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ORIGINAL PAPER

The soil and plant determinants of community structures of the dominant actinobacteria in Marion Island terrestrial habitats, Sub-Antarctica

Tendai Walter Sanyika · William Stafford · Don A. Cowan

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Abstract Marion Island is a Sub-Antarctic island made up of distinct ecological habitats based on soil physiochemical, plant cover and physical characteristics. The microbial diversity and ecological determinants in this harsh Sub-Antarctic environment are largely uncharacterized. Actinobacteria have diverse ecological functions related to soil and plant functioning. This study was aimed at characterizing the diversity and community structures of the dominant actinobacteria in the distinct habitats and to identify their determinant soil and plant characteristics. Using the 16S rRNA gene, the denaturing gradient gel electrophoresis patterns and clone library diversity were correlated with the soil and plant characteristics. Multivariate statistical methods were also used to identify determinant soil and plant characteristics. Salinity and pH were the most important soil determinants, and a number of important site-specific plant species may have been important. The Coastal Fellfield Habitat was dominated by sequences of the suborders *Micrococcineae* (44%) and *Propionibacterineae* (18%), with salinity identified as the principal determinant. The Cotula Herbfield Habitat was dominated by *Frankineae* (37%) and *Streptosporangineae* (38%), which were correlated with organic nutrient concentrations. The Wet Mire Habitat was dominated by *Acidimicrobineae* (61%), with moisture and organic carbon content as principal components. Culture-dependent studies were complementary to culture-independent studies with the majority of actinobacteria isolated not identified in 16S rRNA gene clone libraries. This study demonstrates how the soil physiochemical characteristics and plant species independently determine the community structures of the dominant actinobacteria in distinct ecological habitats. These factors subsequently influence their ecological adaptation, roles and functions.

Keywords Marion Island · Soil · Plants · Actinobacterial community structure · 16S rRNA gene · DGGE

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Introduction

Marion Island (46°54′S, 37°57′E) is a small volcanic island (290 square km), located in the South African zone of sub-Antarctica, approximately 2,300 km south-east of Cape Town in the Atlantic Ocean (Chown and Froneman 2008). The climate is characterised by cold, wet and windy conditions (Chown and Froneman 2008) with a mean annual air temperature of between 5.0 and 6.5°C. The mean daily temperature ranges between 2 and 3°C, with annual rainfall of between 1,900 to 2,800 mm (Smith et al. 1993) and relative humidity averages of around 80% (Chown and Froneman 2008). North-westerly and west-north-westerly gale force winds blow at an average of 107 days per year

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(Smith 1987). The island is heavily vegetated, and extensive biological surveys have identified 23 distinct terrestrial habitats that have been classified into seven Habitat Complexes (Smith and Steenkamp 2001; Smith et al. 2001). The habitats have been subjected to minimal anthropogenic impact and are considerably pristine (Smith et al. 2001) allowing for a comparative study of habitats less influenced by human impact.

Nutrient recycling is the major limiting factor in the mineralization activities of plant litter and consequently to the production of primary energy (Smith 1988). As a result of the absence of macroherbivores in the trophic structure, most of the energy and nutrients are incorporated into the detritus cycle, which is dependent on the activities of soil bacteria and microinvertebrates (Smith 1988; Smith and Steenkamp 1992; Chown and Froneman 2008). The several microbiological studies on Marion Island have largely focused on nitrogen fixing organisms, particularly on cyanobacteria (Smith and Ashton 1981; Smith 1988). Other studies have focused on the isolation and enumeration of bacterial populations using culture-dependent techniques (French and Smith 1986) but were not primarily aimed at diversity studies or comparing community structures between the defined habitats. The soil physiochemical and plant cover characteristics on Marion Island terrestrial habitats have been well characterised (Smith and Steenkamp 2001; Smith et al. 2001) but their influence on the microbial community structures have not yet been described.

Amongst the bacteria, actinobacteria are the most important decomposers of complex material (Pankratov et al. 2006; Vorob'ev et al. 2007). Culture-dependent studies have shown that actinobacteria are amongst the majority of the microorganisms identified in some of the soils from Antarctic and sub-Antarctic terrestrial environments (Moncheva et al. 2000–2002; Kochkina et al. 2001, Smith et al. 2006). Similar results were also obtained using culture-independent studies (Bagatzevska 2000–2002; Brambilla et al. 2001; Shivaji et al. 2004; Smith et al. 2006; Pointing et al. 2009). The well-characterized habitats of Marion Island provide a model for comparative studies on the ecological determinants of actinobacterial diversity and community structures within a typically Sub-Antarctic environment. In this study, we have investigated the actinobacterial diversity of eleven Marion Island terrestrial habitats, undertaken a detailed phylogenetic analysis of three selected habitats that differed substantially in moisture contents, salinities and biotic indices, and linked the diversity to dominant soil physiochemical and plant species cover characteristics. We demonstrate that both soil- and plant-related parameters are important determinants of actinobacterial community structures. These determinants impact on the diversity, adaptation, ecological roles and functions of actinobacteria in these different habitats.

Materials and methods

Description of Marion Island terrestrial habitats soil sampling

Soil samples were collected in June 2005 from eleven Marion Island terrestrial habitats, described by Smith et al. 2001 (Table 1). For each habitat, six 50 g topsoil (1–10 cm deep) samples were aseptically collected, homogenously mixed, redistributed into sterile containers and stored at −80°C until required.

Extraction and purification of soil metagenomic DNA

Soil metagenomic DNA was extracted in triplicate using the FastDNA® SPIN kit (QBIOgene, BIO 101® Systems, Carlsbad, California), based on the bead-beating method, according to the manufacturer's instructions. The DNA extracts were further purified on PVPP in columns (Berthelet et al. 1996) and quality verified using 1% (w/v) agarose gels.

Isolation and culture-dependent cultivation

For each sample, a 1 g suspension of soil was added to 10 ml of sterile water, homogenized and serially diluted up to 10^5 times and 100 µl spread plated onto the respective isolation media in duplicate. All the culture media components were mixed, dissolved, and the pH adjusted as recommended or to 5.0, 5.5 using 5 N NaOH before autoclaving. The isolation media were supplemented with Cycloheximide (50 µg/ml) to inhibit fungal growth. Various media were used at each pH: Middlebrook (7H9) agar (Becton, Dickinson and Co. Sparks, MD, USA), Humic acid agar (HA) (Hayakawa and Nonomura 1987), Czapek (CZ) agar (Waksman 1957) and MC agar (Nonomura and Ohara 1971). The plates were incubated for up to 3 months at 16°C in sealed plastic bags and inspected weekly. For the propagation of purified subcultures, Yeast extract–Malt extract agar (YM) (Shirling and Gottlieb 1966) and modified Yeast peptone agar (Waksman 1957) [2 g of yeast extract, 3 g of malt extract, 3 g of bacteriological peptone, 10 g of Glucose and 15 g of agar adding up to 1 l in distilled water (pH 7.0)] were used. Pure cultures were subcultured from plates into YM broth (pH 7.0) and incubated at 16°C and 100–120 rpm for up to 6 weeks until growth was observed. The cells or mycelia were harvested by centrifuging at 1,200x*g* for 10 min, resuspended in fresh YM medium containing 25% (v/v) glycerol and stored at −80°C until required.

Table 1 The terrestrial ecological habitats of Marion Island used in this study according to (Smith and Steenkamp 2001)

Habitat type	Code	Location/description	Elevation (m)	S	E
Coastal salt–spray complex (Complex 1)					
Coastal herbfield	MI 1.1	Location: Archway, Shore-zone. Fibrous peat with very high Na and Mg. Vegetation dominated by dicots, *C. moschata* and *C. plumose*	22	46°53.892′	37°53.557′
Coastal fellfield	MI 1.2	Location: Archway, Shore-zone. Fibrous peat, volcanic ash with very high Na and Mg. Vegetation dominated by *Dicots, C. moschata* and *A. selago*	20	46°53.895′	37°53.560′
Slope complex (Complex 3)					
Mesic fernbrake	MI 3.3	Location: Archway. Occurs on wet, steep slopes. Deep, highly organic and wet peat with high organic C, low organic P. Vegetation is dominated by *B. penna-marina* and *A. Magellanica*	22	46°53.892′	37°53.557′
Biotic grassland complex (Complex 4)					
Pedestalled Tussock grassland	MI 4.3	Location: Archway, heavily influenced by seabirds and seals. Occurs on compact peat with pedestals, very wet organic mud with very high inorganic N and P. Vegetation dominated by *C. antarctica* and *M. fontana*	30	46°53.868′	37°53.485′
Biotic herbfield complex (Complex 5)					
Cotula herbfield	MI 5.1	Heavily influenced by seabirds and seals. Compact peat, high in total and inorganic forms of N and P and forms of Na and Mg. Vegetation dominated by *C. plumosa* and *P. cookie*	11	46°53.018′	37°52.151′
Biotic mud	MI 5.2	Location: Near Trypot beach, close proximity to seal wallows and penguin rookeries. Very wet, organic mud with vey high inorganic N and P. Vegetation dominated by *C. antarctica* and *M. fontana*	2	46°53.131′	37°52.092′
Biotic lawn	MI 5.3	Close proximity to seal wallows and penguin rookeries Thin fibrous peat which is less organic and drier than 5.2, with higher Ca and Mg levels. Vegetation dominated by *P. annua, P. cookii, C. plumosa, C. antarctica* and *M. fontana*	22	46°53.055′	37°52.044′
Mire complex (Complex 6)					
Dry mire	MI 6.1	Location: Ship's Cove, close proximity to seal wallows and penguin rookeries. Dry oligotrophic peat, drier and less organic with higher Ca and Mg than other mires. Vegetation dominated by *A. magellanica, B. penna-marina* and bryophytes	34	46°52.661′	37°51.567′
Mesic mire	MI 6.2	Location: Ship's Cove. Boggy grassland with wet, organic, dystrophic peat. Vegetation: dominated by Graminoids and bryophytes	64	46°51.532′	37°50.664′
Wet mire	MI 6.3	Location: Ship's Cove. Waterlogged bog with very wet, organic, waterlogged peat. Vegetation: dominated by bryophytes	91 m	46°51.616′	37°50.818′
Mire drainage	MI 6.4	Location: Ship's Cove. A bog in water tracks, on waterlogged peat with very low inorganic P, highest pH for mires. Vegetation dominated by bryophytes	46	46°51.975′	37°51.037′
Biotic mire	MI 6.5	Location: Lake Edge near Swartzkop and influenced by seal and seabird manure. Very wet peat with the highest recorded inorganic N and P for mires. Vegetation dominated by *C. vermicularis, A. magellanica* and *P. cookii*	148	46°55.665′	37°35.665′

Extraction of genomic DNA from bacterial cultures

Genomic DNA extraction from the purified bacterial cultures was performed according to Sambrook et al. (1989).

PCR amplification of 16S rRNA genes

For all PCR reactions, the standard 50 µl PCR reaction solution contained approximately 100 ng DNA template, 0.5 µM of each primer, 200 µM of each dNTP (dATP, dTTP, dCTP, dGTP), 1× PCR buffer (100 mM Tris–HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)SO_4$, 0.1% (w/v) Triton X-100, 2 mM $MgCl_2$) and 1U/µl DNA Taq polymerase. For the universal primers 16S-F1 and 16S-R5 (Table S1), PCR thermo-cycling conditions were according to Weisberg et al. (1991). For the primers E9F (Farrelly et al. 1995) and U1510R (Reysenbach and Pace 1995), the PCR involved an initial denaturation at 94°C for 4 min followed by 25 three-temperature cycles of denaturation at 92°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1.5 min. The final extension was at 72°C for 5 min. Actinobacteria were amplified using the primers S-C-Act-878-a-S-19 and S-C-Act-235-a-S-20 according to (Stach et al. 2003a).

Nested PCR-denaturing gradient gel electrophoresis (PCR-DGGE)

For each triplicate sample, the primary PCR products were used as a template for the nested DGGE-PCR with primers 341F-GC and 534R and separated on a 45–80% urea-formamide denaturing gradient according to Muyzer et al. (1993).

Construction of 16S rRNA gene clone libraries and colony PCR

The PCR products were purified from agarose gels using the GFX PCR DNA and Gel Band purification Kit (Amersham Biosciences) according to the manufacturer's instructions. Ligation reactions were performed using the pTZ57R/T vector (Fermentas) according to manufacturer's instructions and transformed into chemical competent *E. coli* × L1-Blu cells according to the Inoue Transformation method (Sambrook et al. 1989). Colony PCR was used to identify the correct recombinant clones using the universal M13 primers according to Yanisch-Perron et al. (1985).

DGGE screening and dereplication of 16S rRNA gene clone libraries

The M13 PCR products were subjected to nested DGGE-PCR and separated using a 45–80% urea-formamide denaturing gradient according to Muyzer et al. 1993. The different 16S rRNA gene polymorphs identified were used as references to further de-replicate each library.

Sequencing and phylogenetic analysis

DNA sequencing of plasmid DNA was carried out using the 3130 Genetic Analyser and Big Dye terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and BIOLINE Half Dye Mix, based on the Sanger method. The chromatograms were manually checked using the BioEdit software (Hall 1999) and full length sequences assembled. The program CHECK-CHIMERA was used to inspect the sequences for inverted tandem repeats. The amplified DNA sequences were identified through homology searches using BLAST (Altschul et al. 1990) against the NCBI non-redundant database (http://www.ncbi.nlm.nih.gov/) and classified at 80% confidence using the RDP Classifier of the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) Release 10 (Cole et al. 2009). For phylogenetic analysis, the closest matches were retrieved from non-redundant databases and aligned against the sequences from this study using the Greengenes (http://greengenes.lbl.gov) Nearest Alignment Space Tool (NAST). Alignments were manually corrected using BioEdit software. Entire gaps were manually removed and the alignments were trimmed. The alignments were subjected to cluster analysis using *Mega4* (Tamura et al. 2007) and used to generate a phylogenetic tree. The 16 s rRNA gene sequences from known cultured species were used as positive controls. The trees were based on 560 sites using the Maximum Composite Likelihood method and substitution model using Neighbour-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 (Tamura et al. 2004).

Statistical analyses

The AlphaEase FC image processing and analysis software (AlphaInnotech Corporation™ San Leandro, CA) was used for band matching and analysis of DGGE-PCR community profiles. The resulting nominal data were transformed into a presence–absence matrix, and pair-wise comparisons were conducted using the Jaccard coefficient. The formula $Sj = {}_n(A + B)/[{}_nA + {}_nB - {}_n(A + B)]$ was used, where ${}_nA$ and ${}_nB$ are the total numbers of bands in tracks A and B, respectively, and ${}_n(A + B)$ the number of bands common to tracks A and B. Unless otherwise stated, the software Primer V6 (Clarke and Warwick 2001) was used for statistical analyses. Hierarchical clustering and multidimensional scaling (MDS) were based on a similarity matrix generated from standardized and transformed data. The Bray-Curtis index was used for biological data and the

Euclidean distance for environmental data. Hierarchical clustering was conducted using the Unpaired Group Mean Weighted Average (UPGMA, average) pairing method. Multidimensional scaling ordinations were based on 10 iterations, and cluster overlays were based on cluster analysis. Correlation analysis was based on Primer v6 inbuilt functions, BIOENV/BVSTEP (Biota-Environment matching). SIMPER (Similarity/distance Percentages, Species/variable Contributions) analysis was used to measure the ranked correlations and dissimilarities between samples, within and between clusters of samples, based on the squared deviation of averages. For these analyses, variables were first standardized and transformed and normalized for SIMPER analysis. Absolute and presence–absence data of plant species cover were both used. The SIMPER function was used to identify ranked correlations contributing up to a total of 90%. The Multivariate Statistical Package 3.1 (Kovach Computing Services, United Kingdom) was used for cluster and Canonical Correspondence Analysis (CCA). The Kaiser's rule was used to identify the number of axes to extract (Legendre and Legendre 1983), using a square root transformed data matrix, with rare species down-weighted. Eigen analysis was based on the Hill reciprocal averaging algorithm, and the eigenvalue scores were used for the conjoint ordination bi-plots The CCA scores were used for the bi-plot ordination plots.

Accession numbers

The sequence data from this study have been submitted to the GeneBank database under accession numbers FN548 140–FN550803, FN548040–FN548075 and FN548002–FN548039 for 16S rRNA gene library clones and FN55 0112–FN550151 for actinomycete isolates.

Results

Distribution of actinobacterial diversity: DGGE profiles

Eleven samples were analysed using three independent environmental DNA extractions and subsequent DGGE analysis (Fig. S1). The intra-sample replication of duplicate DGGE profiles (Fig. 1) was characterised by high Jaccard correlation coefficients, ranging between 0.83 and 1.00. Three clusters and two habitats with distinct actinobacterial diversity profiles and therefore community structure compositions were identified (Fig. 2).

Determinants of actinobacterial diversity profiles

Based on contributions of the squared deviation of average (SIMPER) soil physiochemical characteristics (Table S2)

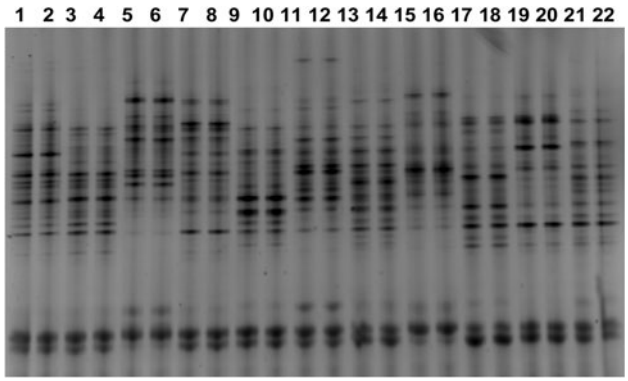

Fig. 1 DGGE analysis of actinobacterial diversity profiles (on 9% polyacrylamide with 30–80% urea-formamide denaturing gradient) in selected Marion Island terrestrial habitats. *Lanes* duplicate samples from Marion Island terrestrial habitats MI 5.3 (*1–2*); MI 5.1 (*3–4*); MI 1.2 (*5–6*); MI 6.5 (*7–8*); MI 6.2 (*9–10*); MI 5.2 (*11–12*); MI 3.3 (*13–14*); MI 1.1 (*15–16*); MI 6.4 (*17–18*); MI 6.3 (*19–20*) and MI 4.3 (*21–22*)

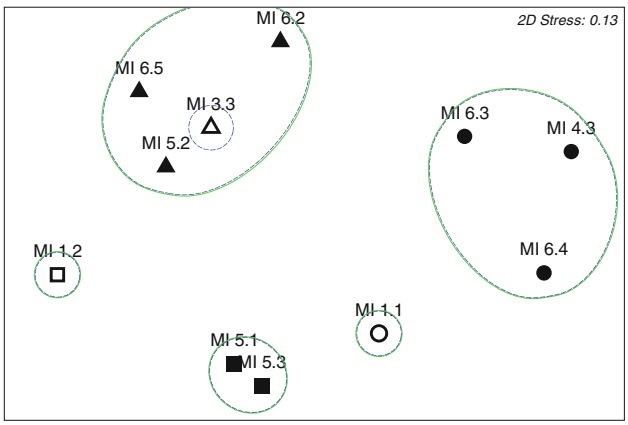

Fig. 2 MDS showing the clustering of habitats based on at least 40 and 42% (*inner circles*) similarity in actinobacterial diversity profiles. Distance between points shows the relative difference between the diversity profiles. *Identical symbols* show habitats that cluster together at 40% identity threshold. Only habitat MI 3.3 appears to have different actinobacterial diversity at 42% threshold

to dissimilarities in actinobacterial diversity profiles using DGGE, the Salt–Spray complex habitats (habitats MI 1.1 and MI 1.2) were most distinct (Table 2). Their soils were characterised by high salt concentrations, and salinity was also the most distinguishing characteristic in these habitats. The analysis of plant cover characteristics (Table S3) using SIMPER was not very informative. Using correlation analysis (BIOENV and BVSTEP) of DGGE profiles, the overall distribution of actinobacteria best correlated with percentage cover of Lichen, Tussock Graminoid, *Poa Annua*, Deciduous Shrub, Mire Graminoid and Bryum/Breutelia amongst the fourteen plant species (Table 3). The soil pH best correlated with actinobacterial diversity profiles or soil pH in combination with organic carbon, bulk density and a variety of salts.

Table 2 The absolute values of soil physiochemical characteristics defining clusters of habitats with similar actinobacterial diversity profiles (presence–absence data) based on SIMPER analysis

Variable	Absolute range of variables			
	Cluster of habitats MI 4.3, MI 6.3 & MI 6.4	Cluster of habitats MI 5.1 & MI 5.3	Cluster of habitats MI 5.2, MI 6.2 & MI 6.5	Cluster of habitats MI 1.2 & MI 1.2
Solution Ca solution	96–119	–	–	–
Solution Na solution	–	–	667–984	–
Solution Mg solution	–	–	71–88	–
Solution Ca solution	–	–	75–85	–
Solution K solution	–	–	Variable	661–687
Total K	0.5–0.7	–	–	1.8–1.8
Total Mg	0.8–2.6	4.3–5.6	1.3–2.1	13.6–15.0
Total Na	1.2–1.4	–	1.3–1.9	14.6–15.0
Total Ca	3–7.6	11.6–19.9	4.0–5.1	49.3–50.0
Exchangeable Na	0.9–1.8	2.6–5.3	1.9–3.0	20.1–22.1
Exchangeable Ca	7.0–9.0	–	5.5–9.1	–
Exchangeable Mg	–	8.9–12	7.7–10.8	–
Exchangeable K	–	–	Variable	–
Ammonium nitrogen	8.8–36.4	–	Variable	8.4–11.0
Nitrite	0.3–0.5	0.5–0.7	Variable	0.4–0.4.0
Nitrate	0.6–5.5	8.7–10.9	Variable	0.4–2.8
Total N	–	2.90–2.90	Variable	1.5–1.7
Total P	Variable	Variable	–	–
Organic P	Variable	259–268	–	55.5–66.5
Organic C	Variable	–	36.7–39.9	–
Bulk density	Variable	143–144	63–81	237–252
Moisture	Variable	658–679	–	–
pH	Variable	–	4.7–5.1	–
Cation exchange capacity	–	Variable	–	78.9–79.6

Each of the characteristics presented as a range contributed at most 1% to the dissimilarity amongst the habitats based on the squared deviation of averages. Moderately dissimilar (up to 5% dissimilarity) soil characteristics are not described (–) and most variable characteristics are indicated. The percentage plant cover characteristics were not informative enough to describe the clusters identified using SIMPER

Phylogenetic positioning of the dominant actinobacterial diversity

A more detailed analysis was performed to identify the different soil- and plant-related factors that influence community structures of the dominant actinobacteria. Three distinctly clustered habitats, Coastal Fellfield Habitat (MI 1.2), Cotula Herbfield (MI 5.1) and the Wet Mire Habitat (MI 6.3), were selected for the analysis based on diversity profiles. A total of 294 sequences represented by 110 phylotypes were identified as actinobacteria, contributing 98% of the entire 16S rRNA gene clones sampled from the three environmental libraries. The non-actinobacterial sequences belonged to the suborders *Gemmatimonadetes*, *Gammaptroteobacteria* and *Chloroflexi (Sphaerobacterales)*.

Habitat MI 1.2 was dominated by members of the suborder *Micrococcineae* (45% of phylotypes, 44% of sequences) (Fig. 3; Table S4). Most of these belonged to the genus *Arthrobacter* and the family *Intrasporangiaceae*. The *Propionibacterineae* accounted for 18% of the phylotypes in this habitat (18% of phylotypes, 17% of sequences).

In Habitat MI 5.1, most of the classified phylotypes belonged to the suborder *Frankineae* (28% of phylotypes, 37% of sequences) and *Streptosporangineae* (31% of phylotypes, 38% sequences). *The Streptosporangineae* were mostly of the family *Thermomonosporaceae*. The suborder *Acidimicrobineae* was also represented amongst the majority of the phylotypes (19% of phylotypes, 14% of sequences) especially the genera *Ferrithrix* and *Iamia*. The *Acidimicrobineae* also dominated the classified phylotypes in Habitat MI 6.3 (53% of phylotypes, 61% of sequences) with only one sequence assigned to the genus *Iamia*.

A number of sequences could not be classified at the genus level. The majority of these were 42% of the *Acidimicrobineae* in Habitat MI 6.3, 25% of the

Table 3 BIOENV and BVSTEP ranked correlations of combinations of soil and plant variables with (1) dominant actinobacterial community structure profiles on DGGE from eleven Marion Island terrestrial habitats and (2) suborder level classification of actinobacterial phylotypes from three habitats based on 16S rRNA libraries

Best variable combinations	Corr.	Significance level (%)
Soil variables and eleven DGGE profiles		
pH, Ca exch	0.32	44
pH, OC		
pH, BD		
pH, Mg sln		
pH (Best overall)	0.37	45
% plant cover and eleven DGGE profiles		
MB, L, TG, DS, BB	0.40	36
MB, L, TG, BB		
MB, L, TG, DS		
MB, TG, DS, BB	0.40	36
MB, L, TG, DS, BB (Best overall)	0.40	37
Soil variables and three 16S rRNA libraries		
CEC	1.00	100
Mg exch		
Na exch		
Mg sln/K sln		
CEC (Best overall)	1.00	68
% cover and three 16S rRNA libraries		
CD, CB	1.00	85
CD, Pt		
CD, RD		
TG, RD		
CD, CB (Best overall)	1.00	83

MB (Mire Bryophyte), L (Lichen), TG (Tussock Graminoid), DS (Deciduous Shrub), BB (Bryum/Breutelia), RD (Rosette Dicot), CB (Cushion Bryophyte), MG (Mire Graminoid), DS (Deciduous Shrub), PA (*Poa Annua*), Ca exch (exchangeable Ca), OC (organic carbon), BD (bulk density), Mg sln (solution Mg), K sln (solution K), CEC (cation exchange capacity), Mg exch (exchangeable Mg), Na exch (exchangeable Na)

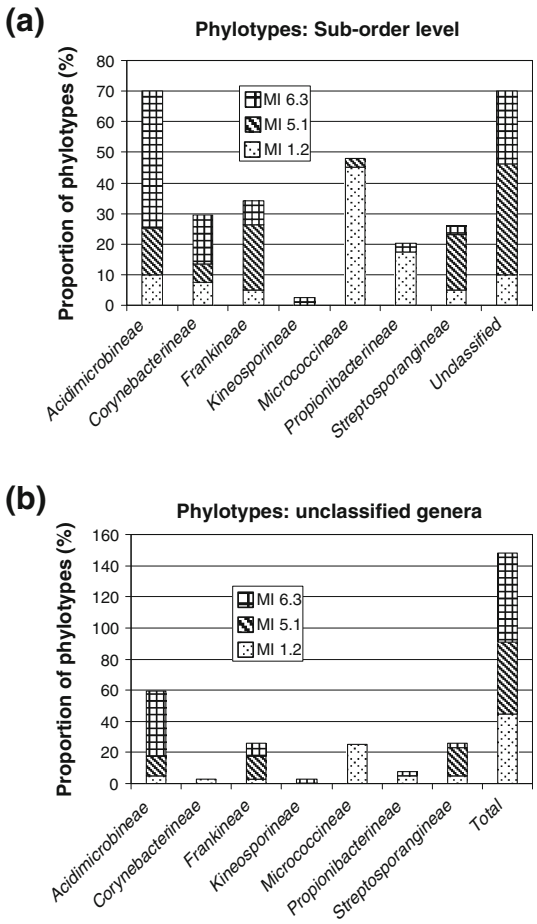

Fig. 3 The distribution of actinobacterial phylotypes in three Marion Island terrestrial habitats using the RDP classifier at 80% confidence interval (bootstraps): **a** suborder level classification, **b** proportion of phylotypes unclassified at genus level amongst the sequences classified to suborder level. A total of 294 actinobacterial clones (109 clones from MI 1.2; 93 clones from MI 5.1 and 92 clones from MI 6.3) represented by 110 phylotypes (40 phylotypes MI 1.2; 32 phylotypes MI 5.1 and 38 phylotypes MI 6.3) were identified

Micrococcineae in Habitat MI 1.2 and 18% of the *Streptosporangineae*, 15% of the *Frankineae* and 12% of the *Acidimicrobineae* in Habitat MI 5.1 (Fig. 3b).

Approximately twenty actinobacterial isolates from this study could be novel based on phylogenetic distance and position with reference to cultured representatives (Fig. S2). Most isolates of the suborder *Streptomycineae* were isolated from Habitat MI 5.1. Isolates MI-5.1 P101, MI-5.1 P202a, MI-5.1 P202b and MI-1.2 P104 were identified as unclassified using the RDP Classifier and had sequence identities ranging between 90.2 and 95.5% to the *S. scabrisporus*. Other sequences related to *S. monomycini,* the genus *Kisatasatospora* and unclassified environmental clones (Fig. 4, Fig. S2). A number of sequences of the suborder *Frankineae*, originating from all three habitats, clustered with environmental clones from Antarctica and anaerobic habitats, especially soil environments. Amongst cultured representatives, the clades were represented by *Acidothermus cellulolyticus, Solicoccus flavidus* and *Blastococcus saxobsidens*. One of the clades contained clones, specifically originating from Habitat MI 5.1, which clustered with *Sporichthya polymorpha.* The other clade contained clones from Habitat MI 6.3 that clustered with *Quadrisphaera granulorum* and a Namibian dolomite marbel *Frankiaceae* isolate. A deeply rooted clade containing unclassified environmental clones originating from lake environments clustered with one sequence, D2-MI 6.3.

Sequences of the suborder *Micrococcineae* clustered with cultured representatives of the genera *Terrabacter,*

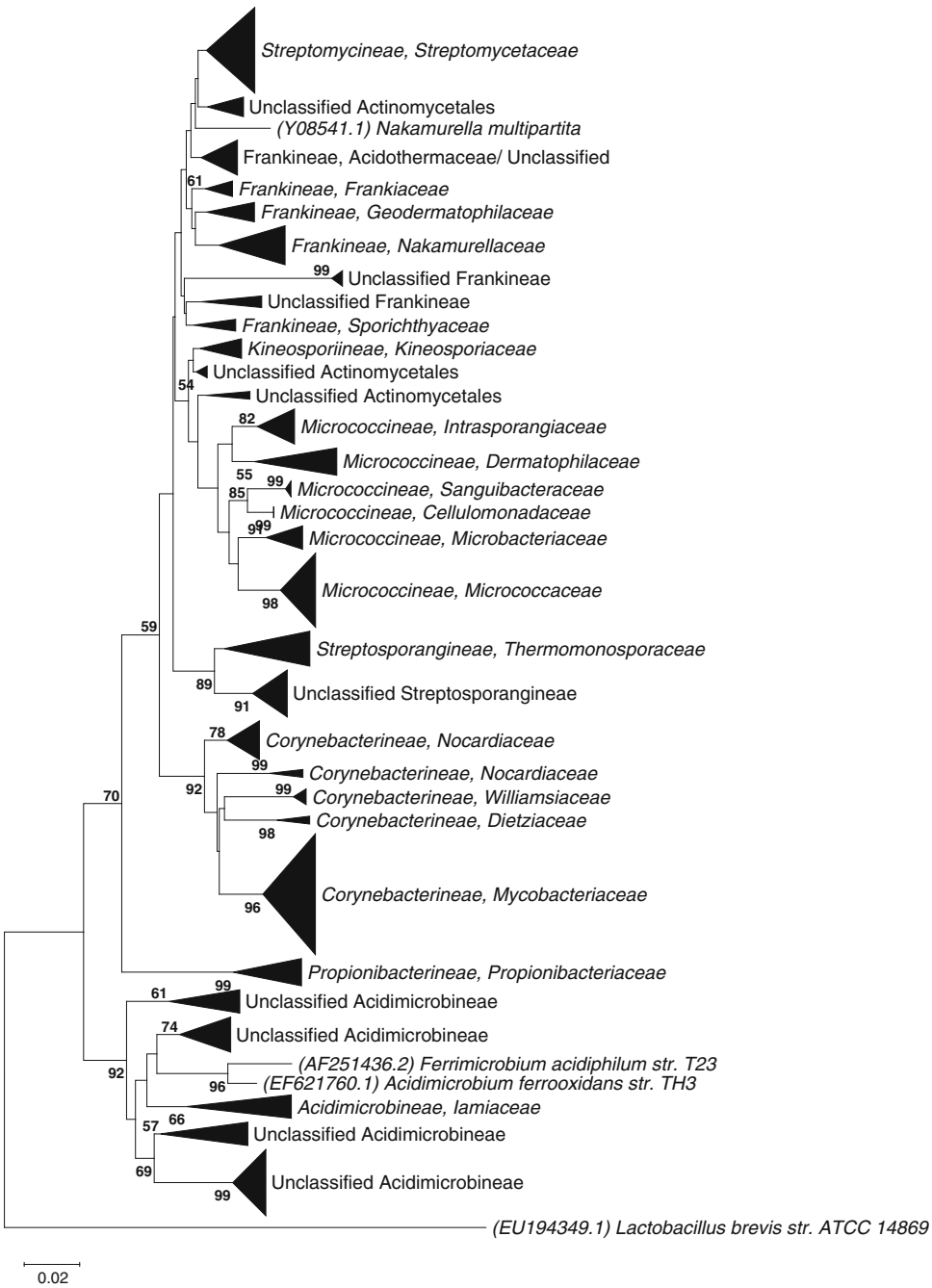

Fig. 4 Overview of the phylogenetic placement of actinobacterial isolates and phylotypes from three Marion Island terrestrial habitats. A detailed phylogenetic tree is provided in supplementary material (Fig. S2). *Scale* shows the number of base substitutions per site

Dermatophilus, Sanguibacter, Cellulomonas, Curtobacterium, Agreia, Microbacterium and *Arthrobacter*. Most of these sequences related to Antarctic derived clones, whilst one novel clade, distantly related to A*rthrobacter,* clustered with a house mattress dust clone. All sequences of the suborder *Streptosporanginea*, except one clone, were novel and uncultured. They were predominantly from Habitat MI 5.1 and clustered with an alpine tundra soil clone. All phylotypes of the suborder *Propionibacterineae* originated from Habitat MI 1.2 and clustered with *Tessaracoccus bendigoensis* and an Antarctic lake ice cover clone. Amongst the phylotypes of the suborder *Corynebacterineae* was a clade containing sequences were represented by *Rhodococcus fascians* and *Rhodococcus coprophilus,* which clustered with a glacial ice bacterium. Other sequences clustered with *Rhodococcus pyridinivorans, Williamsia maris, Dietzia natronolimnaea* and various species of the genus *Mycobacterium.* Sequences of the genus *Mycobacterium* clustered with environmental clones from psychrotrophic habitats such as Antarctica and the Indian Himalayas glacier. Another clade contained *Mycobacterium* clones originating from water bodies including a

reservoir and a river receiving penicillin G production wastewater. The clones of *Acidimicrobiales* were all uncultured and related to *Ferrimicrobium acidiphilum, Acidimicrobium ferrooxidans* and *Iamibacter majanohamensis*. Most of these clones, the majority originating from Habitat MI 6.3, clustered with environmental clones indicative of anaerobic environments, including aspen rhizosphere, forest soil and no tillage soil. Other clones were from acid mine drainage, mining waste material, spring water and a dune field.

Determinants of phylogenetic diversity in three habitats

With all the actinobacterial phylotypes from 16S rRNA gene clone libraries classified using phylogenetic analysis (Table S4), the actinobacterial community structures were dominated by *Micrococcineae* and *Propionibacterineae* in Habitat MI 1.2, *Streptosporangineae* and *Frankineae* in Habitat MI 5.1 and *Acidimicrobidae* in Habitat MI 6.3. At suborder level, the actinobacterial diversity was best explained by the correlation with cation exchange capacity amongst exchangeable magnesium, exchangeable sodium, solution magnesium and solution potassium (Table 3). The best overall determinant of actinobacterial community structure based on DGGE profiles was soil pH. Other non-salinity determinants were bulk density, moisture, ammonium nitrogen, organic carbon and organic phosphate, which were in combination with any of the salts mentioned. A combination of Cushion Dicot with Cushion Bryophyte gave the best correlation for cover of plant species amongst the combinations of Cushion Dicot with Pteridophyte, Cushion Dicot with Rosette Dicot and Tussock Gramminoid with Rosette Dicot (Table 3). Using SIMPER analysis, the actinobacterial community structure of Habitat MI 1.2 was affected by higher mineral salt concentrations compared with MI 5.1 and MI 6.3 and associated with the Cushion Bryophyte, Pteridophyte, Erect Dicot and Rosette Dicot.

Using canonical correspondence analysis (CCA), the specific soil and plant characteristics that correlated with the distribution of the actinobacteria at suborder level were identified (Fig. 5). The distribution of *Micrococcineae* and *Propionibacterineae* in Habitat MI 1.2 correlated with soil salinity, particularly the solution and exchangeable forms of calcium, potassium and magnesium, solution sodium, total sodium and total potassium. The determinant plant species were Cushion Dicot and erect Dicot. The *Frankineae* and *Streptosporangineae* in Habitat MI 5.1 were correlated with total phosphate, organic phosphate, nitrite, nitrate and ammonium nitrogen concentrations. The determinant plant species were Rosette Dicot, Mat Dicot and Tussock Gramminoid. In Habitat MI 6.3, the *Acidimicrobineae* and *Corynebacterineae* were correlated with the soil physical characteristics, particularly organic carbon content, moisture and cation exchangeable capacity. Total plant cover, Mire Gramminoid and Mire Bryophyte were the important plant determinants in this habitat. Amongst 23 soil variables, bulk density, pH, organic carbon, nitrogenous compounds, phosphates and various salts were best correlated with the overall actinobacterial community structure across all the 16S rRNA gene clone libraries (Table 3).

Discussion

This study represents the first analysis of culture-independent studies on diversity of the dominant actinobacteria and their community structures in the distinct, well-characterised Marion Island terrestrial habitats. We established that the Marion island terrestrial habitats, like most Antarctic and Sub-Antarctic environments, contain uncultured and previously unknown bacterial sequences. Some of these sequences have only been identified from Antarctic and Sub-Antarctic environmental samples. DGGE is an important analytical technique for microbial diversity (Muyzer et al. 1993; Fromin et al. 2002) but has a tendency to detect mostly the abundant phylotypes (Kowalchuk et al. 2006; Nakatsu 2007). The diversity described in this study therefore represents the dominant phylotypes. The presence of actinobacterial phylotypes restricted to specific habitats suggests they had specialized ecological functions and were possibly adapted to site specific conditions. In this study, culture-dependent and culture-independent studies were complementary as there was only one potential match between isolates and clone library sequences, represented by a single phylotype.

Culture-dependant techniques do reveal the presence of most bacteria. It is estimated that more than 99% of the total microorganisms unculturable or are uncultured using the standard laboratory techniques (Amann et al. 1995). This is because of lack of appropriate isolation techniques (Amann et al. 1995; Keller and Zengler 2004). Culturing can be improved by using a number of methods including dilution of media to almost extinction or using oligotrophic media (Button et al. 1993; Connon and Giovannoni 2002). Simulating the natural environments in artificial chambers (Kaeberlein et al. 2002; Keller and Zengler 2004), use of filter traps (Gavrish et al. 2008) and application of suitable selective pressure can also improve culturability. Membrane filtration (Hirsch and Christensen 1983) and differential centrifugation (Hopkins et al. 1991; Yamamura et al. 2003; Maldonado et al. 2005) can be used to concentrate actinomycetes from environmental samples. In addition, selective recovery using methods such as the rehydration and centrifugation improves the isolation of zoospore-forming actinomycetes (Hayakawa et al. 2000).

Similar to the findings from this study, Babalola et al. (2008) reported that S*treptomyces* formed the majority of

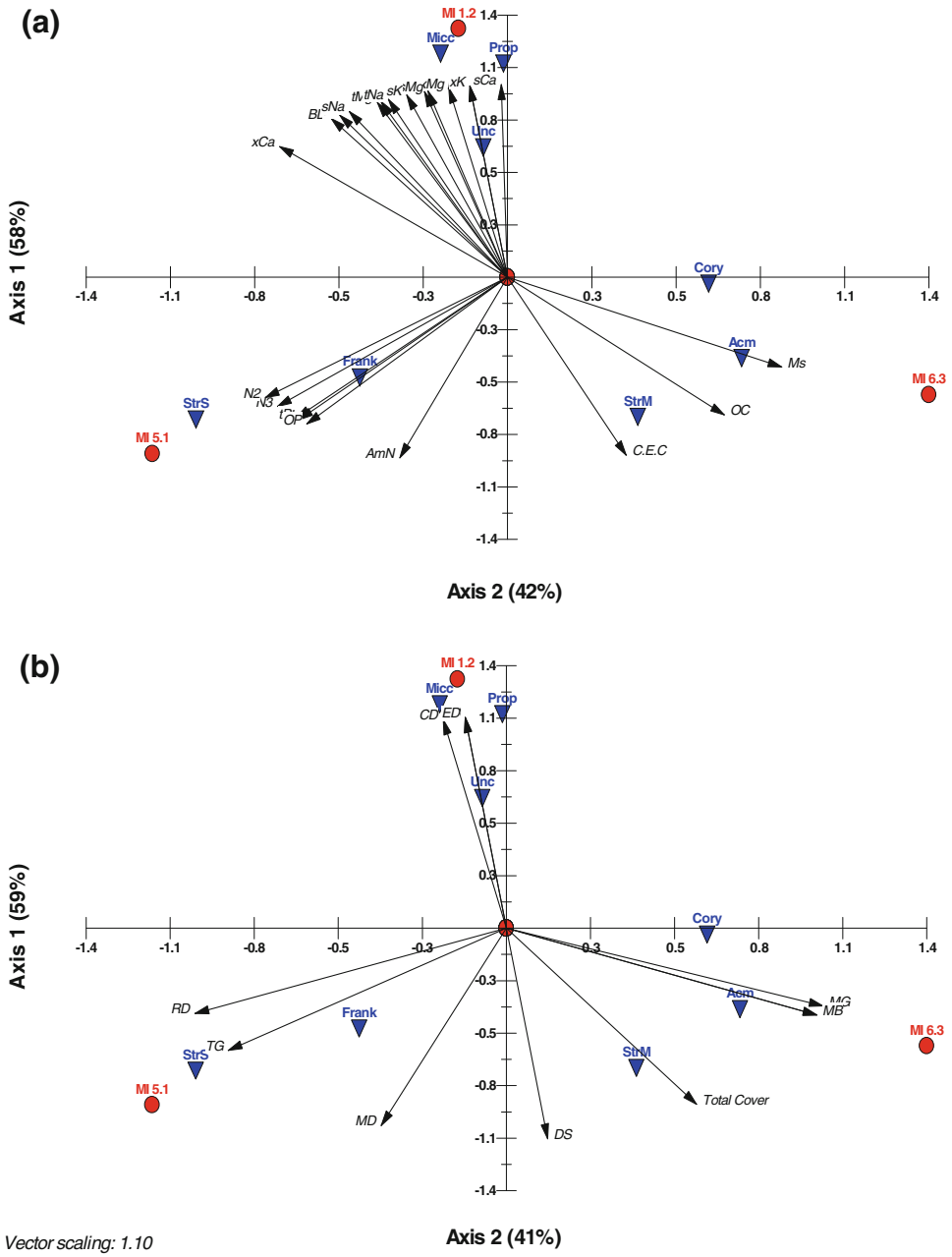

Fig. 5 Canonical correspondence analysis ordination plots correlating **a** the soil physiochemical with **b** the plant cover characteristics from three Marion Island terrestrial habitats (MI 1.2, MI 5.1 and MI 6.3) with the distribution of actinobacterial sequences classified at suborder level. Key: Plant characteristics—ED (Erect Dicot), DS (Deciduous Shrub), magnesium (Mire Graminoid), CD (Cushion Dicot), BM (Brachythecium Moss), RD (Rosette Dicot), TG (Tussock Graminoid), MD (Mat Dicot) and MB(Mire Bryophyte). Soil characteristics: BD (bulk density), pH, Ms (moisture), OC (organic C), tN (total N), amN (ammonium nitrogen), N3 (nitrate), N2 (nitrite), tP (total phosphate), OP (organic phosphate), tCa (total Ca), tMg (total magnesium), tK (total K), tNa (total Na), C.E.C (cation exchangeable capacity), xCa (exchangeable Ca), xMg (exchangeable magnesium), xK (exchangeable K), xNa (exchangeable Na), sCa (solution Ca), sMg (solution magnesium), sK (solution K) and sNa (solution Na). Actinobacterial suborders: Micc (*Micrococcineae*), Prop (*Propionibacterineae*), Unc (unclassified bacteria), Cory (*Corynebacterineae*), Acm (*Acidimicrobidae*), StrM (*Streptomycineae*), Frank (*Frankineae*) and StrS (*Streptomycineae*). The *circles* represent the cases (habitats) and inverted triangles, the variables (actinobacterial suborders). *Arrows* represent the direction and strength of the variables in relation to habitat shown as data points. Distance of *arrows* from the axis centre shows extent of variability based on PCA scores (eigenvalues)

the cultured isolates but were not frequently identified from Sub-Antarctic soil 16S rRNA gene clone libraries. Ancient actinobacteria that have been cultured from Antarctic permafrost soils were thought to have survived the cold conditions as spore or mycelia forms (Kochkina et al. 2001). This suggests that the numbers of actinobacteria

reported from 16S rRNA environmental libraries using culture-independent approaches may be underrepresented. We used combination of chemical or enzymatic lysis accompanied by mechanical methods as recommended for the most efficient lysis of such soil microorganisms (Frostegård et al. 1999). It is most likely the culture conditions were more favourable for spore germination than cell lysis was for DNA extraction. In contrast to culture-independent approaches, culture-independent approaches are based on universal primers that are designed to target known sequences, which make them biased against unknown groups (Amann et al. 1995; Forney et al. 2004). Primer selectivity results in the underrepresentation of some genotypes from environmental samples (Baker et al. 2003). This is supported by the isolation of the actinobacterial cultures that could not be identified using the 16S rRNA gene environmental clone libraries. Even though the actinobacterial-specific 16S rRNA gene primers used in this study were shown to cover more actinobacterial diversity (Stach et al. 2003a, b), members of the suborders *Actinomycineae, Micromonosporineae, Pseudonocardineae* or *Glycomycineae* were not detected in this study.

The important determinants of microbiological community structure, distribution and diversity include soil physiochemical properties (Jaspers and Overmann 2004; Fierer and Jackson 2006) and plant species (Grayston et al. 1998; Bergsma-Vlami et al. 2005). As shown in previous studies (Miethling et al. 2000; Marschner et al. 2001; Garbeva et al. 2004; Berg and Smalla 2009), soil characteristics seem to be the most important drivers of rhizosphere community structure. It has been shown that plant species, genotype and growth stage can also have large impacts on rhizosphere communities (Germida et al. 1998; Grayston et al. 1998; Kowalchuk et al. 2002). In another study, pH was identified as the major determinant of bacterial distribution in a wide variety of habitats (Fierer and Jackson 2006). Similarly, the carbon content has also been identified as a major factor that limits the growth (Demoling et al. 2007) and diversity of bacteria (Bossio and Scow 1995).

The factors that affect the composition of the dominant bacterial community structures seem to differ from those that affect actinobacteria. The community structure of dominant bacteria in Livingston Island (Antarctica), for example, was most affected by total carbon and total nitrogen contents and moisture but not pH (Ganzert et al. 2011). Previous studies have shown that the organic and inorganic amendments of soil may not significantly alter actinobacterial phylogenetic diversity but community structure (Piao et al. 2008). Organic matter and pH were found to control actinobacterial community structure in soils with different physiochemical characteristics due to land use practices (Hill et al. 2011). In our study, pH was also the best overall determinant of actinobacterial community structure. Habitats MI 1.1 and MI 1.2 were distinguished by high salinity indices, also a major determinant of actinobacterial community structure. Indeed, salinity has been identified as the most important factor that influences diversity amongst ecologically diverse microbiological communities (Lozupone and Knight 2007) and lake habitats (Jiang et al. 2010).

The findings of our study are similar to those of Jiang et al. (2010) in which salinity was identified as the most important determinant of actinobacterial diversity and community structure in five lakes on the Tibetan Plateau. These were typically psychrotrophic environments with permafrost conditions (water temperature range, 10–17°C). In their study, the *Micrococcineae* were the dominant group of actinobacterial community structure, accounting for between 80 and 100% of the diversity in four of the habitats (Jiang et al. 2010).

Conclusions

In this study, we have shown that the Marion Island terrestrial habitats differ in phylogenetic diversity of the dominant actinobacteria. We identified the soil- and plant-related factors that determined the dominant actinobacterial community structures in different ecological habitats. Culture-independent studies revealed the presence of mostly uncultured groups and were complemented by the isolation of a number of possibly novel actinobacteria. Smith and Steenkamp (2001) showed that the hypersaline, Salt–spray Complex habitats, MI 1.1 and MI 1.2, had the most distinct soil and plant characteristics. In our study, pH and salinity were the most important determinants of the dominant actinobacterial diversity and community structures. Soil salinity may therefore be an important factor that determines the actinobacterial community structures and life forms in Marion Island terrestrial habitats. Further studies are required in order to gain objective insights into the ecological functions of these phylogenetic groups in Marion Island habitats.

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