagnaceae and rhamnaceae, isolated from root nodules (FE37) of Coriaria arborea collected in New Zealand	96
Lactobacillus brevis ATCC 14687	gi|133711552|gb|EF120367.1|	OUTGROUP	

NB; E-value is zero for all sequences and query coverage at least 98%.

Appendix A11: Some of the actinomycetes isolated in this study

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