Research Article

Actinobacteria from Greenwich Island and Dee Island: Isolation, diversity and distribution

Pek Lim Chu¹, Chai Hoon Khoo¹, Yoke Kqueen Cheah^{1*}¹ Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia.<https://doi.org/10.28916/lsmb.1.1.2017.2>

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Abstract

Actinobacteria from underexplored and unusual environments have gained significant attention for their capability in producing novel bioactive molecules of diverse chemical entities. *Streptomyces* is the most prolific Actinobacteria in producing useful molecules. Rapid decline effectiveness of existing antibiotics in the treatment of infections are caused by the emergence of multidrug-resistant pathogens. Intensive efforts are urgently required in isolating non-*Streptomyces* or rare Actinobacteria and understanding of their distribution in the harsh environment for new drug discovery. In this study, pretreatment of soil samples with 1.5% phenol was used for the selective isolation of Actinobacteria from Dee Island and Greenwich Island. A high number of non-*Streptomyces* (69.4%) or rare Actinobacteria was significantly recovered despite the *Streptomyces* (30.6%), including the genera *Micromonospora*, *Micrococcus*, *Kocuria*, *Dermacoccus*, *Brachybacterium*, *Brevibacterium*, *Rhodococcus*, *Microbacterium* and *Rothia*. Reduced diversity and shift of distribution were observed at the elevated level of soil pH. The members of genera *Streptomyces*, *Micromonospora* and *Micrococcus* were found to distribute and tolerate to a relatively high pH level of soil (pH 9.4-9.5), and could potentially be alkaliphilic Actinobacteria. The phylogenetic analysis had revealed some potentially new taxa members of the genera *Micromonospora*, *Micrococcus* and *Rhodococcus*. Principal Component Analysis of soil samples was used to uncover the factors that underlie the diversity of culturable Actinobacteria. Water availability in soil was examined as the principal factor that shaped the diversity of the Actinobacteria, by providing a dynamic source for microbial interactions and elevated diversity of Actinobacteria.

Keywords: Rare Actinobacteria, distribution, Greenwich Island, Dee Island, Antarctica

1.0 Introduction

Actinobacteria is one of the major and dominant soil inhabitants. Presently, the phylum Actinobacteria comprised of more than 300 genera, representing one of the largest phyla within the domain Bacteria (Gao and Gupta 2012). Furthermore, the members of this phylum, especially the genus *Streptomyces* is the most studied group and also recognized as the most prolific producer of bioactive secondary metabolites with medicinal values, such as anti-tumor, anti-cancer, anti-infection properties (Berdy 2005; Hong et al. 2009). Rare actinobacteria are always referred to as strains that are difficult to isolate, maintain and might correspond to the unmatched source of new natural metabolites (Baltz, 2006). These rare Actinobacteria are contributing to the huge fraction of the commercialised antimicrobial agents. With the objective of discovering rare or novel species of Actinobacteria, selective isolation of Actinobacteria from the environmental samples has been started since few decades ago (Pisano et al. 1986; Pisano et al. 1989; Hayakawa et al. 1997;) and still being practiced extensively in the present (Zakharova et al. 2003; Hayakawa et al. 2004; Hong et al. 2009; Azman et al. 2015).

Antarctica is a pristine region on Earth that serves as a crucial barometer of global climate change. The human contact with this polar continent is less than two centuries of time. Antarctic continent is well known for extreme environmental conditions. These including the surroundings of extremely dryness and cold temperature, strong gusting wind, high UV radiation, continuous cycles of freeze/thawing, limited nutrient accessibility (Onofri et al. 2007; Margesin and Miteva 2011; Jadoon et al. 2013). The driest climate and coldest temperature had attributed to the ice formation that covers most of the lands and lakes of Antarctic continent. About 0.32% is ice-free region and is found to be the soil-forming area on this continent (Ugolini and Bockheim 2008). Microbes are the most abundant life that colonises the Antarctic ecosystems (Wynn-Williams 1996). Since last decades, more attention had received from researchers worldwide to study on the biodiversity of microbes and discovery of novel microbes from Antarctic continent (Nichols et al. 1999; Smith et al. 2006; Teixeira et al. 2010; Li et al. 2011; Muñoz et al. 2011; Gesheva and Negiota, 2012; Duarte et al. 2013; Lee et al. 2013). A general postulate is that the amplified impact of abiotic factors on microbial diversity is expected in the harsh environment of Antarctica, works to drive the community structure and composition (Barrett et al., 2006; Hogg et al., 2006).

* Correspondence: Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia.

e-Mail: ykcheah@upm.edu.my

The phylogenetic marker, 16S ribosomal RNA (rRNA) gene was used to define the species, in characterizing the spatial patterns of microbial distribution. Metagenomics analysis had revealed a high level of bacterial diversity in the Antarctic soils and the dominant phyla of bacteria were Actinobacteria, Proteobacteria and Firmicutes, and a large number of anaerobic bacteria (Teixeira et al. 2010). Actinobacteria was reported to be one of the common components in the Antarctic soil of Lake Wellman area, Darwin Mountains, with three subphyla and 15 genera (Aislabie et al. 2013). This finding also indicates that Antarctic environments presented as a natural reservoir that harbour large number of potentially new and yet-to-be-cultured microbes. Hence, Antarctica has now emerged as a new hope in isolating rare or novel Actinobacteria, enhanced by the elevated environmental stressors in Antarctic continent. The diversity of the difficult-to-culture or rare Actinobacteria influenced by the by abiotic environmental gradients will gain our insight on their survival strategies in colonizing the harsh Antarctic conditions.

Greenwich Island lies between Robert Island and Livingstone Island in the South Shetland Islands (Ivanov et al. 2009). While Dee Island is an ice-free island located between Greenwich Island and Aitcho Island. The presence and diversity of Actinobacteria population on these lands was investigated using selective isolation approach. The factor that is underlying the distribution of these Actinobacteria was also revealed. This study described the diversity and distribution of *Streptomyces* and rare Actinobacteria from Greenwich Island and Dee Island, Antarctica in relation to the abiotic environmental gradients.

2.0 Materials and methods

2.1 Environmental sampling

Soil samples were collected during the XI Ecuadorian Antarctic Expedition to the Research Station “Pedro Vicente Maldonado”, Greenwich Island, South Shetland Islands in 2007 as shown in Figure. 1 (coordinates: 62°24' 18.7"S to 62°24' 32.4"S and 59°44' 13.2"W to 59°45'39.3"W). Top soil samples, the upper 20 cm layer were collected after removing the top 2-3 cm layer, from five different sites within Greenwich Island. Soils were collected into sterile plastic bags by using an aseptic metal trowel and kept in dark for transport to Malaysia. Upon arrival to the research lab, the soils were kept at -20°C. 15 soil samples collected from five different locations at South Shetland Island, Antarctica, were used in this study. The specific sampling sites were: Dee Island (62°25'39.3"S, 59°47'54.3"W); Ambato Point (62°26'30.5"S, 59°39'34.1"W to 62°28'41.4"S, 59°47'22.4"W); Arturo Prat (62°26'53.1"S, 59°39'47.4"W to 62°28'45.7"S, 59°43'41.0"W); Pevima Lake (62°26'51.5"S, 59°43'33.8"W to 62°26'55.1"S, 59°43'38.4"W) and Greenwich Island (62°26'59.5"S, 59°43'53.3"W to 62°27'02.0"S, 59°44'12.1"W). Soil pH was measured by a 1:5 sample-to-deionized water slurry ratio and recording pH using a pH electrode (Hanna, USA). Soil temperature was measured using a digital thermometer (Hanna, USA). The UV-A and UV-B radiation were measured using a radiometer (UVP, UK).

2.2 Selective isolation of Actinobacteria from soils

One gram of each soil sample was air dried for 7 days. The air-dried soils were ground with mortar pestle into fine particles. Selective isolation of Actinobacteria was performed by 1.5% phenol pretreatment of soil samples at 30°C for 30 min (Pisano et al. 1986). The pretreated soils samples were serially diluted up to 10⁻⁴ with sterile 25% Ringer's solution. One hundred microliters of each diluted suspension (10⁻¹, 10⁻², 10⁻³ and 10⁻⁴) was spread onto different selective isolation media. Each diluted soil suspension was spread onto five different isolation media: ISP media 2 (Shirling and Gottlieb 1966), starch casein agar (SCA; Küster and Williams 1964), humic acid-vitamin agar (HVA; Hayakawa and Ohara 1987), *Streptomyces* agar (SA; Atlas, 1993) and Actinomycetes isolation agar (AIA; Atlas 1993). All of the media were supplemented with nalidixic acid (20 mg/L), nystatin (50 mg/L) and cycloheximide (50 mg/L) (Williams and Davies 1965). The isolation plates were incubated at 28°C for one up to eight weeks. Purified bacterial isolates were maintained on ISP medium 2 agar slants at room temperature and in 20% glycerol at -20°C.

2.3 Genomic DNA extraction from pure cultures

Genomic DNA extraction was performed by using a protocol adapted from the Current Protocols in Molecular Biology with some modifications (Wilson 2001). In brief, bacterial broth culture was spun to collect cell pellet. The cell pellet was lysed with Solution I ((50mM glucose; 25mM Tris-HCl; 10mM EDTA; pH 8.0), SDS buffer and proteinase K, and treated with RNase. DNA was extracted from cell lysate through phenol/chloroform/isoamyl alcohol (25:24:1) purification. The aqueous layer obtained was added with sodium acetate to remove histone protein bound to DNA. Finally, the DNA was collected by ethanol precipitation and stored at -20°C.

2.4 23S rRNA PCR amplification and screening for Actinobacteria

PCR amplification of the large insert stretch in 23S rRNA gene that was specific for Actinobacteria was performed. It was carried out in Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). Degenerated primers were used: 23S-f 5'-CCGANAGGCGTAGBCGATGG-3' and 23S-r 5'-CCWGWGTYGGTTTVSGGTA-3' (Gao and Gupta 2005). The final 30 µl PCR reaction mixture contained: 20-200 ng bacteria genomic DNA, 1X PCR buffer with 2 mM MgCl₂, 0.67 mM dNTPs mixture, 0.33 µM of each primer and 1U of Taq polymerase (Genet Bio, Korea). The thermal profile was: pre-denaturation at 97°C for 5 min; followed by 30 cycles of: denaturation at 97°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min; and a final extension at 72°C for 15 min.

Figure 1: Map of the sampling sites, (a) The Antarctic continent with the Antarctic Peninsula in relief. The box indicated the location of Greenwich Island and Dee Island in South Shetland Islands and (b) Greenwich Island and Dee Island. (Adapted from Ivanov LL, 2009).

2.5 16S rRNA PCR amplification of Actinobacteria isolates

From the 23S rRNA gene amplification profile, bacterial isolates with the expected size of products were subsequently selected for 16S rRNA amplification. The PCR primer pair EuBac 27F and EuBac 1492R (Lane 1991) were used to perform PCR amplification of 16S rRNA gene by using the Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). The final PCR reaction mixture consisted: 20-200 ng bacteria genomic DNA, 1X PCR buffer with 2 mM MgCl₂, 0.67 mM dNTPs mixture, 0.33 μM of each primer and 1U of Taq polymerase (Genet Bio, Korea). The thermal profile was: pre-denaturation at

95°C for 5 min; followed by 30 cycles of: denaturation at 95°C for 50 s, annealing at 52°C for 1 min, extension at 72°C for 1.5 min; and a final extension at 72°C for 10 min.

2.6 DNA sequencing of amplified 16S rRNA gene sequences of Actinobacteria isolates and phylogenetic analysis

The PCR products of 16S rRNA gene amplification were purified by the MEGAquick-spin™ PCR and agarose gel DNA extraction kit, using protocols provided by the manufacturer (iNtRON Biotechnology Inc). The purified PCR products were sequenced using BigDye® Terminator Cycle Sequencing Chemistry 3.1 technique and a Genetic Analyzer ABI Prism 3100 (Applied Biosystem). The forward and reverse sequences of 16S rRNA of each Actinobacteria isolates were assembled manually to form an almost complete 16S rRNA gene sequence. The almost complete 16S rRNA gene sequences were subjected to BLASTN program to search for closest identities. The corresponding sequences of the representatives of the relevant genera were retrieved from GenBank/EMBL/DDBJ databases (NCBI, USA). Phylogenetic trees were constructed using MEGA version 6.0 (Tamura et al. 2013) by neighbour-joining method (Saitou and Nei 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and the bootstrap value were calculated by 1000 replicates (Felsenstein 1985). The calculation of pairwise 16S rRNA gene sequence similarities were performed using the EzTaxon-e server (<http://eztaxon-e.ezbiocloud.net/>) (Kim et al. 2012).

2.7 Nucleotide sequence accession numbers

The 16S rRNA gene sequences of Actinobacteria isolates in this study were deposited in the NCBI Genbank database, under accession numbers of KJ743996-KJ744031.

2.8 Statistical analysis

Principal component analysis (PCA) was performed on the relative abundance and diversity of Actinobacteria isolated from each soil samples at genera level, using XLSTAT software version 2014.1.09 (Paris, France MacOS ed). The effect of the presence of water activity, skua area and characteristics of soil were investigated based on the biplot created and examined for the factors that drive the diversity and distribution of Actinobacteria on Greenwich Island and Dee Island.

3.0 Results

3.1 Selective isolation of Actinobacteria

A total of 50 isolates of bacteria were isolated from 15 soil samples collected from South Shetland Island, Antarctica. These isolates were discriminated to be Actinobacteria and other bacteria using the 23S rRNA PCR amplification profiling. The specificity of the 23S rRNA primers was validated by DNA sequencing of representative isolates from the group of non-actinobacteria in 23S rRNA profile. Through the 23S rRNA profiling, 72% (36 out of 50) of the isolates were Actinobacteria, and the remaining 28% (14 out of 50) was of non-Actinobacteria (Table 1).

3.2 Culturable Actinobacteria

Around 40% of the Actinobacteria isolates were visible during the first week of incubation while most of the Actinobacteria isolates were only visible during or after the fourth week of incubation. The numbers of culturable Actinobacteria isolated from each soil sample was found to be higher than the non-Actinobacteria, except for the soil samples labeled as 62, 71 and 96, and there were no culturable isolates of bacteria from the soil samples label as 63, 77 and 103 (Table 1). Low number or absence of culturable bacteria from the soil samples could due to 1) absent of phenol-resistant bacteria, 2) inappropriate culture medium to recover the phenol-resistant bacteria. The highest number of Actinobacteria isolates was recorded on the soil sample labeled as 111, which was collected from Greenwich Island.

Actinobacteria isolates with the colony morphologies characteristic belong to the genus *Streptomyces* were obtained from each sampling site except Dee Island. The phenotypic characteristic included the formation of powdery and chalky aerial mycelium. Besides, orange-pigmented colonies without aerial mycelium formation were also obtained from three sampling sites, which belong to the genus *Micromonospora*. Additional features found on these colonies were blackened colony surface due to the presence of spores (Bredholdt et al. 2007; Becerril-Espinosa et al. 2013).

3.3 Diversity of culturable Actinobacteria

In total, 36 Actinobacteria isolates were recovered from 15 soil samples collected at South Shetland Island. Based on the 16S rRNA gene sequences, the rare Actinobacteria isolates were distributed in the suborders of Micrococceinae, Streptomycineae, Micromonosporineae and Corynebacterineae. The most abundant genus was *Streptomyces* (30.6%). Abundance of non-*Streptomyces* genera were also identified, including the genera *Micrococcus* (22.2%), *Kocuria* (16.7%), *Micromonospora* (8.3%), *Brachybacterium* (5.5%), *Dermaococcus* (5.5%), *Brevibacterium* (2.8%), *Rhodococcus* (2.8%), *Microbacterium* (2.8%) and *Rothia* (2.8%) (Tables 2, 3).

Streptomyces species were recovered from all of the sampling sites except for Dee Island (Table 2). The high species abundance was followed by *Micrococcus* after *Streptomyces*, which were identified from all five sampling sites in this study. In the present work, the HVA was the most efficient isolation medium in recovering Actinobacteria from soil samples, by recovering 8 out of 10 genera of Actinobacteria identified (Table 3).

Table 1: Physical and chemical properties of soils and numbers of Actinobacteria and bacteria isolates from 15 soil samples

Sampling site	Soil reference	Soil temperature (°C)	pH	Solar radiation (mV/cm ²)		Numbers of isolates		Total
				UVA (20)	UVB (20)	Actinobacteria	Other bacteria	
Dee Island (62°25'39.3"S, 59°47'54.3"W)	53	10.2	8.7	5.83	6.84	2	1	3
Ambato Point (62°26'30.5"S, 59°39'34.1"W 62°28'41.4"S, 59°47'22.4"W)	62	16.1	9.5	3.57	4.82	1	3	11
	63	11.8	9.4	4.12	6.81	-	-	
	to 64	9.5	9.5	4.08	2.4	5	2	
Arturo Prat (62°26'53.1"S, 59°39'47.4"W 62°28'45.7"S, 59°43'41.0"W)	68	7.3	8.4	5.69	16.1	3	-	9
	70	7.3	8.2	4.71	4.54	3	-	
	to 71	9	8.2	5.12	4.2	1	2	
Pevima Lake (62°26'51.5"S, 59°43'33.8"W 62°26'55.1"S, 59°43'38.4"W)	96	4.3	7.8	0.65	0.53	-	2	14
	97	9.5	7.7	0.56	0.42	3	-	
	to 98	8.9	7.5	0.58	0.42	5	1	
	100	8.9	7.6	0.94	1.08	3	-	
Greenwich Island (62°26'59.5"S, 59°43'53.3"W 62°27'02.0"S, 59°44'12.1"W)	76	2.9	7.6	11.01	1.32	3	1	13
	77	2.6	7.6	1.61	0.01	-	-	
	to 103	5	7.5	3.52	4.86	-	-	
	111	4.8	7.4	3.46	4.63	7	2	
Total						36	14	50

The influence of soil alkalinity on the distribution of Actinobacteria species was studied. As shown in Figure. 2, 9 out of 10 genera of Actinobacteria identified in this study were successfully recovered from soils of slightly alkaline (pH 7.4-7.8), accounted for the highest diversity of soil Actinobacteria. While for the soils of moderately alkaline (pH 7.9-8.4), five Actinobacteria genera were isolated. The least count of Actinobacteria genera (three) was obtained from soils of highly alkaline (pH ≥ 8.5). The members of *Kocuria* were found in the soil of slightly and moderately alkaline. While the members of *Rothia* were only recovered from the soil of moderately alkaline. Interestingly, the members of *Streptomyces*, *Micrococcus* and *Micromonospora* were found in the soils of all three types of alkalinity.

Table 2 List of Actinobacteria isolates in this study

Sampling site	Isolate (accession no.)	Closest relative	Similarity (%)
Dee Island	53S4a (KJ743996)	<i>Micrococcus yunnanensis</i> YIM 65004 ^T (FJ214355)	99.78
	53H3a (KJ743997)	<i>Micrococcus yunnanensis</i> YIM 65004 ^T (FJ214355)	99.71
Ambato Point	62SC2a (KJ743998)	<i>Micrococcus yunnanensis</i> YIM 65004 ^T (FJ214355)	99.93
	64A3a (KJ743999)	<i>Micrococcus yunnanensis</i> YIM 65004 ^T (FJ214355)	99.57
	64H3b (KJ744000)	<i>Micromonospora aurantiaca</i> ATCC 27029 ^T (CP002162)	99.93
	64H3c (KJ744001)	<i>Streptomyces somaliensis</i> NBRC 12916 ^T (AB184243)	100
	64H3d (KJ744002)	<i>Streptomyces somaliensis</i> NBRC 12916 ^T (AB184243)	100
	64H3e (KJ744003)	<i>Streptomyces abidoiflavus</i> DSM 40455 ^T (Z76676)	100
Arturo Prat	68A3b (KJ744004)	<i>Kocuria marina</i> KMM 3905 ^T (AY211385)	99.79
	68I3c (KJ744005)	<i>Rothia amarae</i> JCM 11375 ^T (AY043359)	99.79
	68S4a (KJ744006)	<i>Micrococcus yunnanensis</i> YIM 65004 ^T (FJ214355)	99.71
	70H1b (KJ744007)	<i>Streptomyces albidoflavus</i> DSM 40455 ^T (Z76676)	100
	70H1c (KJ744008)	<i>Streptomyces albidoflavus</i> DSM 40455 ^T (Z76676)	100
	70H1d (KJ744009)	<i>Streptomyces albidoflavus</i> DSM 40455 ^T (Z76676)	100
	71H2b (KJ744010)	<i>Micromonospora aurantiaca</i> ATCC 27029 ^T (CP002162)	99.71
Pevima Lake	97H2a (KJ744011)	<i>Kocuria marina</i> KMM 3905 ^T (AY211385)	99.78
	97H2b (KJ744012)	<i>Brevibacterium ptyocampae</i> DSM 21720 ^T (EU484189)	99.26
	97H2c (KJ744013)	<i>Kocuria marina</i> KMM 3905 ^T (AY211385)	99.79
	98S1a (KJ744014)	<i>Kocuria rhizophila</i> DSM 11926 ^T (Y16264)	100
	98S1b (KJ744015)	<i>Brachybacterium paraconglomeratum</i> LMG 19861 ^T (AJ415377)	99.64
	98H2a (KJ744016)	<i>Micrococcus yunnanensis</i> YIM 65004 ^T (FJ214355)	99.93
	98H4a (KJ744017)	<i>Streptomyces rutgersensis</i> NBRC 12819 ^T (AB184170)	100
	98H4c (KJ744018)	<i>Streptomyces rutgersensis</i> NBRC 12819 ^T (AB184170)	100
	100S2a (KJ744019)	<i>Dermacoccus nishinomiyaensis</i> DSM 20448 ^T (X87757)	99.71
	100S4a (KJ744020)	<i>Kocuria palustris</i> DSM 11925 ^T (Y16263)	99.93
	100S4b (KJ744021)	<i>Kocuria palustris</i> DSM 11925 ^T (Y16263)	100
Greenwich Island	76H2a (KJ744022)	<i>Dermacoccus nishinomiyaensis</i> DSM 20448 ^T (X87757)	99.78
	76SC3a (KJ744023)	<i>Micrococcus yunnanensis</i>	99.71

76A3b (KJ744024)	YIM 65004 ^T (FJ214355) <i>Micrococcus yunnanensis</i>	99.86
111S1a (KJ744025)	YIM 65004 ^T (FJ214355) <i>Brachybacterium nesterenkovi</i> DSM 9573 ^T (X91033)	99.64
111H2a (KJ744026)	<i>Micromonospora eburnea</i> LK2-10 ^T (AB107231)	98.83
111H2c (KJ744027)	<i>Rhodococcus yunnanensis</i> YIM 70056 ^T (AY602219)	99.13
111H3b (KJ744028)	<i>Microbacterium saccharophilum</i> K-1 ^T (AB736273)	99.42
111H3g (KJ744029)	<i>Streptomyces albidoflavus</i> DSM 40455 ^T (Z76676)	100
111H3h (KJ744030)	<i>Streptomyces albidoflavus</i> DSM 40455 ^T (Z76676)	100
111H3I (KJ744031)	<i>Streptomyces albidoflavus</i> DSM 40455 ^T (Z76676)	100

Table 3: Taxa of Actinobacteria recovered on five different isolation media

Culture media	Families	Genera	Number of isolates
ISP2 agar	<i>Micrococcaceae</i>	<i>Rothia</i>	1
Starch casein agar	<i>Micrococcaceae</i>	<i>Micrococcus</i>	2
Actinomycetes isolation agar	<i>Micrococcaceae</i>	<i>Micrococcus</i>	2
		<i>Kocuria</i>	1
<i>Streptomyces</i> agar	<i>Micrococcaceae</i>	<i>Micrococcus</i>	2
		<i>Kocuria</i>	3
	<i>Dermacoccaceae</i>	<i>Dermacoccus</i>	1
	<i>Dermabacteraceae</i>	<i>Brachybacterium</i>	2
Humic acid-vitamin agar	<i>Streptomycetaceae</i>	<i>Streptomyces</i>	11
	<i>Micromonosporaceae</i>	<i>Micromonospora</i>	3
	<i>Micrococcaceae</i>	<i>Micrococcus</i>	2
		<i>Kocuria</i>	2
	<i>Brevibacteriaceae</i>	<i>Brevibacterium</i>	1
	<i>Dermacoccaceae</i>	<i>Dermacoccus</i>	1
	<i>Nocardiaceae</i>	<i>Rhodococcus</i>	1
	<i>Microbacteriaceae</i>	<i>Microbacterium</i>	1

Figure. 2 Percentage of total recovery of genera of Actinobacteria from soils of different alkalinity. Abundance of each genus recovered from different alkalinity was showed in each bar strip

Phylogenetic trees were constructed based on 16S rRNA sequences to investigate the evolutionary relationships between the Actinobacteria isolates in this study and their closest taxa members. *Streptomyces* isolates from Ambato Point, Arturo Prat and Greenwich Island were forming a distinct subclade with the type strains of *Streptomyces somaliensis* and *Streptomyces albidoflavus* (Figure. 3a), delineated from other *Streptomyces* species. The *Streptomyces* isolates from Pevima Lake were forming a cluster that was represented by *Streptomyces rutgersensis*. Other than that, putative new species of non-*Streptomyces* were identified in this study. All of the isolates of *Micrococcus* were found to be closely related to *Micrococcus yunnanensis* and shared 16S rRNA gene similarity ranging from 99.5-99.93% (Figure. 3b). Interestingly, three isolates, namely 64A3a, 62SC2a and 76A3b, formed a distinct cluster out of the subclade of *M. yunnanensis* that comprised of all other isolates of *Micrococcus*, which could represent new taxa of *M. yunnanensis*. A putative new species of *Micromonospora* were identified, namely, isolate 111H2a, by sharing <99% of 16S rRNA gene similarity with *Micromonospora eburnea*, and formed a phyletic lineage out from the subclade of *M. eburnea* (Figure 3c). A *Rhodococcus* isolate with no mycelial growth was closely related to *Rhodococcus yunnanensis* by sharing 99.13% 16S rRNA gene similarity. However, it formed a phyletic lineage out from the subclade represented by type strain of *Rhodococcus yunnanensis* in the phylogenetic tree (Figure 3d), representing a putative new taxon. By sharing 99.78-99.79%, 99.71-99.93% and 100% 16S rRNA gene similarities, the isolates were clustered within the subclades represented by *Kocuria marina*, *Kocuria palustris* and *Kocuria rhizophila* respectively (Figure 3e). The isolates of the genera *Brachybacterium*, *Dermacoccus*, *Brevibacterium*, *Microbacterium* and *Rothia* were clustered together with their closest relatives (Figure 3f, 3g, 3h, 3i and 3j).

Figure 3: Neighbour-joining tree based on partial 16S rRNA gene sequences showing phylogenetic relationships between culturable rare Actinobacteria isolates and their relative type strains, (a) *Streptomyces*, (b) *Micrococcus*, (c) *Micromonospora*, (d) *Rhodococcus*, (e) *Kocuria*, (f) *Brachybacterium*, (g) *Dermaococcus*, (h) *Brevibacterium*, (i) *Microbacterium* and (j) *Rothia*.

3.3 Principal component analysis (PCA)

In order to investigate the factor underlying the distribution of Actinobacteria from different biogeographical areas at Greenwich Island and Dee Island, PCA was performed according to the total species abundance data at the genus level. The result of PCA performed on soil samples based on actinobacterial community composition was presented in Figure 4. The first principal component explained 41.36% of the total variance, and the second principal component explained 17.95% of the total variance. By representing 59.32% of the total variance, a two-dimensional scores plot was presented. From the observation, the soil samples were distributed uncorrelated to the geographical locations. When the presence of water activity examined as the explanatory variable, the soil samples of Pevima Lake showed the highest degree of

variation of the actinobacterial community. The soil sample 97 (of the middle of the lake) was found to be distantly clustered with the other soil samples of lakeside. Furthermore, the variation of actinobacterial community underlie by the presence of water activity was also observed on the soils from Arturo Prat, in which the actinobacterial community in the soil sample with water activity (soil sample 68) was distantly related to the soil samples without water activity (soil samples 70 and 71). Although the water activity is absent in the soil sample 111, it was highly deviated from the cluster of all other soil samples and comprising of the most divergent actinobacterial community.

Figure 4: Ordination biplot generated by principal component analysis (PCA) of actinobacterial communities at genus level.

4.0 Discussion

Actinobacteria are widely distributed in the Antarctic environments (Smith 2006; Babalola et al. 2009; Foong et al. 2010; Ganzert et al. 2011; Pearce et al. 2012; Fan et al. 2013; Teo and Wong 2014). In the previous studies, the research of biodiversity on Greenwich Island was on mesophilic and psychrotrophic microfungi and most recently on the tellurite-resistant bacteria (Arenas et al. 2014). The main objective of this study was to isolate the pretreated Actinobacteria that were distributed in the soils of Greenwich Island and Dee Island.

As demonstrated by Pisano and co-workers, the pretreatment of environmental samples with phenol solution had greatly reduced the contamination of bacteria and fungi, in turn, favours development of slow-growing Actinobacteria. On the other hand, the percentage of recovery of Actinobacteria was increased by the inclusion of nystatin and cycloheximide to inhibit the growth of fungi, and nalidixic acid to eliminate the growth of non-mycelial bacteria. The success of selective isolation of Actinobacteria from soils will provide a more precise and significant enumeration of this soil microflora, and more importantly, capable of representing the composite picture of the population of rare Actinobacteria in the soil ecological studies. Then only after the well-established isolation techniques and enumeration of Actinobacteria from the environmental samples, numerous unknown taxa could be uncovered and thus improve our understanding of the diversity and distribution of Actinobacteria in Antarctic environment.

In the effort of isolating Actinobacteria from Greenwich Island, HVA was the most efficient isolation medium and capable of recovering the highest diversity of *Streptomyces* and rare Actinobacteria. In contrast with a study demonstrated by Li et al. (2012), HAV was found to be the least efficient isolation medium for the isolation of endophytic actinobacteria from environmental plant samples. The differences in between suggested selection of culture media is an important criterion for the isolation tasks with different environmental

samples. In this study, 62% of the Actinobacteria isolates were recovered from HVA. Interestingly, all of the *Streptomyces* and *Micromonospora* isolates were only recovered on HVA. This finding could relate to the natural ecological role of Actinobacteria in recycling and decomposition of organic materials in soils, and their prevalence distribution in humus-rich soils (Goodfellow and Williams 1983; Ventura et al. 2007). Furthermore, Actinobacteria have been shown to utilize humic acid, a biological decomposition-resistant polymer, as the carbon and nitrogen source (Hayakawa 2008). HVA alone or with the supplement of antibiotics were shown to be efficient in the selective isolation of specific genera of Actinobacteria from various physical and chemical pretreatment of soil samples. This is due to the advantage of HVA offers proper growth and good sporulation of Actinobacteria in laboratory conditions.

The pH value is one of the key chemical parameters that shaped the distribution of microbes in soil (Lee 1991). Previous studies have shown that pH was affecting the community structure and diversity of bacteria in acidic soil (Lauber et al. 2009; Chu et al. 2010). Results from the present work on alkaline soils indicate the highest diversity of actinobacterial taxa was observed in the soils of slightly alkaline (pH 7.4-7.8). This finding is in accordance with the finding reported by Basilio et al. (2003), in which Actinobacteria were most prevalent in neutral and slightly alkaline conditions. The complexity of genera of Actinobacteria was reduced significantly as the alkalinity of soil increased to the extreme level. However, some isolates of the genera *Streptomyces*, *Micrococcus* and *Micromonospora* were viable and well-adapted to the extreme pH level (up to pH 9.5). Some of the members of these genera were previously found to be viable in various extreme alkaline environments and reported as alkaliphiles that produce novel biological substances (Jiang and Xu 1996; Vasavada et al. 2006; Mitsui et al. 2010; Bull 2011). Although *Micrococcus* was reported as one of the readily isolatable taxa from the Antarctic soils (Cameron et al., 1972), this study presented the first isolation of *M. yunnanensis* which is tolerant at the pH 9.5, above the tolerance pH range (pH 6-8) reported previously (Zhao et al., 2009). This success of colonisation in high pH could probably due to the present of mechanisms in maintaining cytoplasmic pH (Padan et al. 2005). The employment of alkaline pH homeostasis is essential for alkaliphilic bacteria to have their proteins function optimally while sustaining the integrity of the structure. Recently, novel species of *Streptomyces* was isolated from saline-alkaline soils and were identified as alkaliphiles (Jiang et al. 2011; Zhang et al. 2013). This indicates the care selection of soil samples with elevated stressors of chemical parameters (such as pH) will increase the novelty of isolation of Actinobacteria from targeted environments (Bull 2011). The nature characteristics of the soil, in this case, the pH level, had selected for different bacterial species which dominate the bacterial communities. Further work needs to be conducted to investigate the roles of these Actinobacteria in the soil microbial communities. This present work, therefore, provided a baseline platform for the selective isolation of Actinobacteria from Greenwich Island and Dee Island.

The present work was supported by the findings of other researchers that *Streptomyces* species was one of the most abundant, wide distributed and dominant Actinobacteria in the Antarctic soils (Saul et al. 2005; Pan et al. 2013). The high frequency of *Micrococcus* species isolated from total soil samples implied the well-adaptation in the harsh cold environment and played a significant role in the native niche of bacterial communities. Tiwari and Gupta (2012) had reviewed on the bioactive compounds reported from different genera of rare Actinomycetes isolated from various natural habitats and conclude that many of the successful antimicrobial agents currently available on the market are produced by rare Actinomycetes. The effort of isolating rare Actinobacteria is now gaining more attention and successful isolations of numerous new taxa of Actinobacteria from Antarctic were reported, such as new species of *Nocardiopsis*, *Micromonospora*, *Streptomyces* and new the genera *Friedmanniella* and *Barrientosimonas* (Yassin et al. 1993; Schumann et al. 1997; Hirsch et al. 2004; Le Roes-Hill et al. 2009; Li et al. 2011; Lee et al. 2013). Low sequence similarity of 16S rRNA gene and distant phyletic lineage from the closest relative type strain showed by the rare Actinobacteria isolates in this study had assigned them to be putative new species of Actinobacteria. Furthermore, the rare Actinobacteria derived from the genus *Micromonospora* was recognized as one of the most prolific producers of novel secondary metabolites (Weinstein et al. 1963; Arcamone et al. 1980; Antal et al. 2005). Further investigation is needed for the validation of new species, such as DNA-DNA hybridization, which is one of the important criteria for the delineation of bacterial species, with the recommendation of relatedness values fall below 70% cut-off point (Wayne et al. 1987).

From the PCA analyses presented in Figure4, fluctuation of the diversity of Actinobacteria was correlated with the presence of water activity. The soil sample from the middle lake was distinctly clustered from the soil samples of lakeside. This implies the different nutrient composition and physicochemical parameters of the center part of the lake which favour the changes in the bacterial community structure and diversity, compared to the lakeside. On the other hand, the effects of water activity and extreme UV index on the formation of divergent community structure were also examined (soil sample 68). Soil sample 111 was a soil mixture of decaying rock and near to the skua nest. The high divergent community structure observed could represent a nutrient-rich microhabitat for a high diversity of Actinobacteria to carry out decomposition and recycling processes of soil organic matters (Crawford 1988).

This study provides a base overview of the diversity of Actinobacteria at Greenwich Island and Dee Island. The metagenomics studies had demonstrated the prevalence of Actinobacteria in Antarctic soil (Teixeira et al. 2010; Aislabie et al. 2013). However, the number of culturable Actinobacteria only representing a tiny fraction of the total community of soil Actinobacteria, indicating numerous taxa of this group of bacteria are yet-to-be-cultured from Antarctic environments. Application of selective isolation can lead to the discovery of novel rare Actinobacteria from soil ecologies. The soil pH had exerted strong selection on the distribution of Actinobacteria in the soil. While an elevated diversity of actinobacterial community was examined with the presence of water activity in soil that served as a dynamic source for microbial interactions. Hence, in the future, it will be interesting to investigate the biosynthesis potential of the Actinobacteria recovered, especially the rare Actinobacteria. There is a hope where rare Actinobacteria may bear biosynthesis capacity of new antibiotics to combat the multidrug-resistant human pathogens and emerging deadly diseases. The discovery of novel chemotypes with significant biological activity will become potential leads for drug discovery, which benefit the biotechnological and pharmaceutical industries.

5.0 Declaration

The authors declare no conflicts of interest in this work.

6.0 Acknowledgements

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