AMBIO

ANNEXE 1

5 Cyanobacteria in cold environments

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5.1 Introduction

Perennially cold environments in which temperatures remain below 5°C are common throughout the biosphere (Margesin and Häggblom 2007). In these habitats, the persistent cold temperatures are often accompanied by freeze-thaw cycles, extreme fluctuations in irradiance (including ultraviolet radiation), and large variations in nutrient supply and salinity. As a result of these constraints, polar and alpine environments contain a reduced biodiversity, with prokaryotes contributing a major component of the total ecosystem biomass as well as species richness. Cyanobacteria are of particular interest because they often represent the predominant phototrophs in such ecosystems. Current research shows that a diverse range of cyanobacteria can be found in polar and alpine habitats, and that they show a remarkable ability to tolerate the abiotic stresses that prevail in these cold environments. Their presence was already observed during the early explorations of the polar regions at the end of the 19th century (Vincent 2007).

The widespread distribution in cold habitats of communities dominated by cyanobacteria, particularly in ice-based environments, makes them of great interest for the reconstruction of microbial life and diversification on early Earth (Vincent et al. 2004b). These ice-based habitats with their sustainable microbial communities are potential analogues for biotopes present during the major glaciation events of the Precambrian. The fossil record suggests that cyanobacteria would have been present throughout these Proterozoic events, and perhaps during earlier periods of global cooling (Schopf 2000). Polar microbes, including cyanobacteria, are also of interest to astrobiologists studying the prospect of life beyond our planet. Antarctica has been proposed as an analogue to an early stage of Mars where liquid water occurred and where life could have evolved at a similar time to the development of cyanobacteria on early Earth (Friedmann 1986).

In this chapter, we first introduce the taxonomical status and the general characteristics of cyanobacteria. We then examine cyanobacterial diversity in antarctic, arctic and alpine habitats, focusing on the molecular approaches. The ecophysiological traits of cyanobacteria that allow them to survive and often thrive in such cold environments are also presented. We conclude this review by consideration of the biogeographical distribution of polar cyanobacteria, an active topic of current research.

5.2 Taxonomy and diversity

Cyanobacteria are Gram-negative oxygenic photosynthetic bacteria that, according to the fossil record, achieved most of their present morphological diversity by two billion years ago (Schopf 2000). Cyanobacteria were initially described as algae in the 18th century and the first classification system was based on the International Code of Botanical Nomenclature as described by Oren (2004). In the botanical taxonomy, two major works can be noted. Firstly, Geitler (1932) produced a flora that compiled all European taxa, which already encompassed 150 genera and 1500 species based on the morphology. Secondly, the recent revisions by Anagnostidis and Komárek (e.g. Komárek and Anagnostidis 2005) aimed to define more homogeneous genera, still based on the morphology. After the prokaryotic nature of cyanobacteria became more obvious on the basis of ultrastructural and molecular studies, it was proposed that their nomenclature should be governed by the International Code for Nomenclature of Bacteria (Stanier et al. 1978). Currently, the phylum of Cyanobacteria encompasses 5 subsections (corresponding to the 5 orders in the botanical classification) in the Bergey's Manual of Systematic Bacteriology (Castenholz 2001):

I. Chroococcales (unicellular);
II. Pleurocapsales (large cells subdividing into smaller baeocysts);
III. Oscillatoriales (simple filamentous);
IV. Nostocales (filamentous, non-branching heterocyst-forming);
V. Stigonematales (filamentous, branching, heterocyst-forming).

To date, only a few names of cyanobacterial taxa have been validly published according to bacterial rules, reflecting not only technical difficulties but also the confusion due to the existence of two nomenclatural systems (Oren 2004). Current taxonomical studies on cyanobacteria are now adopting a polyphasic approach, which combines genotypic studies with morphological and phenotypic analyses.

Early studies on the diversity and biogeographical distribution of cyanobacteria were based on the identification of the organisms entirely on the basis of morphological criteria. Cyanobacteria often have quite simple morphologies and some of these characters exhibit plasticity with environmental parameters, so that their taxonomic usefulness can be limited. Moreover, a number of botanical taxa have been delimited based on minute morphological differences (e.g., sheath characteristics, slight deviations in cell dimensions or form), and many authors have shown that the genetic diversity does not always coincide with that based on morphology (e.g. Rajaniemi et al. 2005; Taton et al. 2006b). To address these problems, studies on environmental samples (natural mixed assemblages of microorganisms) are typically based at present on clone libraries or DGGE (Denaturating Gradient Gel Electrophoresis) using molecular taxonomic markers, most often the 16S rRNA gene. The obtained 16S rRNA sequences are compared and generally grouped into OTUs (Operational Taxonomic Units) or phylotypes on the basis of their similarity (e.g. 97.5% similarity for Taton et al. 2003 or 98% for de la Torre et al. 2003). With such similarity values, each OTU might correspond to one or more bacterial species but is clearly distinct from other OTUs at the species level (Stackebrandt and Göbel 1994). This therefore provides a conservative estimate of the diversity, following bacterial criteria.

5.3 General characteristics

Cyanobacteria possess photosystems I and II, which are located on thylakoid membranes (except in the genus *Gloeobacter*). The cells usually have a characteristic blue-green coloration due to the phycocyanin (blue), allophycocyanin (blue) pigments in addition to chlorophyll *a*, although some species may additionally contain phycoerythrin that colours the cells red. In a few taxa, other chlorophylls have been observed, including chlorophylls *b* and *d* (Miyashita et al. 1996; Castenholz 2001). Some cyanobacteria are also able to fix atmospheric nitrogen. Furthermore, cyanobacteria have various storage bodies for carbon, nitrogen, phosphate and the enzyme ribulose 1,5-biphosphate carboxylase/oxygenase (RubisCO) (Castenholz 2001). Cyanobacteria are also known to produce a great variety of secondary metabolites with diverse activities. To date, 600 bioactive molecules have been described, most of which have been found in the Oscillatoriales and Nostocales orders (Welker and von Döhren 2006).

5.4 Antarctic habitats

Studies on the molecular cyanobacterial diversity in Antarctica, using a culture-independent approach, have focused on the following regions to date: the Prydz Bay region (Bowman et al. 2000; Smith et al. 2000; Taton et al. 2006a), the McMurdo Dry Valleys (Priscu et al. 1998; Gordon et al. 2000; Christner et al. 2003; de la Torre et al. 2003; Taton et al. 2003; Smith et al. 2006; de los Rios et al. 2007), the McMurdo Ice Shelf (Jungblut et al. 2005) and the Antarctic Peninsula region (Hughes and Lawley 2003; Hughes et al. 2004). Strains have been isolated from the same regions, as well as from the Dronning Maud Land (Rudi et al. 1997; Vincent et al. 2000; Smith et al. 2000; Billi et al. 2001; Nadeau et al. 2001; Casamatta et al. 2005; Taton et al. 2006b; Comte et al. 2007).

5.4.1 Ice-based habitats

Cyanobacteria dominate microbial consortia formed in ice-based habitats such as cryoconite holes and melt water ponds. Cryoconite (literally “cold rock dust”) gives rise to vertical, cylindrically formed holes in the ice surface that contain a thin layer of sediment overlain by water. The formation of these habitats is initiated through the absorption of solar radiation by the sediment and the subsequent ablation of the surrounding ice (Wharton et al. 1985). Studies of these holes on the Canada Glacier, McMurdo Dry Valleys, show that they contain cyanobacteria as well as heterotrophic bacteria, eukaryotic micro-algae and colourless protists and even metazoans such as rotifers, nematodes and tardigrades (Mueller et al. 2001).

An analysis of 16S rRNA sequences from the Canada Glacier cryoconite communities showed the presence of cyanobacteria phylogenetically related to *Chamaesiphon* (96.2% 16S rRNA sequence similarity) (Christner et al. 2003), a genus that is known to occur in the periphyton that forms over rocks in European mountain streams. Other polar sequences in this lineage (ca 96% similarity) originated from the ice cover of Lake Bonney (Priscu et al. 1998) and a deglaciated glacier area in the Peruvian Andes (Nemergut et al. 2007). A second group of sequences is 99.2% similar to a clone from a meltwater in Livingston Island (Antarctic Peninsula) (unpubl. data) and the third sequence group is 99.7% similar to clones from lake Fryxell, lakes in the Prydz Bay area and the Antarctic

Peninsula. Notably, sequences of the third group are also 99.2% similar to one sequence from the Peruvian Andes (Nemergut et al. 2007).

Another important class of ice-based habitat is the meltwater ponds that form on ice shelves. These contain liquid water during the summer months, but completely freeze over the winter. The biota of these habitats must therefore contend with extreme temperature changes, freezing and desiccation stress, and high salinities. The ponds on the McMurdo Ice Shelf have low nutrient concentrations, especially nitrogen, due to the marine origin of the sediments (Hawes et al. 1993) and their characteristics are described by Wait et al. (2006). Thick benthic cyanobacterial mats comprise a diverse community of Nostocales and Oscillatoriales as well as other bacterial phyla and microeukaryotes (Howard-Williams et al. 1989; Nadeau et al. 2001; Jungblut et al. 2005). In one of these mats, the presence of microcystin was detected (Jungblut et al. 2006).

5.4.2 Soils and rock

Cyanobacteria are often the primary colonisers of permafrost soils in areas where melt water flushes occur through snow melt or retreated glaciers. In the Dry Valleys of Antarctica, the soils are old, weathered and have low carbon and nutrient concentrations (Vincent 1988). Thus, the colonisation by cyanobacteria increases soil stability and nutrient concentrations through, for example, nitrogen fixation. Terrestrial dark crusts are found throughout Antarctica and are commonly dominated by cyanobacteria (e.g., Broady 1996; Mataloni and Tell 2002; Adams et al. 2006).

Cyanobacteria are also often identified in biofilms below and within the rocks where the microclimate gives protection against environmental stresses such as high UV radiation, temperature extremes, desiccation and physical removal by wind. They can be found in depth below the rock surface depending on the optical characteristics of the rocks and the level of available photosynthetically active radiation (PAR). Depending on the spatial location of the communities, they are hypolithic (beneath rocks), endolithic (in pore spaces of rocks), chasmoendolithic (in cracks and fissures of rocks), or cryptoendolithic (in the pore space between mineral grains forming sedimentary rocks) (Vincent 1988; Hughes and Lawley 2003).

Molecular analysis of such communities revealed a few cryptoendolithic cyanobacterial sequences in beacon sandstone of the Dry Valleys (de la Torre et al. 2003), and in granite boulders of Discovery Bluff (de los Rios et al. 2007). Interestingly, in the latter study, one 16S rRNA sequence was related (98.9%) to a DGGE band from Swiss dolomite (Sigler et al. 2003) and the second sequence is identical to one hypolithic sequence from quartz rocks in the Vestfold Hills (Smith et al. 2000). This group of sequences also has affinities (93.5% similarity) to the chlorophyll *d* containing *Acaryochloris marina* (Miyashita et al. 1996) and de los Rios et al. (2007) hypothesized that some cryptoendoliths could possess this pigment and that its particular absorption spectrum would be beneficial in environments with little light. Another well-known cryptoendolithic cyanobacterium belongs to the genus *Chroococcidiopsis* and was found in sandstones of the Dry Valleys (Friedmann 1986). It is remarkably resistant to desiccation and has close relatives in hot deserts (Fewer et al. 2002).

5.4.3 Ponds, lakes, rivers and streams

Similar to their presence in ice-based habitats, cyanobacteria form large biomass accumulations in Antarctic ponds, lakes, rivers and streams (Vincent 1988). They often form thick, cohesive, highly pigmented mats that coat the benthic environments. A large variety of lake types are present in Antarctica and span a wide range of environmental conditions (Gibson et al. 2006). Many of them are covered with ice for most of the year or even have a perennial ice cover. Studies on perennially ice-covered Lake Hoare in the McMurdo Dry Valleys have shown that PAR irradiance exerts an overall control on microbial photosynthetic production, composition and mat structure (Vopel and Hawes 2006). Other characteristics such as nutrients and salinity also influence the cyanobacterial diversity (Gibson et al. 2006). The diversity and function of the microbial lake communities have been reviewed by Ellis-Evans (1996). At the molecular level, Taton et al. (2003, 2006a,b) showed a large cyanobacterial diversity in the benthic mats from five different Antarctic lakes in two regions (see Sect. 5.7). Cyanobacteria also form biofilms and microbial mats in rivers and streams (Vincent 1988).

In addition to the benthic communities, cyanobacteria are also found in the water column of lakes, and picoplanktonic forms often dominate the plankton. The abundance of planktonic picocyanobacteria is dependent on nutrient availability and light (Vincent 2000), The 16S rRNA sequences of S*ynechococcus*-like picocyanobacteria were obtained from lakes in the Vestfold Hills (lakes Ace, Pendant, Clear). They appeared to be related, but distinct from other *Synechococcus* genotypes such as *Synechococcus* PS840 from the Russian marine coast (Waleron et al. 2007).

5.4.4 Marine ecosystems

The abundance of picocyanobacteria decreases markedly from temperate latitudes to the polar regions (Marchant et al. 1987; Fouilland et al. 1999). This decrease is assumed to be due to temperature-limitation (Marchant et al. 1987) as well as continuous losses due to grazing, advection and mixing (Vincent 2000). The 16S rRNA sequences of picocyanobacteria retrieved at the Subantarctic Front (51°S) were closely related to temperate oceanic *Synechococcus*, as WH8103 and WH7803 (Wilmotte et al. 2002).

5.5 Arctic habitats

Most available studies focused on the Canadian Arctic so far, whereas no information is yet available from the Russian Arctic.

5.5.1 Ice-based habitats

Similar to the South Polar Region, ice shelves as well as glaciers, provide a variety of habitats for cyanobacteria in addition to other biota (Säwström et al. 2002; Mueller et al. 2005). However, the total area of ice shelves is lower than to Antarctica, where 40% of coastline is fringed by ice shelves. Furthermore, the recent break up of the Ward Hunt Ice Shelf (Mueller et al. 2003) signals the massive reduction of these habitats through global warming. Arctic ice-based habitats can be differentiated into cryoconite holes, melt water ponds and sediment patches without continuous coverage by water. Microbial mats can be

prolific (Vincent et al. 2004a), but are less developed than in Antarctica, and this may be due to the increased grazing pressure in the Arctic (Vincent 2000).

5.5.2 Lakes, streams and ponds

Cyanobacteria play an important role in Arctic lakes, ponds and streams and have been well studied in the Canadian High Arctic (Bonilla et al. 2005).The most common groups are Oscillatoriales and Nostocales, with some Chroococcales. The benthic microbial mats in lakes often have a cohesive layering, which is established through an extra polysaccharide matrix and often have a characteristic pigment stratification (Bonilla et al. 2005). Planktonic picocyanobacterial communities of these lakes comprise mainly *Synechococcus* (Vincent 2000) and can be separated into fresh and saline ecotypes (unpubl. data). In these lakes, primary production is only nutrient limited in the planktonic communities, whereas the microenvironments of the benthic mats result in increased nutrient availability and sufficiency (Bonilla et al. 2005).

5.5.3 Soils and rock

Terrestrial cyanobacteria in the Arctic are also a major primary coloniser of soils and can be found within soil crusts, symbiotic in lichens and within rocks. They are an important source of nitrogen for the nutrient limited soils of the Arctic (Zielke et al. 2005). Cryptoendolithic communities are common in sandstone outcrops of Eureka, Ellesmere Island, and consist of similar cyanobacterial morphotypes as in Antarctic rocks (Omelon et al. 2006). However, their diversity seems higher than in comparable habitats of the Dry Valleys. This may be due to higher average temperatures, higher humidity due to close spatial distance to open water and longer periods with available liquid water relative to the McMurdo Dry Valleys. Hypolithic cyanobacteria are commonly observed under opaque rocks subjected to periglacial movements (Cockell and Stokes 2004).

5.5.4 Marine ecosystems

In the arctic marine environment, similarly to the Southern Ocean, picocyanobacteria are rare, in contrast with their abundance in temperate and tropical oceans. A study of their molecular diversity in the Beaufort Sea showed that the picocyanobacteria were affiliated with freshwater and brackish *Synechococcus* lineages, but not to the oceanic ones. Their origin, therefore, seemed allochthonous, as the Arctic Sea is much influenced by large riverine inputs (Waleron et al. 2007).

5.6 Alpine habitats

5.6.1 Streams and lakes

In alpine streams, water chemistry, geochemical conditions, hydraulic conditions and permanence of flow are the key factors defining taxonomic diversity. Cyanobacteria have been found as part of microbial mats, epiphytic on mosses and endosymbiotic in lichens in stream habitats of many alpine regions but there are no specific studies on their molecular diversity or adaptations (McClintic et al. 2003; Rott et al. 2006). Nutrient concentrations show large variations during the year with peaks in late winter and autumn. PAR and UV radiation also range from

low levels in presence of ice and snow cover to high levels during summer months, creating a need for protective mechanisms to survive. Rott et al. (2006) have described different colonisation patterns for several cyanobacterial morphotypes in alpine streams.

Cyanobacteria in alpine freshwater lakes can be found as benthic and planktonic communities. Plankton communities are mostly comprised of *Synechococcus* morphotypes and their abundance is correlated to nutrient availability, particularly nitrogen and phosphorus. Benthic communities were studied by Mez et al. (1998), and Sommaruga and Garcia-Pichel (1999). Interestingly, the presence of cyanotoxins was demonstrated by Mez et al. (1998).

5.6.2 Rocks and soils

Cyanobacteria are also dominant components of alpine soil crusts, and rock-associated communities as described for endolithic communities of dolomite rocks in the Swiss Alps (Sigler et al. 2003) and soils from recently deglaciated areas in the Peruvian Andes (Nemergut et al. 2007). The 16S rRNA sequences obtained from the Alpine dolomite layers show high similarities with Antarctic cryptoendoliths (see Sect. 5.4.2), or up to 97.7% similarity with the Andean cyanobacteria (Nemergut et al. 2007), or appear novel (less than 93% similarity with database sequences). Other Andean soil sequences are related to the sequences of *Chamaesiphon* PCC7430 (ca 96% sequence similarity), of diverse *Nostoc* strains (a. o. 97.4-98.5 % with the Antarctic ANT.L52B.1), of the Antarctic *Leptolyngbya frigida* ANT.LH52.2 (98.5%) and ANT.LH52B.3 (99.6%), or are quite different from database sequences and thus represent a novel diversity.

5.7 Ecophysiology

5.7.1 Coping with the cold

In general, high latitude and high altitude cyanobacteria tend to be cold tolerant (psychrotrophs), with suboptimal growth under low temperatures, rather than psychrophiles that grow optimally at low temperature (Tang and Vincent 1999). They have a variety of mechanisms that allows them to tolerate and continue to grow, albeit often at slow rates, in the cold and to tolerate freeze-thaw conditions (Vincent 2007). To maintain membrane fluidity at low temperatures, polyunsaturated fatty acids with decreased chain-lengths are incorporated into the membrane. In addition, the production of compatible solutes (e.g. trehalose) helps to reduce the freezing point of the intracellular fluid. This strategy also reduces cell desiccation as less water is needed to retain the osmotic equilibrium (Welsh 2000). Furthermore, extracellular compounds such as polymeric substances can reduce ice nucleation around the cells (Vincent 2007). Cyanobacteria must also withstand prolonged seasonal dormancy phases in frozen and liquid water. Freeze-dried cyanobacterial mats in Antarctica have been shown to resume photosynthesis within minutes to hours after rethawing (Vincent 2007).

5.7.2 Osmotic stress

Typical hypersaline environments are saline ponds and lakes and brine channels in the sea ice (Vincent 1988). Sudden increases in salt concentration are

counterbalanced by a rapid accumulation of salts to maintain the osmotic equilibrium. Long-term survival strategies involve uptake of inorganic ions, to balance the extracellular ion concentrations, as well as the production of organic osmolytes (Oren 2000).

5.7.3 High and low irradiance

UV radiation and high energy PAR can induce photo-inhibition, phycobiliprotein degradation, chlorophyll-bleaching and DNA damage, or the production of reactive oxygen species, and the net damage may be exacerbated at low temperatures (Vincent 2007). Cyanobacteria have evolved a variety of DNA repair mechanisms, such as excision repair and photo-reactivation, to cope with UV induced DNA damage (Castenholz and Garcia-Pichel 2000). However, these processes are reduced at lower temperatures. Furthermore, the cyanobacteria produce photoprotective screening and quenching pigments (gloeocapsin, scytonemin, mycosporine) and many Antarctic cyanobacteria seem to avoid radiation by migrating to deeper layers within the microbial mats (Castenholz and Garcia-Pichel 2000). High concentrations of scytonemin can lead to a black coloration in many cyanobacterial mats and soil crusts (Vincent 2007).

Conversely, phototrophs in polar and alpine regions must also contend with low irradiances caused by prolonged snow and ice cover. The cyanobacteria utilise highly efficient light capturing complexes, with photosynthetic quantum yields close to the theoretical maximum (Hawes and Schwartz 2001; Vincent 2007).

5.8 Biogeography

The question of endemism and distribution of cyanobacterial taxa is still a topic of much debate. A long-standing theory of microbial distribution is that “everything is everywhere, but the environment selects” and that local habitats select for specific microbial flora that is globally distributed (Baas-Becking 1934). Castenholz (1992) noted the slow rates of speciation in the cyanobacteria together with their large dispersal abilities, and this in combination with the relatively young age of most polar ice-free environments suggest that endemism is likely to be rare amongst polar cyanobacteria. Morphological identifications seemed to support this hypothesis. However, such characterisation is limited due to morphological plasticity (see Sect. 5.2). In addition, Komárek (1999) noted that a number of identifications of Antarctic cyanobacteria had been made with flora written for temperate countries without taking into account their ecology, which could give the false impression that mostly cosmopolitan taxa were found on this continent. Indeed, by avoiding such ‘force-fitting’, Komárek (1999) has found about 60% of endemic species amongst the 68 morphospecies found in various microbiotopes of ice-free areas of King George Island.

Several features of Antarctica suggest that endemism may be possible there, although it has yet to be demonstrated convincingly (Vincent 2000): (i) Antarctica has been more isolated than other parts of the world for several million years; (ii) dispersal processes which favour local species are more efficient than long-range dispersal processes; (iii) there has probably been strong environmental selection for adaptive strategies. As a step towards addressing this question, the molecular characterisation of cultured and uncultured cyanobacterial diversity has been carried out in a number of Antarctic biotopes (see above Sect. 5.4). The results

from these studies suggest the presence of OTUs/phylotypes with cosmopolitan and bipolar distributions, but also the presence of some genotypes that seem to be restricted to specific Antarctic sites. However, there is never complete 16S rRNA sequence identity for bipolar or cosmopolitan organisms that are members of the same OTU. The use of ITS sequences, which are more variable than the 16S rRNA, could increase the resolution of the distributional patterns (Taton et al. 2006a).

Taton et al. (2003, 2006a,b) have analysed the molecular diversity in Lake Fryxell (Dry Valleys), four coastal lakes in the Prydz Bay area (East Antarctica) and two meltwater samples from Livingston Island (Antarctic Peninsula). Using clone libraries based on 16S rRNA sequences, a total of 63 OTUs were detected, of which 44 were only found in Antarctica (70%). This suggests a high degree of endemism, even if a portion of this uniqueness could be due to geographic gaps in the database. A higher proportion of the cosmopolitan genotypes are found in several Antarctic regions (47% compared to 16% for the potentially endemic sequences). Thus, if they were able to disseminate and colonise habitats in different continents, this could be due to resistance capacities that are also helpful to spread to different Antarctic regions. Furthermore, there appears to be an on-going exchange between freshwater and terrestrial biotopes (Gordon et al. 2000), which could also explain why temperature flexibility (psychrotrophy, see above Sect. 5.7.1) is more common than psychrophily in cyanobacteria. Another conclusion is that each new sample brings new genotypes and this suggests that much diversity still awaits discovery.

5.9 Conclusion

Cyanobacteria evolved under the harsh conditions of the Precambrian and their modern representatives retain a remarkable ability to adapt to and survive within extreme conditions. They dominate terrestrial and freshwater cold ecosystems of the Arctic, Antarctic and alpine regions, even though they do not seem to be specifically adapted to optimal growth at low temperatures. They play a major ecological role as they often are primary colonisers of substrates and major primary producers in these ecosystems.

The application of molecular tools in combination with classic morphological techniques has begun to provide new insights into the real diversity of cyanobacteria and their biogeographical distribution in cold environments. Our survey of recent studies suggests complex distributional patterns of cyanobacteria, with cosmopolitan, endemic, and habitat-specific genotypes. This ongoing research will help to identify specific geographical areas that have unique microbial communities. However, many more studies are needed to unravel the enormous diversity of cyanobacteria and to better define their biogeographical patterns in cold environments. This is an urgent task in view of the climatic changes that will undoubtedly alter the structure and functioning of microbial communities in polar and alpine ecosystems.

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Hidden levels of phylodiversity in Antarctic green algae: further evidence for the existence of glacial refugia

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Hidden levels of phylodiversity in Antarctic green algae: further evidence for the existence of glacial refugia

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Recent data revealed that metazoans such as mites and springtails have persisted in Antarctica throughout several glacial–interglacial cycles, which contradicts the existing paradigm that terrestrial life was wiped out by successive glacial events and that the current inhabitants are recent colonizers. We used molecular phylogenetic techniques to study Antarctic microchlorophyte strains isolated from lacustrine habitats from maritime and continental Antarctica. The 14 distinct chlorophycean and trebouxiophycean lineages observed point to a wide phylogenetic diversity of apparently endemic Antarctic lineages at different taxonomic levels. This supports the hypothesis that long-term survival took place in glacial refugia, resulting in a specific Antarctic flora. The majority of the lineages have estimated ages between 17 and 84 Ma and probably diverged from their closest relatives around the time of the opening of Drake Passage (30–45 Ma), while some lineages with longer branch lengths have estimated ages that precede the break-up of Gondwana. The variation in branch length and estimated age points to several independent but rare colonization events.

Keywords: Antarctica; biogeography; endemism; glacial refugia; green algae; molecular phylogeny

1. INTRODUCTION

Recent work on terrestrial metazoans in Antarctica has questioned the long-held 'recolonization hypothesis' that expanded ice-cover during successive Neogene and Late Pleistocene glacial maxima has resulted in almost complete extinction of biota, followed by extensive colonization after glacial retreat (e.g. Stevens *et al.* 2006; Convey & Stevens 2007; Convey *et al.* 2008; McGaughran *et al.* 2008; Pugh & Convey 2008). Instead, the data support the 'glacial refugia hypothesis', with various groups of organisms, including mites, springtails and copepods, showing a high degree of endemism and regionalization, suggesting that they were able to survive in isolated ice-free refugia (see Convey & Stevens 2007; Convey *et al.* 2008 for an overview). To date, the focus has largely been on terrestrial organisms for which coastal oases and nunataks, the Transantarctic mountains and specific regions such as the McMurdo Dry Valleys—some of which are known to have been ice-free since at least the Mid-to-Late Miocene (up to approx. 14 Ma; Boyer 1979; Prentice *et al.* 1993)—probably acted as important refuges.

Evidence for the widespread persistence of refugia in which aquatic organisms could survive glaciation (e.g. coastal low-latitude regions) is, however, still elusive (Hodgson *et al.* 2001, 2005; Gibson & Bayly 2007). Life in Antarctic aquatic ecosystems is largely microbial and confined to benthic mats consisting of cyanobacteria, and to a lesser extent diatoms and green algae (Sabbe *et al.* 2004; de los Rios *et al.* 2004). Micro-organisms form an interesting test case in the light of the refugium hypothesis in that they are expected, within the limits of their environmental tolerance, to show ubiquitous distribution patterns as a result of their virtually unlimited dispersal capacity (but see ongoing discussion on microbial biogeography in, for example, Martiny *et al.* 2006). Molecular studies of cyanobacteria, using 16S rRNA gene sequencing, suggest that most isolates have a long association with the Antarctic environment (Taton *et al.* 2003, 2006). Likewise, morphological studies on diatoms suggest that in some areas at least 40 per cent of the species are Antarctic endemics (Schmidt *et al.* 1990; Sabbe *et al.* 2004; Spaulding *et al.* in press). These high levels of endemism may point to the importance of relatively low dispersal rates and long-term survival in suitable habitats confined to isolated glacial refugia. In contrast to diatoms and cyanobacteria, the green algal component of microbial mats has remained virtually unstudied. The available data are largely restricted to morphological taxonomic inventories on the continent, such as Victoria Land (Cavacini 2001; Adams *et al.* 2006), the Antarctic Peninsula (Mataloni & Pose 2001) and maritime Antarctica (Fermani *et al.* 2007; Zidarova 2007). Broady (1996) suggested that most Antarctic

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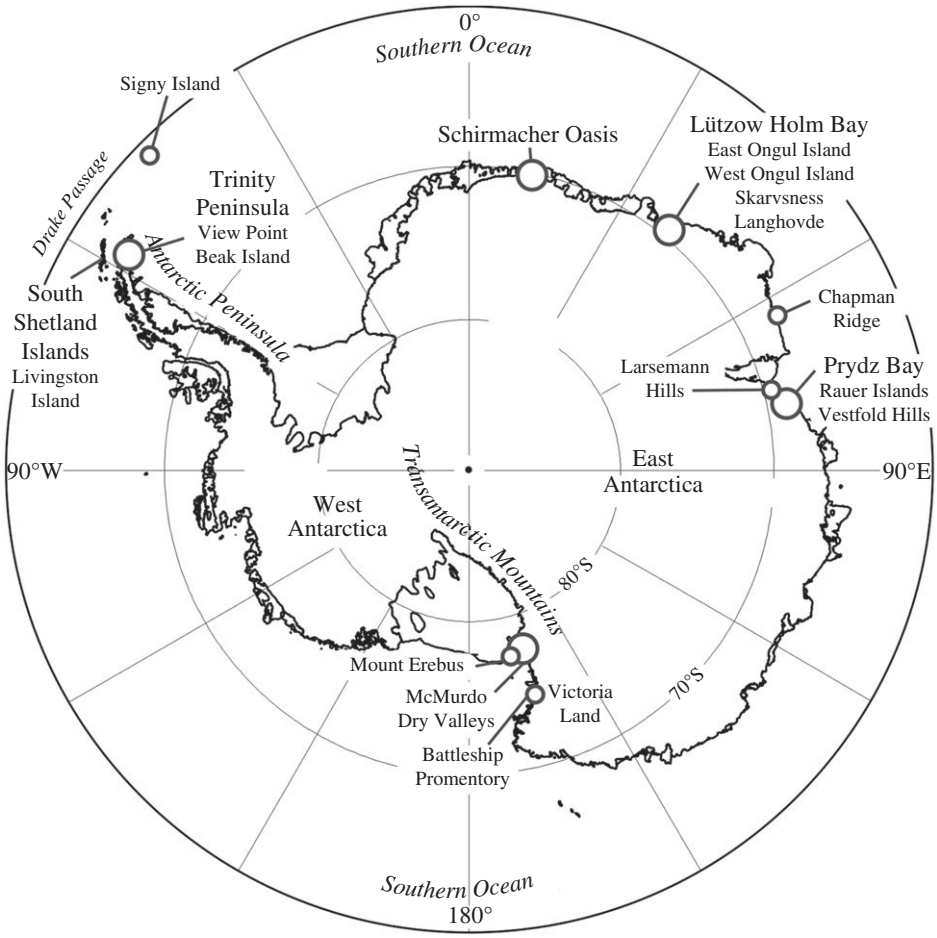


Figure 1. Map of Antarctica highlighting the five major sampling areas (large circles) and the original isolation source for the sequences from GenBank (small circles).

terrestrial green algae are cosmopolitan taxa. Morphological delimitation of green algal species and higher taxa is, however, contentious, especially in groups that are morphologically depauperate and exhibit convergent evolution towards reduced morphology (e.g. coccoid forms). Several cases of incorrect taxon delineation and cryptic species diversity are being disclosed by application of molecular phylogeny. A well-known example is that of the coccoid alga *Chlorella*, which has been shown to be polyphyletic, with the different taxa placed in separate green algal classes (Huss *et al.* 1999). DNA sequence data are therefore well placed to provide more insight into the green algal diversity on the Antarctic continent. The currently available molecular data are, however, fragmentary, consisting of a number of isolated taxonomic and ecophysiological studies on individual taxa (e.g. *Pyramimonas australis*: Moro *et al.* 2002; *Scenedesmus* sp.: Lesser *et al.* 2002; Pocock *et al.* 2004; lichen symbionts: Romeike *et al.* 2002; eukaryotic clone libraries: Christner *et al.* 2003; de la Torre *et al.* 2003; Fell *et al.* 2006).

The current study was designed to assess whether Antarctic freshwater green algae are mainly cosmopolitan species as a result of their fast colonization rates (Broady 1996), supporting the recolonization hypothesis, or are endemic to Antarctica or particular Antarctic regions, supporting the 'glacial refugia hypothesis' (cf. Convey *et al.* 2008). To this end, we employed molecular phylogenetic techniques, including molecular clock analysis of nuclear encoded 18S rRNA gene sequences of original Antarctic microchlorophyte isolates, along with an extensive set of other green algal sequences.

2. MATERIAL AND METHODS

(a) *Sampling and culturing*

Samples were obtained from 33 lakes in maritime and continental Antarctica: The Rauer Islands and Vestfold Hills (Prydz Bay, Princess Elizabeth Land), the McMurdo Dry Valleys (Victoria Land), View Point and Beak Island (Trinity Peninsula, Antarctic Peninsula), Schirmacher Oasis (Dronning Maud Land) and East Ongul, West Ongul, Langhovde and Skarvsness in the Lützow Holm Bay area (Enderby Land) (figure 1, table 1). Microbial mats or sediment samples were obtained using a custom-made scoop or a UWITEC gravity corer (Mondsee, Austria). Sample containers were topped up with water, cooled during transport and stored at 5°C.

Samples were incubated in liquid Bold modified basal freshwater medium (commercial stock Sigma-Aldrich, USA), liquid or agarized WC (Guillard & Lorenzen 1972) and desmid medium (recipe at utex.org). Cultures were grown at 12–15°C, with a 14 : 10 day : night cycle and a light intensity of 25–35 μmol photons $m^{-2}\,s^{-1}$.

From each sample a number of single cells or colonies with different morphology were picked out for monoclonal growth (table 1). These isolates were initially screened using light microscopy and amplified ribosomal DNA restriction analysis (ARDRA).

Table 1. Overview of the studied samples, collection and incubation data and the number of isolates obtained.

sample codes	lake name	region	region code	sampling date	number of isolates	number sequenced (unique)
R8	Rauer Island Lake 8	Rauer Islands, Prydz Bay (Princess Elisabeth Land)	PB	22 Dec 1997	2	1
ACE [1–5]	Ace Lake	Vestfold Hills, Prydz Bay	PB	18 Feb 1999	45	8 (1)
FRY [1/2]	Lake Fryxell	McMurdo Dry Valleys (Victoria Land)	McM	Feb 1999	39	10 (3)
B4/6/6L	Beak Island Lakes 4 and 6	Trinity Peninsula/Prince Gustav channel, Antarctic Peninsula (Graham Land)	TP	16 Jan 2006	14	4
VPL1/1A/2/3/4/5/6/9/9B/M	View Point Lakes 1–6, 9	Trinity Peninsula/Prince Gustav channel, Antarctic Peninsula (Graham Land)	TP	12 Jan 2006	83	14 (4)
EO2/3/4/5/7	East Ongul Lakes 2–5, 7	Lützow Holm Bay (Enderby Land)	LB	08 Jan 2007	44	11 (1)
LA1/3/4/6/8	Langhovde Lakes 1, 3–4, 6, 8	Lützow Holm Bay (Enderby Land)	LB	17–19 Jan 2007	15	1
SK2/5/6/9	Skarvsness Lakes 2, 5–6, 9	Lützow Holm Bay (Enderby Land)	LB	11–16 Jan 2007	25	3
WO1/4/8S/8L/10	West Ongul Lakes 1, 4, 8, 10	Lützow Holm Bay (Enderby Land)	LB	21–25 Jan 2007	35	6
SC1/2/6	Schirmacher Oasis Lakes 1, 2, 6	Schirmacher Oasis (Dronning Maud Land)	SC	29 Jan 2007	6	3 (1)

(b) *DNA extraction, PCR amplification, amplified ribosomal DNA restriction analysis screening and sequencing*

DNA was extracted as described in Zwart *et al.* (1998). The 18S rRNA gene was amplified using the primers P2 (5′-CTGGTTGATTCTGCCAGT-3′) and P4 (5′-TGAT CCTTCYGCAGGTTCAC-3′; Moon-van der Staay *et al.* 2000). The PCR reaction mixtures contained 2 µl of DNA, 0.5 µM of each primer, 200 µM of each deoxynucleoside triphosphate, 2.5 U of Taq DNA polymerase (Ampli Taq), 10× PCR buffer (100 mM Tris/HCl, pH: 8.3; 500 mM KCl; 15 mM $MgCl_2$; 0.01 per cent (w/v) Gelatin) and 400 ng of bovine serum albumin. The PCR reaction consisted of an initial denaturation of 2 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 55°C and 1.5 min at 72°C, and a final extension of 10 min at 72°C. ARDRA screening was performed using the restriction enzymes *Alu*I, *Hin*fI and *Taq*I following the protocol by Ventura *et al.* (2001). ARDRA images were analysed using BioNumerics 4.6 (Applied Math, Kortrijk, Belgium) with a curve-based (Pearson's correlation) clustering technique in order to define groups for further analysis. At least one strain from each ARDRA group (identical patterns) or morphological group (based on general characteristics such as size, shape and pigmentation) from each region was selected for sequencing.

Sequencing of the 18S rRNA gene was performed using the primers P2 and P4 (see above) and the internal primers P5, P12, P14, P15 and P16 (respectively, 300>, 1055<, 528>, 960> and 536<; Huss *et al.* 1999). Sequences were automatically assembled and visually checked in BioNumerics 4.6 and were deposited in GenBank under accession numbers FJ946881–FJ946908.

(c) *Sequence alignment and phylogenetic analyses*

The newly obtained Antarctic microchlorophyte sequences were combined with the closest NCBI BLAST hits, along with previously published sequences from Arctic and Antarctic regions and a broad diversity of sequences, representing the main classes and orders of Chlorophyta. Five representatives of Streptophyta were selected as outgroup. Taxa, locality details and GenBank numbers are listed in table S1 in the electronic supplementary material. While taxon sampling for the construction of the phylogenetic tree may influence our results, this is mainly constrained by the availability of sequences on GenBank. Arctic and alpine microchlorophytes sequences remain relatively under-represented in the public databases and, to our knowledge, no comparable studies have been carried out in these regions. The Antarctic sequences II22, II11 and V13 contained two putative group I introns: S516 and S943 (numbers reflect the homologous position in *Escherichia coli* 16S rRNA gene; Haugen *et al.* 2005). After introns had been removed, the sequences were aligned using Muscle 3.6 with standard parameters (Edgar 2004) and visually inspected. A FASTA-file of the alignment can be requested from the authors.

Phylogenetic analyses consisted of maximum likelihood (ML) and Bayesian inference (BI) tree searches under a general time-reversible model with a proportion of invariable sites and gamma distribution split into four categories ($GTR + I + \Gamma$), as determined by the Akaike Information Criterion in PAUP/Modeltest 3.6 (Posada & Crandall 1998; Swofford 1999). ML analysis was carried out with PhyML 2.4.4 (Guindon & Gascuel 2003). The reliability of each internal branch was evaluated based on 1000 bootstrap replicates. For BI, two independent runs, each consisting of four incrementally heated, Metropolis-coupled chains, were run for seven million generations using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). Parameter values and trees were sampled every thousand generations. Convergence and stationarity of the runs was assessed using Tracer 1.4 (Rambaut & Drummond 2007), and a burn-in sample of

Table 2. Overview of the sequenced strains, with GenBank accession number (acc), detailing the regions (codes as in table 1) from which strains with identical 18S rRNA gene sequence were isolated.

strain in tree	strain name(s) identical 18S sequences	acc.	region				
			PB	McM	TP	LB	SC
VII3	Lake Fryxell (VII3, VII4)	FJ946904		x			
VI12	Lake Fryxell (VI12)	FJ946905		x			
II4	Ace Lake (II4) Lake Fryxell (VI8)	FJ946902	x	x			
VPL9-6	View Point (VPL9-6)	FJ946907			x		
VPL9-5	View Point (VPL9-5)	FJ946901			x		
I5	Ace Lake (I5, I6)	FJ946892	x				
VPL4-4	View Point (VPL4-4)	FJ946900			x		
II11	Ace Lake (II11, II12, II22, III1, V13), Rauer (R8-2), West Ongul (WO1L-3, WO1S-2, WO1S-5, WO8L-2, WO8L-6), East Ongul (EO2-2, EO2-3, EO2-6, EO2-11, EO2-14, EO3-3, EO4-4, EO4-10 EO5-7), View Point (VPL1-5, VL2-4, VL2-6, VPL6-4), Beak (B4-1, B6-1, B6-6, B6-8), Skarsness (SK2L-4, SK2L-11, SK5S-6), Langhovde (LA6L-6)	FJ946893	x		x	x	
VI11	Lake Fryxell (VI11, VI13, VI26, VI4), West Ongul (WO10-1), View Point (VPL1-1, VPL1A-2, VPL9B-2, VPL9B-5, VPL9B-6), East Ongul (EO5-4c)	FJ946884		x	x	x	
VI2	Lake Fryxell (VI2, IX4)	FJ946883		x			
VPL1-3	View Point (VPL1-3)	FJ946890			x		
EO7-4	East Ongul (EO7-4)	FJ946882				x	
SC2-2	Schirmacher (SC2-1, SC2-3)	FJ946881					x
VPL5-6	View Point (VPL5-6), East Ongul (EO2-17)	FJ946891			x	x	

1000 trees was removed before constructing the majority rule consensus tree.

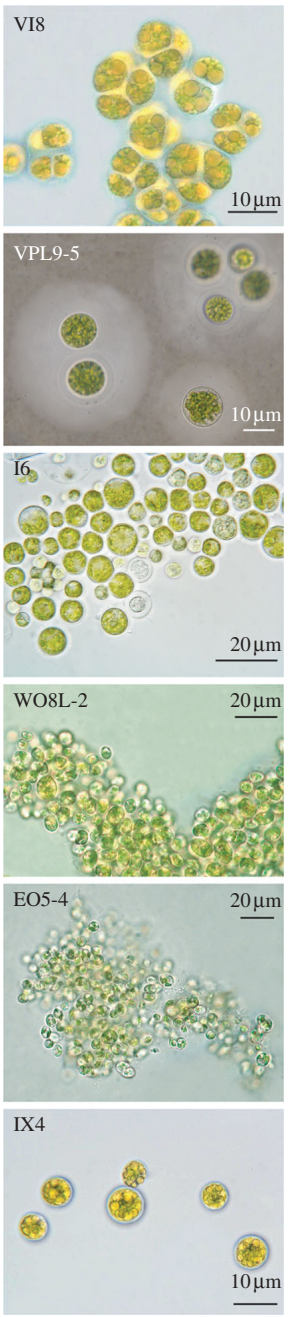
Relative node ages were estimated using r8s 1.7 (Sanderson 2002) by rate-smoothing the ML tree using penalized likelihood and a log-smoothing parameter of 5, selected as optimal by cross-validation (Sanderson 2003). Absolute ages were estimated by setting the minimum and maximum age of the Chlorophyta–Streptophyta split at 700 and 1500 Ma, based on the fossil record and molecular clock estimates (Douzery *et al.* 2004; Hedges *et al.* 2004; Yoon *et al.* 2004; Berney & Pawlowski 2006; Cavalier-Smith 2006; Roger & Hug 2006; Zimmer *et al.* 2007; Herron *et al.* 2009).

3. RESULTS

We characterized 61 isolates of green algae from 13 maritime and 30 continental Antarctic samples (table 1). All strains were isolated as non-flagellated unicells (coccal morphology). Sequencing of the 18S rRNA gene yielded 14 distinct sequences. Ten of these were detected in one Antarctic region only, two were detected in two regions and two were detected in three regions (table 2). No sequences were found in more than three of the five regions sampled. Twelve additional GenBank sequences of freshwater and terrestrial green algae from different Antarctic regions were incorporated in our dataset, along with a broad representation of green algae from other regions. The alignment of 191 sequences was 1854 sites in total, including 950 phylogenetically informative characters. ML and BI phylogenetic analyses yielded similar tree topologies, which are congruent with published 18S phylogenies of green algae (Lewis & Lewis 2005; Pröschold & Leliaert 2007). The ML phylogram, with indication of ML bootstrap values and BI posterior probabilities, is shown in fig. S1 in the electronic supplementary material. The chronogram, obtained by rate-smoothing the ML tree using penalized likelihood and calibrated by a conservative and more liberal node age of the Chlorophyta–Streptophyta split (700 and 1500 Ma, respectively), is shown in figure 2.

Phylogenetic analysis shows that the freshwater Antarctic isolates are mainly distributed among the chlorophytan classes Chlorophyceae and Trebouxiophyceae, and only a single Antarctic strain (*Trichosarcina mucosa*, a filamentous green alga) belongs to the Ulvophyceae (figure 2). Apart from the *Chlorella vulgaris* NJ-7 strain, identical to an isolate from a eutrophic pond in The Netherlands, none of the Antarctic sequences was identical to non-Antarctic sequences currently available in GenBank. Uncorrected *p*-distances with the most closely

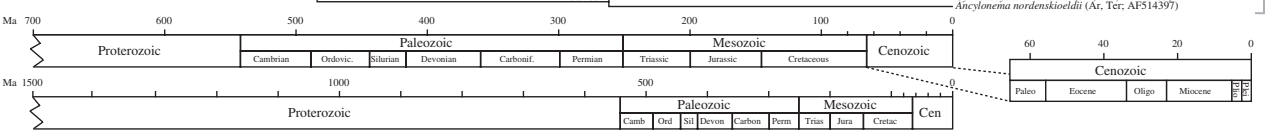
Figure 2. (*Opposite.*) ML tree of the green algae inferred from 18S rDNA sequences with branch lengths fitted to a molecular clock using penalized likelihood, and absolute ages estimated by setting the split of Chlorophyta and Streptophyta at 700 and 1500 Ma, respectively. Numbers at nodes indicate statistical support: ML bootstrap proportions (more than 50) and BI posterior probabilities (more than 85). Phylogenetic positions of freshwater and terrestrial Antarctic sequences are indicated in red, Arctic sequences in blue. Strains obtained during this study are highlighted and indicated by strain name. In addition to species names, climatic region (NT, north temperate; ST, south temperate; Tr, tropical; Ar, Arctic; An, Antarctic), environment (FW, freshwater; Mar, marine; Ter, terrestrial) and GenBank accession numbers are given within parentheses. Regions from which identical sequences were retrieved are indicated in parentheses (McM, McMurdo Dry Valleys; VH, Vestfold Hills; VP, View Point; R, Rauer Islands; WO, West Ongul; EO, East Ongul; Beak, Beak Island; SK, Skarvsness; LA, Langhovde; SC, Schirmacher Oasis). Inset: light microscopic images of a selection of Antarctic strains.



Colour legend of taxa in tree
Antarctic freshwater and terrestrial (bold: this study)
Arctic

Lake Fryxell VII2 (McM; FJ946904)
Lake Fryxell VI12 (McM; FJ946905)
Lake Ace II4 (VH, McM; FJ946902)
cf. *Chlorococcum* sp. 105-99 (Ar, Ter; AF514408)
Chlorococcum oleofaciens (NT, Ter; U41176)
Chloromonas perforata (NT, FW; U70794)
Pleurastrum insigne (NT, Ter/L; Z28972)
Chlamydopodium vacuolatum (Tr, Ter; M63001)
chlamydomonad sp. Pic 8/18 P-17w (NT, FW; AY220091)
VPL9-6 (VP; FJ946907)
Ettlia minuta (Tr, Ter; M62996)
Ascochloris multinucleata (NT, Ter, U63106)
Spongiochloris spongiosa (NT, FW; U63107)
Chlorosarcinopsis minor (NT, FW; AB049415)
Haematococcus zimbabwiensis (Tr, Ter; U70797)
Chlamydomonas sp. (Palmer station, An, Mar; EF106784)
Palmellopsis sp. BCP-EM1VF1 (NT, Des; AY271673)
Chlorogonium euchlorum (ST, Ter; AJ410443)
Haematococcus pluvialis (NT, FW; AF159369)
Dunaliella salina (Ar, Saltlake; EF473743)
Chlamydomonas subcaudata (NT, FW; AJ781310)
Chlamydomonas applanata (NT, Ter; U39984)
Characiosiphon rivularis (NT, Ter; AF395437)
Chlamydomonas pseudopulsatilla (Ar, Ter; AF514405)
Microglena monadina (NT, FW; AY220559)
Chlamydomonas pulsatilla (Ar, Ter; AF514404)
Pseudotetracystis sp. NB1-VF11 (NT, Des; AY614714)
Chlamydomonas chlamydogama (NT FW; AY220560)
Chlamydomonas noctigama (NT, Ter; AF008241)
Chlamydomonas hedleyi (NT, FW; AJ781312)
uncultured chlorophyte (NT, Mar; AY180047)
Chlamydomonas sp. SAG 73.94 (Ar, Ter; AF514399)
Chlamydomonas raudensis (Lake Bonney, An, FW; AJ781313)
Chloromonas nivalis (Ar, Ter; AF514409)
Chloromonas cf. *platystigma* (Ar, Ter; AF514401)
Chloromonas reticulata (NT, FW; AJ410448)
Actinochloris sp. BCP-LG3VF20 (NT, Des; AY271674)
Chlorococcum cf. *tatrense* (Ar, Ter; AF514407)
uncultured chlorophyte (NT, Ter; EF023280)
uncultured chlorophyte (NT, Ter; EF024027)
Chlamydocapsa sp. MX219-VF21 (NT, Des; AY614713)
chlorococcalean sp. 102/98 (Ar, Ter; AF514411)
VPL9-5 (VP; FJ946901)
chlamydomonad sp. Pic8/18T-9d (NT, FW; AY220589)
Asterococcus superbus (NT, FW; AB175836)
Asterococcus korschikoffii (NT, FW; AB175837)
Lobochlamys segnis (NT, FW; AJ410464)
Gloeococcus maximus (Battleship Promontory, An, Ter; U83122)
Chlamydomonas baca (NT, FW; U70781)
Tetracystis sp. *BCP-ZNP3-36* (NT, Des; AY377439)
Chlamydomonas reinhardtii (NT, FW; M32703)
Paulschulzia pseudovolvox (NT/Ar, Ter; U83120)
Tetraspora sp. (NT, FW; U83121)
Spermatozopsis similis (NT, FW; X65557)
Carteria obtusa (NT, Ter; AF182818)
Sphaeroplea robusta (NT, FW; U73472)
Trochiscia hystrix (NT, FW; AF277651)
Cylindrocapsa geminella (NT, FW; U73471)
Golenkinia longispicula (NT, FW; AF499923)
Schizomeris leibleinii (NT, FW; AF182820)
Uronema belkae (NT, FW; AF182821)
Chaetophora incrassata (NT, FW; D86499)
Dictyochloris fragrans (NT, Ter; AF367861)
Chaetopeltis orbicularis (NT, FW; U83125)
Radiococcus polycoccus (NT, FW; AF388378)
Lake Ace I5 (VH; FJ946892)
Pseudomurtiella aurantiaca (NT, FW; AB005748)
Bracteacoccus giganteus (NT, Ter; U63099)
Planktosphaeria gelatinosa (NT, FW; AY044648)
Pseudoschroederia antillarum (NT, FW; AF277649)
VPL4-4 (VP; FJ946900)
chlorophycean sp. Tow 2/24 P-12d (NT, FW; AY197633)
Kirchneriella aperta (NT, FW; AJ271859)
Pseudodictyosphaerium jurisii (NT, FW; AF106074)
Pediastrum duplex (NT, FW; M62997)
Characiopodium hindakii (Tr, Ter; M63000)
Bracteacoccus sp. ZNP1-VF32 (NT, Des; AF513379)
Scenedesmaceae sp. Tow 9/21 P-14w (NT, FW; AY197639)
Desmodesmus subspicatus (NT, FW; AJ249514)
Neodesmus pupukensis (ST, FW; X91267)
Scenedesmus pectinatus var. *pectinatus* (NT, FW; AB037092)
Coelastrum sphaericum (NT, FW; AF388376)
Scenedesmus obliquus (NT, FW; X56103)
Scenedesmus deserticola (NT, Des; AY510462)
Scenedesmus sp. SEV3VF49 (NT, Des; AF513373)
Scenedesmus regularis (NT, FW; AB037095)
Coelastrella terrestris (Mt. Erebus, An, Ter; AB012847)
Scenedesmus sp. CCMP 1625 (Mt. Erebus, An, Ter; AF339735)
Coelastrella saipanensis (AB055800)
Scenedesmus obtusus (Ar/NT, FW; AB037091)
Scenedesmus arcuatus (NT, FW; AY170311)
Scenedesmus sp. Antarctic (Gondwana Lake, An, FW; AM419228)
Lake Ace II11 (VH, R, WO, EO, VP, Beak, SK, LA; FJ946893)
Oedogonium cardiacum (NT, FW; U83133)
Bulbochaete hiloensis (NT, FW; U83132)
Oltmannsiellopsis viridis (NT, FW; D86495)
Trichosarcina mucosa (Vestfold Hills, An, FW; AM109906)
Monostroma grevillei (NT, Mar; AF015279)
Pseudendoclonium akinetum (NT, FW; DQ011230)
Hazenia mirabilis (NT, FW; AF387156)
Ulva intestinalis (NT, Mar; AJ000040)
Blidingia dawsonii (NT, Mar; DQ001138)
Acrosiphonia arcta (NT, Mar; AY303600)
Protomonostroma undulatum (NT, Mar; DQ821517)
uncultured Urospora (Terra Nova Bay, An, Mar; AJ626846)
Chlorothrix sp. ChloPac47SI (NT, Mar; AY476827)
Lake Fryxell VI11 (McM, WO, EO, VP; FJ946884)
Chlorella sp. NJ-18 (An, Ter; DQ377324)
Chlorella sp. (NT, FW; AY197624)
Lake Fryxell VI2 (McM; FJ946883)
Chlorella pyrenoidosa (NT, FW; AB240151)
Chlorella sp. YEL (NT, Ter; DQ377322)
Chlorella lobophora (NT, Ter; X63504)
Chlorella vulgaris (NT, FW; X13688)
Chlorella vulgaris NJ-7 (An, Ter; DQ377323)
Dictyosphaerium pulchellum (NT, FW; AY323838)
Chlorella sp. FACHB831 (Tr, Ter; DQ377321)
Chlorella sorokiniana (NT, Ter; X73993)
Muriella terrestris (NT, Ter; AB012845)
Chlorella minutissima (NT, Ter; X56102)
VPL1-3 (VP; FJ946890)
Nannochloris sp. ANR-9 (NT, FW; AY220081)
Koliella spiculiformis (NT, FW; AF278744)
Marvania geminata (NT, FW/Ter; AF124336)
Picochlorum eukaryotum (NT, Mar; X06425)
Nannochloris sp. Ant-1 (An, Ter; EF440182)
Nannochloris sp. JL 4-6 (NT, FW; AY195983)
Prototheca wickerhamii (NT, Ter; X74003)
Radiofilum conjunctivum (NT, FW; AF387155)
Eremosphaera viridis (Tr, FW; AF387154)
Oocystis solitaria (NT, FW; AF228686)
Oocystis heteromucosa (Tr, FW; AF228689)
Amphikrikos cf. *nanus* (NT, FW; AF228690)
Stichococcus sp. NB2VFF10 (NT, Des; AF513370)
Stichococcus chodati (NT, Ter; AB055867)
Stichococcus sp. FACHB753 (Tr, EU045358)
Prasiola stipitata (NT, Mar; EF200524)
Stichococcus bacillaris (NT, Ter; AB055866)
EO7-4 (EO; FJ946882)
Chlorella sp. (Ar, Ter; AF514413)
Koliella longiseta (NT, Ter/FW; U18520)
Raphidonema brevirostre (Ar, Ter; AF514410)
Pabia signienais (Signy Island, An, Ter; AJ416108)
Elliptochloris subsphaerica (Chapman Ridge, Ant, Ter; AB006050)
Chlorella ellipsoidea (NT, FW; X63520)
Chlorella mirabilis (NT, Ter; X74000)
Coenocystis inconstans (NT, Ter; AB017435)
Parietochloris pseudoalveolaris (NT, Ter; M63002)
Coleochlamys perforata (NT, Ter/Snow; M62999)
Leptosira terrestris (NT, Ter; Z28973)
Treboxia sp. (McMurdo dry valleys, An, Ter; AY250848)
Treboxia asymmetrica (NT, Ter/L; Z21553)
Treboxia usneae (NT, Ter; Z68702)
Friedmannia israeliensis (NT, Ter; M62995)
Treboxia magna (NT, Ter/L; Z21552)
SC2-2 (SC; FJ946881)
Watanabea reniformis (NT, FW; X73991)
Dictyochloropsis reticulata (NT, Ter; Z47207)
Lobosphaera tirolensis (NT, Ter; AB006051)
Choricystis sp. (NT, FW; AY195970)
Choricystis minor (NT/Ar, FW; X89012)
Botryococcus sp. (NT, FW; AY197640)
Coccomyxa glaronensis (NT, Ter/L; AM167525)
Coccomyxa pringsheimii (NT, Ter/L; AY762603)
Paradoxia multiseta (NT, FW; AY422078)
Paradoxia sp. 294-GA206 (Ar, Ter; EU282454)
VPL5-6 (VP, EO; FJ946891)
Scherffelia dubia (NT, FW; X68484)
uncultured prasinophyte (Ar, Mar; EF100340)
Tetraselmis striata (NT, Mar; X70802)
Picocystis salinarum (NT, Saltern ;AF125167)
Pseudoscourfieldia marina (NT, Mar; X75565)
Nephroselmis olivacea (NT, FW; X74754)
Pyramimonas disomata (Tr, Mar; AB017121)
Pyramimonas austrialis (Terra Nova Bay, An, Mar; AJ404886)
Pterosperma cristatum (NT, Mar; AJ010407)
Cymbomonas tetramitiformis (NT, Mar; AB017126)
Pycnococcus provasolii (NT, Mar; X91264)
Dolichomastix tenuilepis (NT, Mar; AF509625)
Crustomastix stigmatica (NT, Mar; AJ629844)
Ostreococcus tauri (NT, Mar; AY329635)
Mamiella sp. (NT, Mar; AB017129)
Mantoniella squamata (NT, Mar; X73999)
Mantoniella antarctica (Lützow Holmbukta, An, Mar; AB017128)
Micromonas pusilla (An, Mar; DQ025753)
Micromonas pusilla (NT, Mar; AY954999)
Mesostigma viride (NT, FW; AJ250109)
Chaetosphaeridium globosum (NT, FW; AF113506)
Chlorokybus atmophyticus (NT, FW; AF408244)
Cylindrocystis brebissonii (NT, FW; AF154439)
Ancylonema nordenskioeldii (Ar, Ter; AF514397)

Chlorophyceae
Ulvophyceae
Trebouxiophyceae
Prasinophyceae
Streptophyta



related non-Antarctic sequences ranged from 0.002 to 0.034 (table S2, electronic supplementary material). Of the 26 distinct Antarctic sequences, 15 are Trebouxiophyceae. Within this clade, six strains (VI11, VI2, VPL1-3, NJ-18, NJ-7 and Ant-1) belong to the Chlorellaceae (Huss *et al.* 1999) and are intermixed with temperate members of the clade. Likewise, the remaining trebouxiophycean Antarctic isolates are scattered among temperate freshwater green algae. One strain, VPL5-6, was most closely related to a *Paradoxia* isolate from the Siberian permafrost, although its exact phylogenetic position could not be determined with satisfactory statistical support. The 18S sequence of isolate SC2-2 from the Schirmacher Oasis was extremely divergent, resulting in a deep branch with uncertain phylogenetic affinity within the trebouxiophycean lineage. Fourteen unique sequences are distributed in the Chlorophyceae clade. Within the Chlorococcales clade, two isolates from Lake Fryxell (VII3 and VI12) are most closely related to strain II4 from Ace Lake, which was found to be sister to a snow alga from Spitzbergen. Strain VPL9-6 is also resolved within the Chlorococcales but its phylogenetic affinity within this clade is uncertain. Four distinct sequences fall within the *Scenedesmus*/*Desmodesmus* clade along with temperate and Arctic members. The remaining Antarctic chlorophycean strains are spread mainly among temperate freshwater representatives.

While the phylogram (fig. S1, electronic supplementary material) reflects the different levels of sequence divergence found between the Antarctic sequences and their closest non-Antarctic relatives, the time-calibrated phylogeny (chronogram; figure 2) provides absolute age estimates for evolutionary events. Estimated ages of the Antarctic lineages (determined as the node separating the Antarctic sequence from its non-Antarctic sister) range from 2.7–9.9 Ma for a number of chlamydomonad isolates (Chlorophyceae) to over 17–84 Ma for the majority of the sequences and 330–708 Ma for the trebouxiophycean isolate SC2-2.

4. DISCUSSION

From five well-distributed regions in both maritime and continental Antarctica, 14 distinct microchlorophyte sequences were recovered. Although there is no absolute threshold of 18S sequence divergence for defining green algal taxa, most Antarctic sequences are divergent enough to be considered distinct species, genera or even higher-order taxa (Lewis & Flechtner 2004; Lewis & Lewis 2005). Our results thus indicate a wide phylogenetic diversity of apparently endemic Antarctic lineages at different taxonomic levels, which has several important implications.

First, our results clearly contrast with morphological studies, which have suggested that Antarctic green algal communities are dominated by cosmopolitan species (Broady 1996), and instead support the notion that Antarctica has developed a distinct regional flora. Given that convergent evolution has led to high morphological similarity between unrelated microchlorophyte species, it is not unusual that molecular data contrast with morphological data. For example, Fawley *et al.* (2004) detected a high number of novel 18S rRNA genotypes during a molecular diversity study in freshwater systems in North America. Similarly, in desert areas of western North America, Lewis & Lewis (2005) discovered that green algae are not merely accidental visitors from aquatic environments, but long-term inhabitants of these extreme environments. Our results thus corroborate the recent studies that have revealed a high degree of endemism in other Antarctic micro-organisms such as cyanobacteria (e.g. Taton *et al.* 2006) and diatoms (Sabbe *et al.* 2003), and refute the hypothesis that for micro-organisms everything is everywhere (e.g. Finlay & Clarke 1999).

Second, our results point to several independent but rare colonization events over a long time frame and long-term survival in glacial refugia as evidenced by the different branch lengths of the Antarctic lineages. Owing to the lack of reliable green algal fossils, our phylogenetic tree is only calibrated at a single node, using a minimum and maximum age of the Chlorophyta–Streptophyta split at 700 and 1500 Ma, respectively (Douzery *et al.* 2004; Hedges *et al.* 2004; Yoon *et al.* 2004; Berney & Pawlowski 2006; Cavalier-Smith 2006; Roger & Hug 2006; Zimmer *et al.* 2007; Herron *et al.* 2009). Nonetheless, this approach allowed us to obtain rough estimates for the divergence times of the different Antarctic microchlorophyte strains. The majority of the lineages (16 out of 26) have estimated ages between 17 and 84 Ma and, based on available sequence data, probably diverged from their closest relatives around the time of the opening of Drake Passage (30–45 Ma) during the Eocene, which initiated the first transient glaciations on the continent. The lineages with longer branch lengths, including SC2-2 (330–708 Ma), have estimated ages that precede the break-up of Gondwana (65–100 Ma). These findings therefore support the hypothesis of the existence of refugia being present during successive glacial cycles (Convey & Stevens 2007; Convey *et al.* 2008) and conflict with the recolonization hypothesis, which proposes that fast colonization rates have resulted in the dominance of cosmopolitan species on Antarctica. Green algal refugia may include ice-free terrestrial habitats as well as ice habitats such as cryoconites and supraglacial ponds. Geological data do not rule out the presence of glacial refugia for aquatic micro-organisms in at least three out of the five major regions included in our study. The McMurdo Dry Valleys have probably had ice-free areas since at least the Mid-to-Late Miocene (up to approx. 14 Ma; Boyer 1979; Prentice *et al.* 1993), with large glacial lakes being present since the Last Glacial Maximum (LGM; Wagner *et al.* 2006). In Prydz Bay, parts of the Larsemann Hills, less than 80 km away from the Rauer Islands and the Vestfold Hills, were ice-free during the LGM (Hodgson *et al.* 2001). Some lakes in this region were shown to contain relict populations of diatoms and copepods (Hodgson *et al.* 2005; Cromer *et al.* 2006), while in other lakes taxa currently present in other Antarctic and sub-Antarctic regions became locally extinct during the LGM (Hodgson *et al.* 2006). In the Lützow Holm Bay region, ice-free conditions in some coastal areas during the LGM can be inferred from ^{14}C dates of *in situ* fossils in raised beach deposits (Miura *et al.* 1998). However, whether freshwater habitats escaped glaciation in this region remains unclear.

Third, our data shed some light on green algal dispersal within the Antarctic continent. Most phylotypes (10 out of 14) were only retrieved from a single ice-free region. This implies that, on the one hand, dispersal

rates within Antarctica could be low and that immigrants are competitively excluded (priority effects), and/or, on the other hand, that our sampling of the Antarctic microchlorophyte flora is currently limited by a small number of sampling sites that may differ in their limnological properties. Only two taxa (II11 and VI11), belonging to the genera *Chlorella* and *Scenedesmus*, were detected in three of the five regions, suggesting that these taxa may have more easily dispersed over the Antarctic continent. This is in agreement with Lawley *et al*. (2004), who found a similar lack of overlap between the eukaryotic biota of 'patterned ground soils' using a clone library approach from several widely separated Antarctic locations, with only a few taxonomic units showing apparently wider distributions. However, extended taxon sampling and more variable molecular markers (such as rDNA ITS) will be required to elucidate phylogeographic patterns of green algal taxa within Antarctica.

While the methods used for both cultivation and tree construction may have biased our results, they clearly highlight that (Antarctic green algae show a remarkable divergence from taxa from other regions). We are therefore confident that our main conclusion, namely that Antarctica is characterized by a distinct microchlorophyte flora, probably as a result of ancient patterns of isolation and long evolutionary history on the continent, will hold as new data become available.

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Issue #3 March 2009

A Comment from the EBA Co-Chairs

As we draw to the end of the massive effort that has been the International Polar Year (IPY), it is opportune to consider what impact it has had on Antarctic biology and its research community. At the outset we need to remember that biology was not a significant component of any of the three previous IPYs. So already a major achievement of this IPY has been its emphasis on a comprehensive cross-disciplinary approach to polar science.

As demonstrated by the familiar 'honeycomb' diagram illustrating the linkages between the almost 200 programmes involved, there has been unprecedented involvement from the international science community, based on whatever measurement of financial, personnel, international or disciplinary involvement is employed.

Particular features have been the number of cross-disciplinary and bipolar programmes involved, and the prominence given to outreach and education activities. This is clearly a reflection of the diversity and energy that currently characterises the polar research communities, within which biology is no exception.

The encouragement of young scientists has led to the development of APECS, the Association of Polar Early Career Scientists, which has already established itself with a very active and engaging presence at last year's SCAR Open Science Conference in St Petersburg, and is a real contribution to planning 'succession' in our community (to use an ecological analogy!).

The opportunities provided by IPY programmes on the shorter term, and SCAR science programmes on the longer term, can develop and integrate those of the often more fragmented efforts of individual researchers, research groups and national programmes. Without trawling through all of the programmes within the IPY honeycomb which have a biological element, we have already seen in previous issues of this Newsletter how some of these programmes are taking forward this aim, with good examples provided by for instance CAML, MERGE, and the many international collaborations facilitated by the Spanish national programme this season on Livingston Island.

Now that IPY is drawing to a close, we face the serious but vitally important challenge of how to sustain the level of new activities that have been fostered. An important element of IPY planning has always been 'legacy'. For example, and from a scientific perspective, a new research station is indeed a solid investment in the future, and several national programmes have invested significant sums of 'new' money in infrastructure developments. However, the dividends of such investment will only be realised if these same operators commit to supporting the research, both nationally and through international collaborations, that these facilities enable.

We look forward to a successful and productive 2009 and hope to see many of you in Sapporo for the SCAR Biology Symposium.

Pete Convey & Guido di Prisco
EBA Co-Chairs

Welcome from the SCAR President - Chuck Kennicutt

Colleagues, I appreciate this opportunity to address the EBA community. I have instituted a monthly "Notes from the President" to communicate with SCAR Delegates on a regular basis*. The topic of the January note was the excellent outcome of the first, 4-year external review of SCAR's Scientific Research Programs, including EBA. By now most of you should have been provided the external review comments. If you have not, the SCAR Secretariat can provide them.

It can not be said often enough that at the core, SCAR is an international, interdisciplinary scientific organization. Everything SCAR does and how SCAR is perceived as an organization is rooted in the quality and timeliness of SCAR's scientific portfolio. While SCAR has an equally important mission in its advisory capacity to the Antarctic treaty System, this can only be effective if we are scientifically strong.

In such dynamic times the renewal of SCAR's scientific programs is essential to the continuing health of the organization. Continuous improvement of SCAR's scientific portfolio is assured by procedures for program planning, proposing, implementation, reporting and review. A Cross-Linkages workshop, which was an incubator for the generation of new ideas, was held in early February 2009. The report from this workshop can be found on the SCAR website at: www.scar.org/researchgroups/ Under 'Cross-Linkages Workshops'.

The positive review of EBA is a reflection of the excellence of the science conducted, how that science is communicated to the larger world, and the international partnerships generated. I will not repeat the external reviews of EBA but a few items are important.

Reviewers stated that EBA has resulted in many publications that engage all fields of biology. EBA has contributed directly to increasing knowledge and has led to international efforts to synthesize knowledge. EBA has contributed to public understanding of Antarctica and is excellent value for the money invested. These comments are illustrative of the strength and quality of the EBA program.

In closing my note, I state that SCAR's excellent science is achieved by volunteers, from the scientists that come together to propose and implement the programs to those that provide assessments, review, and oversight. I can think of few other more meaningful metrics of the health of an organization than the willingness of the community it serves to participate in assuring success.

I congratulate the EBA team on a job well done and I look forward to even greater scientific accomplishments in the future! Keep up the excellent work!

*** "Notes from the President" can be viewed on the SCAR website at: www.scar.org/communications/presidentsnotes/**

Personal Highlights of 2008 from some of the EBA Work Package Leaders

WP 1: Evolutionary history of Antarctic organisms

Dominic Hodgson

In the last newsletter we described a recent review paper by EBA participants (Convey et al. 2008, Biological Reviews 83: 103-117), which describes the evolutionary history of Antarctic organisms in the terrestrial realm from Gondwana to the present. This paper is important as it shows that much of the contemporary Antarctic terrestrial biota has been continuously isolated in situ on a multi-million year, even pre-Gondwana break-up timescale. In the last ten months we have been reviewing parallel evidence in the marine realm. Recent analyses confirming a high incidence of species-level endemism, marked regionalisation of fauna and decreasing South American affinity in a circumpolar manner away from the Antarctic Peninsula, all point to a long and continuous existence of a marine fauna in at least some locations on the shelf. In a direct analogy to the terrestrial realm, it is clear that while much geophysical evidence demonstrates that large areas of the continental shelf were over-ridden by grounded ice or rendered inhospitable by permanent ice-shelf coverage or frequent iceberg-scouring during the last glacial period (and by inference at previous glacial maxima), clear biological evidence points to the persistence of at least some elements of the marine fauna in dynamic refugia.

Looking forward to the 2009 SCAR Biology Symposium in Japan, more than 30 abstracts have been submitted in the Antarctic Evolutionary History sub-theme. These cover a wide range of the latest research linked to EBA Work Package so it should be an excellent session.

WP 3: Patterns of gene flow and consequences for population dynamics: Isolation as a driving force

Ian Hogg

As the International Polar Year draws to a close, there has been considerable activity in the Ross Sea region. The New Zealand-led Terrestrial Biocomplexity Programme (nzTABS), has recently completed its second, and major, field season. Over 30 scientists from 8 countries were involved including NZ, US, UK, Canadian, German, Spanish, Austrian and Australian personnel. This is one of the largest terrestrial biology programmes ever undertaken by Antarctica New Zealand and was a resounding success. Over 450 samples were collected by the field teams and are currently being analysed for a range of biota including bacteria, fungi, lichens, moss, and micro- and macro-invertebrates. This combined with detailed geochemical analyses will be combined into a GIS model to determine factors influencing the distribution of the biota. Genetic analyses will be used to determine connectivity and gene flow among the locations. Next year's field season will be used as an opportunity to ground-truth predictions based on the GIS model, and to supplement sampling from this season. Much of the gene flow work is being led by Byron Adams (Brigham Young University) – microinvertebrates, Ian Hogg (University of Waikato) – arthropods, and Allan Green and co. (Universities of Waikato and Madrid) – lichens and mosses. This recent work combined with previous/existing data will see a number of EBA and WP3-related publications from several sources, including work from the Italian programme from Francesco Frati and colleagues at the University of Siena. We look forward to a productive conclusion to the IPY.

WP 5: Impact of past, current and predicted future environmental change on biodiversity and ecosystem function

David Renault

I would like to highlight the publication of the book: The Biology of Polar Regions (DN Thomas, GE Fogg, P Convery, CH Fritsen, JM Gili, R Gradinger et al.)

"There is now an increased awareness of the importance of polar regions in the Earth system, as well as their vulnerability to anthropogenic derived change, including of course global climate change. This new edition offers a concise but comprehensive introduction to polar ecology and has been thoroughly revised and updated throughout, providing expanded coverage of marine ecosystems and the impact of humans. It incorporates a detailed comparison of the Arctic and Antarctic systems, with a particular emphasis on the effects of climate change, and describes marine, freshwater, glacial, and terrestrial habitats."

Julian Gutt

The World Marine Biodiversity Conference was held in Valencia in November 2008 with several contributions of high scientific value on Antarctic topics; they were mainly related to CAML. From the perspective of Work Package 5 it might be interesting to know that the "Valencia Declaration on the Protection of Marine Biodiversity" was made. The major objective is evident from the title. For details see: www.marbef.org

Another highlight was the formation of the Cross-SSG Action Group on Prediction of Changes in the Physical and Biological Environments of the Antarctic, of which I am a co-chair. See page 8 for more details on this.

WP 2: Evolutionary adaptation to the Antarctic environment

Takeshi Naganuma

See page 6 For a description of Takeshi's exciting work on the Byers Peninsula last season in collaboration with a Spanish IPY project.

WP 4: Patterns and diversity of organisms, ecosystems and habitats in the Antarctic, and controlling processes

Satoshi Imura

My highlight is that I spent the 2007-2008 austral summer in Syowa Station, not as a scientist, but as the leader of the 49th Japanese Antarctic Research Expedition team. Also, on Jan. 26, 2009, a scientific meeting on the biodiversity under extreme environments (Japanese local meeting) was held in the National Institute of Polar Research, Tokyo, Japan. 19 scientists from various research organizations joined in the meeting, exchanged their newest outcomes and discussed future plans.

Other EBA Committee Members

Co-Chairs:
Guido di Prisco
Peter Convey
Secretary & SCADM Representative
Shulamit Gordon
CO LSSSG (ex officio)
Kathy Conlan
Members:
Dana Bergstrom
Angelika Brandt
Marc Lebouvier (conservation matters)
Other Work Package Leaders
Brigitte Ebbe (WP1)
Daniel Costa (WP2)
Elie Poulin (WP3)
Lucia Campos (WP4)
(go to www.eba.aq for emails)

The Latitudinal Gradient Project (LGP)

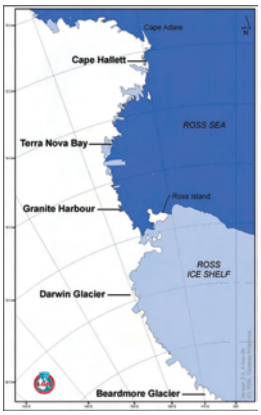
www.lgp.aq

Shulamit Gordon (s.gordon@antarcticanz.govt.nz) - Project Manager, Antarctica New Zealand

The Latitudinal Gradient Project (LGP), is an international, multi-disciplinary project that involves researchers mostly from New Zealand, Italy, and the United States. The aim of the project is to look at how marine, terrestrial and freshwater ecosystems vary along the north-south latitudinal gradient of the Victoria Land coast in the Ross Sea region.

The LGP encourages collaboration between different disciplines, particularly by concentrating research at specific sites, thus enabling direct comparisons between sites and allowing for a "complete ecosystem" picture to be built.

In the late 1990s researchers from various disciplines working in the Ross Sea region began to seriously discuss the possibility of using the latitudinal gradient along the Victoria Land coast from Cape Hallett at 72°S to the southern tip of the Ross Ice Shelf at 86°S, as a proxy for climate change. Several workshops were held on the issue, beginning at the 1998 SCAR biology symposium in Amsterdam, and over the course of a few years the LGP framework was developed.



Approximately 18 terrestrial, marine or freshwater research projects are being conducted at five pre-selected locations: Cape Hallett (72°S), Terra Nova Bay (75°S), Granite Harbour (76° S), Darwin Glacier (79° S), and Beardmore Glacier (83°S), and there are plans for an additional site at New Harbour (77°S). So far, over half of these sites have been visited.

The LGP's general hypothesis is that *ice-driven dynamics control the structure and function of marine, terrestrial and freshwater ecosystems along the Victoria Land coast*. Supporting this hypothesis, eight key questions were formulated:

1.How does ecosystem structure and function change with latitude, and why?
2.What is the role of persistent, large-scale ice structures in defining community composition?
3.How do snow and ice dynamics influence ecosystems and ecosystem processes?
4.How does climate affect the availability and composition of free water?
5.How does climate affect the predictability, persistence and extent of sea ice cover?
6.How are key marine biological processes influenced by sea ice conditions?
7.How does soil development influence terrestrial ecosystems?
8.To what extent are past conditions preserved in paleoindicators?

All research conducted under the LGP umbrella is working towards answering at least one of the eight key questions.

Each nation's approach and contribution to the LGP are different. New Zealand has nine LGP-related research projects and Antarctica New Zealand has set aside money and manpower to be used specifically for the LGP with the appointment of a Project Manager, Shulamit Gordon, to coordinate New Zealand's LGP-related research activities. The Italians have four terrestrial programmes and several marine programmes within the Terra Nova Bay region, and have cooperated with New Zealand on a number of projects.

The United States' contribution to the LGP is mainly through the Long Term Ecological Research (LTER) Network and it's site in the McMurdo Dry Valleys (www.mcmlter.org). A group from the LTER has also cooperated with New Zealand scientists at Cape Hallett, the northernmost LGP site. What unifies all the scientists from the different national programmes are the main hypothesis and the eight key questions that the LGP is trying to answer.

The scientists working within the framework of the project won't be able to make a full comparison of sites until all the sites identified have been visited. However, each project is already building a picture of the differences or similarities between sites that have been visited so far. Examples of these finding are:

- Prof. Allan Green from the University of Waikato and his collaborators from Germany, Austria, Spain and Australia have found that lichen species numbers are high in the maritime Antarctic, but relatively constant and low on the continent. They have also found no apparent decline in the number of species found along the Victoria Land coast as latitude increases.

- Dr. Mark Stevens, formerly at Massey University, has shown that a large proportion of invertebrate animals living on the continent have survived throughout multiple glacial cycles, over millions or tens of millions of years, in refugia. The observed distributions may have nothing to do with the latitudinal gradient, but rather pockets of some species surviving under unusual conditions.
- Dr Jenny Webster-Brown from the University of Auckland has observed that nitrate and calcium concentrations of inland and coastal aquatic ponds increase both with latitude and distance from the coast.
- The McMurdo LTER team have found that the distribution of metazoan and microbial communities in N. and S. Victoria Land are linked to the source of organic matter and soil geochemisty.
- Dr. Vonda Cummings and her team from New Zealand's National Institute of Water and Atmospheric Research have determined that a variety of characteristics of marine benthic communities along the Victoria Land coast are linked to differences in ice conditions between locations.

- Dr Ken Ryan from Victoria University of Wellington has found that low salinity, high visible and UVB radiation, and changing temperatures impact the productivity of sea ice microbial communities and this may influence summer bloom events.

A significant compilation of 18 LGP-related papers was published in a 2006 special edition of *Antarctic Science*. A second LGP publication is set for 2010 with about 30 papers envisaged - this time including British work from the Peninsula.

Building on the success of the LGP, an EBA sponsored workshop was held in May 2008 to explore gradient comparisons between Victoria Land, the Antarctic Peninsula and other areas in Antarctica. This is the next step in the development of an Antarctic Gradients concept, creating linkages and providing the means for comparisons across the continent.

The LGP is a key project of the EBA programme, contributing to work packages 4 and 5.

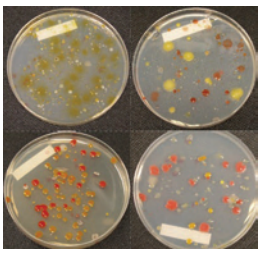
Text kindly contributed from articles on the LGP written by Joseph Cheek for the International Polar Foundation's SciencePoles website www.sciencepoles.org/

Images: Centre - Lichen at Cape Hallett (Catherine Beard K024 04/05). Above - Marine benthic organisms at Terra Nova Bay (Rodd Budd K082 06/07) - Antarctica New Zealand Pictorial Collection

AMBIO: Antarctic Microbial Biodiversity: The Importance of Geographical and Ecological Factors www.ambio.ulg.ac.be

Annick Wilmotte (awilmotte@ulg.ac.be), Wim Vyverman (wim.vyverman@UGent.be), Anne Willems (anne.willems@UGent.be)

AMBIO is a 'Science for a Sustainable Development' project funded by the Belgian Federal Science Policy (2007-2011) and forms a partnership between three Belgian laboratories from the University of Liège and Ghent University.



AMBIO aims to improve our knowledge of the biodiversity, community composition and taxonomic turnover of cyanobacteria, heterotrophic bacteria and protists (diatoms and green algae) in Antarctic terrestrial aquatic ecosystems (including lakes, cryoconites and seepages). To this end, samples were collected from regions at a circum-Antarctic level and along a latitudinal gradient ranging from high-latitude continental sites in East and West Antarctica (including the site of the future Belgian Base near the Utsteinen nunatak), to the Sub-Antarctic islands.

The aims of the project include:

1.To expand the existing database of rRNA operon sequences and the culture collections of bacteria, cyanobacteria and microalgae;

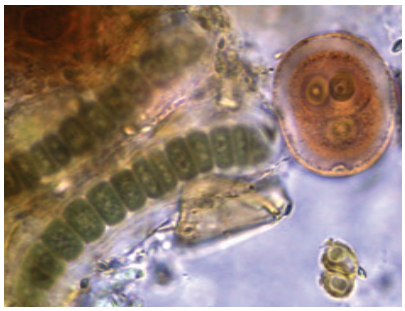
2.To study the microbial diversity in wet terrestrial habitats in the three biogeographic regions of the continent: Sub-Antarctica, Maritime and East Antarctica;

3.To study the taxonomic turnover between different habitats and among comparable habitats along ecological and geographical gradients to analyse the congruence and disparity in patterns of diversity and turnover observed for different taxa;

4.To study more in-depth distribution of selected taxa;

5.To identify regions of unique microbial diversity that deserve to be protected as Antarctic Specially Protected Areas.

The AMBIO project explicitly contributes to the IPY initiative MERGE and the SCAR EBA program (e.g. data from AMBIO are deposited in the EBA database). First results reveal the presence of genotypes with a restricted distribution, potentially endemic in all groups, as well as microbial diversity that had not previously been found.



Images from left to right: bacterial cultures; diatom (Pinnularia microstauron); cyanobacteria from the Penguin mountains (Asterocapsa and Coleodesmium genera are respectfully unicellular and filamentous)

The Evolution of Octopuses in and Around Antarctica

Louise Allcock (louise.allcock@gmail.com), Martin Ryan Marine Science Institute, National University of Ireland, Galway

Over 10 years ago it first became apparent from detailed morphological taxonomy that there were curious similarities between octopus genera endemic to Antarctica and genera normally associated with the deep ocean floor. The lack of hard anatomical structures means that octopus systematics is notoriously chaotic and, at that time, the prevailing view was that these genera were not particularly closely related.

To overturn these opinions, and investigate the relationship further, it was clearly going to be necessary to employ molecular techniques, and what followed was the very slow process of collecting tissue samples from deep-sea specimens. Octopuses have good vision and can move quickly, so catching them in deep trawls is not easy.

However, research programmes worldwide, including programmes such as ANDEEP, slowly began to yield specimens. In 2004, the UK's Natural Environment Research Council funded the research and Jan Strugnell joined the project as a post-doc, bringing expertise in 'relaxed phylogenetics'. These are cutting edge techniques that supersede the problematic 'molecular clock' methods and allow co-estimation of phylogenies and divergence times. The techniques are computationally expensive and the parallel computing clusters at the British Antarctic Survey and Queen's University Belfast were put to work.

We determined that the deep-sea octopuses had their evolutionary origins in Antarctica, and that they had evolved and diversified during periods of intense climate change in Antarctica. We hypothesise that the development of the Antarctic Circumpolar Current and the accompanying strengthening of the thermohaline circulation were drivers of evolution (Strugnell et al., 2008).

This research was highlighted in the 4th Census of Marine Life report and as a consequence brought evolution and Antarctica into the headlines for a few days. In addition, the results of this study have been included in the new 'Google Oceans' release within Google Earth 5.0 (http://earth.google.com/). Jan Strugnell (jan.strugnell@gmail.com) is now an independent fellow at Cambridge University but we continue to work closely and we have been looking at the microevolution of some endemic Antarctic species using a combination of the COI barcode gene and microsatellites. We hope to be able to use these data in the future to explain some of the microscale processes driving evolution in Antarctica.

Strugnell, J., Rogers, A.D., Prodöhl, P.A., Collins, M.A. & Allcock, A.L. (2008) The thermohaline expressway: the Southern Ocean as a centre of origin for deep-sea octopuses. *Cladistics*. 24:853-860.

Images courtesy of Dave Barnes.

Polish Projects Contributing to the EBA Programme

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As a member of the Antarctic Treaty, Poland, through the Polish Academy of Sciences, runs the Henryk Arctowski Scientific Station, which has been active year-round since 1977. The station is situated on King George Island in the South Shetlands Islands. In the vicinity of the station there are protected areas recognised by SCAR, COMNAP, and the Antarctic Treaty.

H. Arctowski Station's Buildings

Henryk Arctowski Station is managed by The Department of Antarctic Biology of the Polish Academy of Sciences (DAB PAS) and supports studies in Antarctica in the fields of ecology, oceanography, limnology and trophochemoreception in polar region.

The DAB PAS has undertaken a scientific program entitled "Changes and variability of polar ecosystems" from the Henryk Arctowski Station over the past few years. The program was started by Prof. Stanisław Rakusa - Suszczewski (the former director of DAB PAS). The main subjects of the program were:

1. Interactions in Admiralty Bay within the coastal zone (King George Island);
2. Monitoring of selected flora and fauna and trophic interaction of coastal ecosystems;
3. Admiralty Bay coastal zone (King George Island);
4. Invertebrates and fish chemoreception;
5. Management of protected areas – ASPA 8 and ASPA 34;
6. Monitoring of tourism and its influence on the environment in the neighborhood of Arctowski Station.

Nowadays the DAB PAS cooperates with other scientific institutions that work in the H. Arctowski Station area in the fields of:

- Marine and terrestrial microbiology, genetics, botany and ecology;
- Marine phyto- and zooplankton investigations;
- Geology and glaciology.

The DAB PAS has also taken part in the IPY programs:

- ClicOPEN: ecology, microbiology – reaction of biota on climate changes and glacier retreat;
- ACE: geology – Antarctic Climate Evolution;
- ALIENS: biology – Aliens in Antarctica;
- AMES: marine biology, avian and mammal monitoring.

Another programme Poland is involved in is POLARCAT – bipolar monitoring of the atmospheric precipitations contaminations.

Tourist visit on Polish Antarctic Station

In the Antarctic summer season of 2008/2009 the following projects were undertaken from the H. Arctowski Station:

1. *Impact of deglaciation on polar ecosystems formation and development by microorganisms.* Project participants: the DAB PAS, the University of Warsaw, Gdańsk University of Technology, the University of Warmia and Mazury in Olsztyn – co-ordinator Dr M. Zdanowski (DAB PAS). The project is a part of international program in IPY framework – ClicOPEN No 34;
2. *Microbiocoenosis formation under conditions of deglaciation in polar fiords* – co-ordinator Dr K. Jankowska from Gdańsk University of Technology;
3. *Impact of climate warming on terrestrial and marine ecosystems in the Antarctic maritime* – leader Prof. A. Tatur (DAB PAS), grant connected also with ClicOPEN No 34. Project participants are from 7 polish scientific centers. Main topics of the project are: Ecophysiology of marine invertebrate in climatic stress ; Dynamics of benthic ecosystems near glaciers; Dynamic of bay's water in mixing zone; Succession of fauna and flora in new areas exposed by retreating glaciers; Impact of climate on allopathic genetic dispersion seals and penguins;
4. *Biological monitoring of birds and pinnipedian mammals* – co-ordinator Dr M. Korczak (DAB PAS);
5. *Alien species in Antarctica* – co-ordinator Dr K. Chwedorzewska (DAB PAS), grant in the framework of IPY projects – ALIENS No 170;
6. *Heavy metals and radionuclides in Antarctic environment* – co-ordinator Prof. M. Olech (Jagiellonian University in Krakow, DAB PAS);
7. *Maps of flora in the areas of planned reserves* - Turret Point, Three Sisters, Penguin Island – co-ordinator Prof. M. Olech (Jagiellonian University in Krakow, DAB PAS).

All projects and grants will be continued in the following years.

The H. Arctowski Polish Antarctic Station is the converging point of great scientific potential from Poland and it is open for collaboration with scientists from all over the world.

Main building of H. Arctowski Station

If you would like to collaborate with, or contact, any of the Polish programmes listed above, please contact Agnieszka Pociecha at pociecha@iop.krakow.pl

Polar Barcode of Life Initiative (PolarBOLI)

Ian Hogg (hogg@waikato.ac.nz) - School of Biological Sciences, University of Waikato, New Zealand

The International Barcode of Life Initiative (iBOL) seeks to ultimately obtain short sequences of the mitochondrial DNA COI gene from representatives of all eukaryotic life. The purposes of the initiative are to streamline routine identification of taxa (e.g. for biomonitoring and biosecurity), and to assist with the identification and cataloguing of the Earth's biodiversity. In order to facilitate this venture, a number of smaller initiatives have been developed to draw upon existing taxonomic expertise and collection capacity (e.g. sampling expeditions, museums etc.).

One of these initiatives is the Polar Barcode of Life (PolarBOLI) which will focus on biodiversity in both polar regions. All major ecosystems and animal-life south of the Antarctic convergence are included in the initiative. Much work has already been undertaken with the various IPY Southern Ocean cruises and terrestrial programmes. However, the EBA community can assist greatly and further contribute to this venture. For more information on iBOL or PolarBOLI please visit www.dnabarcoding.org (iBOL), or contact Ian Hogg (hogg@waikato.ac.nz).

Biodiversity, Function, Limits and Adaptation for Molecules to Ecosystems (BIOPEARL)

Dominic Hodgson (daho@bas.ac.uk) - British Antarctic Survey, UK

The BIOPEARL project led by Katrin Linse at the British Antarctic Survey, with collaborators from across EBA, has recently published a series of papers on a cruise to the Scotia Sea (*Antarctic Science* 2008, 20, 3 – special issue) looking at the evolutionary history of Antarctica and its marine fauna. The cruise took place in 2006 aboard RRS *James Clark* with researchers studying the faunal assemblages, investigating molecular phylogenetic relationships in selected species groups and examining the Holocene sediment records.

Research highlights include surveys of the faunal richness in marine, intertidal, freshwater, terrestrial and parasitic realms in the South Orkney Islands published by Barnes et al. (*Journal of Biogeography* 2008) which reveal a rich regional biodiversity with more species than the Galapagos! As part of the Census of Antarctic Marine Life they collated results of SCUBA, trawl and terrestrial surveys with a century of literature and modern databases to investigate patterns in species accumulation, endemism, and faunistic affinities in each realm. Results of just 11 benthic samples yielded 19 classes and 158 species. Nearly a third were new to the area, whilst five species and one genus were new to science. As suspected but never quantified, the marine benthos dominates polar biodiversity, at least at the South Orkney Islands. Marine species there constitute 20% of those recently listed for the entire Southern Ocean, whilst > 60% of terrestrial species are known from Antarctica.

Dr Jan Strugnell with one of the octopus specimens from the BIOPEARL cruise

Including samples from the same cruise the work of Strugnell et al. (*Cladistics* 2008, 24, 853–860) has identified the Southern Ocean as a centre of origin for deep-sea octopuses with the initiation of the global thermohaline circulation providing the mechanism for the radiation of Southern Ocean fauna into the deep sea (see article on page 4).

Using a relaxed phylogenetic approach to coestimate phylogeny and divergence times the Authors trace the evolutionary origins of the deep-sea Octopus lineage to Antarctica, and identify lineage diversion at 33 million years ago (Ma) with a subsequent radiation at 15 Ma. As both of these dates are critical in development of the global thermohaline circulation the research suggests that this has acted as an evolutionary driver enabling the Southern Ocean to become a centre of origin for deep-sea fauna. This is the first unequivocal molecular evidence that deep-sea fauna from other ocean basins originated from Southern Ocean taxa.

Participating inthe Spanish IPY Campaign on the Byers Peninsula 2008-2009

Takeshi Naganuma (takn@hiroshima-u.ac.jp) - School of Biosphere Science, Hiroshima University, Japan

I had a chance to participate in the Spanish Antarctic IPY campaign on the Byers Peninsula (08-09), kindly organized by Prof. Antonio Quesada, Universidad Autonoma de Madrid (UAM), Spain. The campaign was the major field activity of the Spanish program LIMNOPOLAR that has invited a number of foreign researchers (and I was the first Japanese participants). Prof. Ana Justel (camp leader) and Prof. Eugenio Rico, UAM, and Dr. Dermot Antoniades, Laval University, Canada, and I (along with two Spanish technicians) spent eight days in Byers Peninsula, Livingston Island, maritime Antarctica. The peninsula has been specified as Antarctic Specially Protected Area (ASPA) 126. The island is known for a simple weather forecast "today, windy; tomorrow, windy; always, windy", and we enjoyed a simply cheerful and fruitful atmosphere throughout the stay.

The title of my activity in Byers was "phylogeographic characterization of cosmopolitan bacterial species" and I collected more than 80 rock and sand samples to isolate my target microbial cosmopolitans. Cosmopolitans are species that show global occurrences. Diversity of certain cosmopolitan species (that differ from each other at sub-species levels) are regarded to reflect evolution or divergence caused by relatively recent environmental (*e.g.*, biological, ecological, geological, astronomical and/or climatological) events. A derived hypothesis is that evolutionary divergences and geographical distributions of "endemic strains" of cosmopolitan species provide 1) implications for past (but not too remotely past) global/local events, and 2) predictions for potential bio-/ecological influence of current global changes. This hypothesis is tested by collection of strains from as many habitats as possible, including the Byers Peninsula.

An idea behind the hypothesis is the well-known Baas Becking's laws: "everything is everywhere" and "the environment selects". The laws have been mainly tested by isolation and characterization of "endemic" species, whose occurrences have often been correlated to specific environmental parameters as controlling factors. In contrast, my approach to Baas Becking is to collect "cosmopolitan" species from as many and various sites as possible to see the extents of their global-wide occurrences.

One example of cosmopolitans at my lab is *Bacillus licheniformis*. Two strains, one from the Gobi Desert and the other from the Gobi-originated yellow dust storm show a 100% identity of near-full-length 16S rRNA gene sequences, while less-than 100% (but >97%) similarities are shown with many other *B. licheniformis* strains from other parts of the Earth. These strains provide information about the extents of "unit regions" of airborne transportability.

My lab has also isolated *B. pumilus* from the Greenland glacier surface. Of course, this species is known to be a real cosmopolitan, but detailed genotypic and phenotypoic comparisons have not been made so far. A 100% identity in 16S rRNA gene sequence, for example, is needed to discuss realistic phylogenetic-biogeographic (*i.e.*, phylogeographic) distribution of a single species, while strain-to-strain variability (*i.e.*, less than 100% but >97% similarities) would also provide evolutionary and climatic dispersal processes.

Therefore, the major purpose of this study is to isolate and characterize cosmopolitan species from the maritime Antarctic. Isolation of representative cosmopolitan species from Byres will greatly facilitate understanding evolutionary and climatic dispersal processes. Target organisms are euryhaline halophiles of both spore-formers and non-spore-formers as follows: spore-forming genera belonging to the spore-forming genera of *Bacillus*, *Gracilibacillus*, *Halobacillus*, *Paenibacillus* and *Virgibacillus*; and non-spore-forming genera of *Chromohalobacter*, *Halomonas* and *Marinobacter*. My particular target organisms are *B. licheniformis* and *B. pumilus* that are known to be the most die-hard organisms against various stresses.

My students and I expect to contribute to EBA in terms of: 1) isolations and phylogenetic-phenotypic characterizations of moderately halophilic microorganisms as candidates for "cosmopolitans"; 2) biogeography of selected "cosmopolitans"; and, 3) description of new species, together with the wonderful Spanish and Canadian comrades.

DATA AWARENESS

SCAR-MarBIN: free and open access to Antarctic Marine Biodiversity Data

Bruno Danis - bruno.danis@scarmarbin.be
www.scarmarbin.be

To date, the two main achievements of MarBIN are:

1. The development of the Register of Antarctic Marine Species (RAMS); and
2. The development of a distributed network of interoperable databases holding information on Antarctic marine biodiversity. MarBIN now offers access to 120 datasets, including almost a million distribution records, and information on more than 13,000 taxa.

For the end of the IPY, SCAR-MarBIN has committed to achieve the following goals:

1. A complete Species Register (almost done);
2. Design a new data portal (almost done);
3. Have 100 interoperable datasets (done);
4. Have 1000000 occurrence records (almost done).

The new data portal includes many new features, two of which I'd like to focus on here:

DNA data taken onboard: SCAR-MarBIN now directly interfaces with GenBank, through deeplinking. You will be able to search for sequenced-only records and confront occurrence data with the corresponding sequences (e.g. how do pycnogonids from the Weddell Sea genetically compare to those sampled along the Scotia Arc?). Thousands of sequenced records will be immediately available, thanks to the efforts of Rachel Grant, the CAML barcode manager.

3D visualization: the new SCAR-MarBIN data portal includes new visualisation tools, in 2D (using Openlayers) and 3D (using the new Google Earth plugin). These tools are extremely powerful and are free. The 3D version will allow us to take advantage of Google Earth's new feature, which allows us to display items in 3 dimensions, including depth.

SCAR-MarBIN V2.0 includes a lot of cool features, which should satisfy our most advanced users as well as new visitors and even non-specialists. Jump in! Visit the website, play around and help us improve it. You could also check out:

MarBIN on Facebook: http://www.facebook.com/group.php?gid=41137217190&ref=ts

MarBIN on YouTube: http://www.youtube.com/user/scarmarbin

Terrestrial Biodiversity Database Functions

Dave Watts - Dave.Watts@aad.gov.au
http://data.aad.gov.au/aadc/biodiversity/index.cfm

One of the key functions of the Antarctic Biodiversity Database that is hosted by the Australian Antarctic Data Centre is the search and discovery link across various databases such as the SCAR Map Catalogue and the SCAR Composite Gazetteer of Antarctica. Here are two examples to illustrate these links:

Example 1 - I need to find all taxa observations near Transkriptsii Gulf, Bunger Hills:

1.Go to the SCAR Composite Gazetteer at http://data.aad.gov.au/aadc/gaz/scar/;
2.Use the search page with the word 'Transkriptsii';
3.Click on the Australian entry;
4.This page shows details of the location. Scroll down the page to where it gives links to species seen within one degree of this location;
5.Click on 'All' to find observations or additional taxa details.

The same approach to list nearby taxa is on the SCAR Map catalogue where you can list taxa within the bounds of the map (along with any place names).

Example 2 – I want to list all species within the order 'Lepicoleales' (There are two approaches to this):

1.Use the Advanced Taxonomy Search page at http://data.aad.gov.au/aadc/biodiversity/search_taxon.cfm;
2.Enter the order name 'Lepicoleales' and select the search option of 'Hierachy search - Kingdom/Phylum/Class/Order';
3.The results are shown as a tree-like structure. Follow the link on the required order name to drilldown into the family, genus links.

OR

1.Use the 'Quick name search' option on the 'Taxonomy' tab at http://data.aad.gov.au/aadc/biodiversity/index.cfm;
2.This searches for genus, species so we enter a known genus of this order such as 'Trichocolea';
3.Click on any of the resultant 5 species names to see an individual page about that species. At the top is a taxonomic hierarchy where you can use these links to the required order.

From these examples you are continually being linked between the Biodiversity Database, the map catalogue and the composite gazetteer. This is all possible by directly hosting and managing the map catalogue and the gazetteers within the one database. Please feel free to explore these connections and their usefulness and if you have any suggestions for improvements, please contact the Data Centre via the request form seen on each page at the top right.

Message from Kim Finney - Chair of SCAR's Standing Committee on Antarctic Data Management (SCADM)

http://scadm.scar.org

In January 2009 the Joint Committee on Antarctic Data Management (JCADM) underwent a name change after COMNAP withdrew sponsorship for the group. It is now the SCAR Standing Committee on Antarctic Data Management (SCADM). Kim Finney, Manager of Australia's Antarctic Data Centre is the new Chief Officer and Shulamit Gordon is the group's new Deputy Chief Officer, along with Helen Campbell (British Antarctic Survey).

Following the St Petersburg delegates and business meetings held in July 2008, the SCAR Executive appointed an Ad-hoc Action Group to finalise the SCAR Data and Information Strategy, prepared for SCAR by JCADM (now SCADM). Dr Sergio Marenssi is the Chair and Dr Kathy Conlan is representing the Life Sciences Group. The SCAR Data and Information Strategy was presented and discussed at the recent SCAR Cross-Linkages Workshop held in Modena, Italy (February 2009). Dr Dominic Hodgson (British Antarctic Survey) represented EBA at the workshop. The final draft of the Strategy will be presented to EXCOM for endorsement at the August business meetings in Punta Arenas. Development of an implementation plan, which will follow, will be undertaken collaboratively with a range of partner organisations under the umbrella of a funded CODATA sponsored project aimed at developing a Polar Information Commons.

What's New with the Standing Scientific Group on Life Sciences

Kathy Conlan - Chief Officer SSG-LS
kconlan@mus-nature.ca
www.scar.org/researchgroups/lifescience/

Expert Group on Human Biology and Medicine
HB&M members continue to undertake the majority of the medical research carried out in the Antarctic and three new members have recently been recruited. HB&M is currently developing a cooperative project on bone density loss in the Antarctic and is investigating the potential for Arctic collaborations. The results on Antarctic seabirds as vectors for Lyme disease is going to publication.

Expert Group - Continuous Plankton Recorder (CPR)
About 90 CPR tows around Antarctica were made in 2007/8 off 8 vessels, which covered 25,000 nautical miles and produced 5000 sample records. New Zealand will add CPR's to the toothfish fishing vessels that run between NZ and the Ross Sea while the South American LA-CAML consortium will add CPR tows across the Drake Passage. The CPR results show a large scale change from krill to small copepods and a large increase in foraminiferans. Globally, a world-wide reduction in copepod size has been found. Some of the important applications of CPR studies are in monitoring distribution patterns and assisting CCAMLR for its fisheries needs.

Cross-SSG Action Groups
Three new Action Groups have recently been formed with Life Sciences participation:
1. Prediction of Environmental Changes: Julian Gutt (Ger), Martin Riddle (Aus), (see article this page);
2. Subglacial Aquatic Environments: Warwick Vincent (Chair, Can), Guido di Prisco (Italy), Takeshi Naganuma (Japan);
3. King George Island: Doris Abele (Ger).

New Cross-Linkages:
Geological and Life Sciences:
The new Action Group-Seeps and Vents aims to locate, describe and compile guides for the identification of cold seep features and hydrothermal vents and their associated fauna (see article on this page).

Physical and Life Sciences:
Ocean Acidification is of great concern for the Southern Ocean ecosystem and research has been expanding rapidly. Within SCAR, this issue has been handled by the Expert Group on Oceans. The SSG-Life and Physical Sciences have recently become co-partners in the EG-Oceans.

Environmental Code of Conduct for Terrestrial Field Work
After extensive consultation within SCAR and COMNAP, the Environmental Code of Conduct for Terrestrial Field Work will be submitted as an Information Paper to the ATCM and CEP in June 2009.

Seeps and Vents Action Group (SAVANT – Seeps and Vents ANTarctica)

Dr Philip O'Brien - Action Group Coordinator (Phil.OBrien@ga.gov.au)

The Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR) has been charged with developing management practices for Vulnerable Marine Ecosystems (VMEs) in Antarctic waters. The VMEs identified as having a high priority by CCAMLR (Conservation Measure 22-06) are: biological communities associated with seamounts, cold seeps and hydrothermal vents, cold water coral and sponge communities.

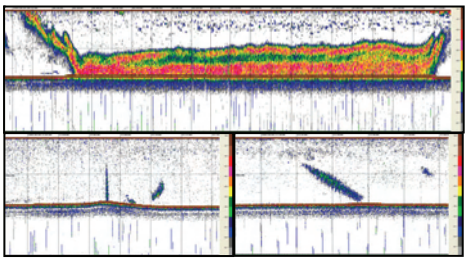
Mapping of seamounts is a fairly straightforward exercise that can be accomplished using global data sets such as satellite gravity, for seamounts over a certain size and compilations of ship-based bathymetry. These activities are underway under the auspices of International Bathymetric Chart of the Southern Ocean Project. Accurate location of cold seep and hydrothermal vent communities is more difficult and will require protocols using a range of ship-based techniques. However, existing geophysical data can be used to identify areas more likely to contain such features.

To assist CCAMLR, the SCAR Geoscience and Life Science SSGs have started an Action Group that aims to identify areas within the CCAMLR region likely to contain Vulnerable Marine Ecosystems around cold seeps and hydrothermal vents. The coordinator is Philip O'Brien of Geoscience Australia.

The Action Group aims to:

- Compile a guide for the identification of fluid escape features to assist in the detection of possible seep sites;
- Compile a guide for the identification of cold seep and hydrothermal vent organisms;
- Review seismic reflection data to detect possible areas of shallow and leaking gas;
- Review echo sounder data for evidences of possible gas flares from active vents;
- Review multibeam and sidescan data for evidence of fluid escape structures on the sea floor;
- Review biological data for evidence of organisms associated with cold seeps or hydrothermal vents;
- Provide locations of areas of possible fluid seepage and biological communities to CCAMLR for incorporation in a GIS.

Activities so far include contacting potential participants, particularly those involved in research into seeps and hydrothermal vents. A number of people already involved in programs such as ChEss (Biogeography of Deep-water Chemosynthetic Ecosystems) have expressed interest. A pilot study reviewing Antarctic echo sounder data for evidence of gas flares in the water column has also started.



120 kHz echograms showing a variety of gas bubble flares from active methane seeps from the Yampi Shelf, north western Australia. a) shows a large mass, b) illustrates a vertical flare, c) images a flare inclined in the direction of tidal current flow

Cross-SSG Action Group on Prediction of Changes in the Physical and Biological Environments of the Antarctic

Julian Gutt - Co Chair (Julian.Gutt@awi.de)

A Cross-SSG Action Group on Prediction of Changes in the Physical and Biological Environments of the Antarctic was formed at the end of last year and the first meeting was held in Cambridge. The chair for the biological side is Julian Gutt and John Turner (BAS) for the physical side.

The decision to form such a group was based on the following situation. Predictions of physical parameters (atmosphere, ice, ocean) are possible and available. In several cases the results still differ quite considerably from each other, but they can be evaluated and can be improved. On the biological side it is especially difficult to take such approaches and they are extremely rare focussing on selected aspects, e.g. productivity, compared to the enormous ecological complexity.

It will be very hard, if not impossible, to model and simulate the next 100 years for the entire Antarctic ecosystem. But this does not mean that with some effort - simple future scenarios for several ecological aspects can't be developed. The terms of reference of the Action group are to:
1. Assess our current ability to predict how the environment of the Antarctic will evolve over the next century;
2. Determine the parameters needed from climate models to predict changes in the biosphere;
3. Consider the issues involved in downscaling from the resolution of climate models to those required for prediction of biological systems;
4. Produce improved predictions of selected physical parameters and estimate the changes to marine and terrestrial biota;
5. Identify areas where future research is needed.

The first product of the Action Group is a major contribution to the Antarctic Climate Change and Environment Report to be published by SCAR this year (see page 10).

Workshops Partly Sponsored by EBA

EBA is keen to sponsor meetings and workshops that bring people together to facilitate collaboration. To apply for EBA support to hold such a meeting/ workshop, contact Shulamit, the EBA Secretary for further information.

SCAR-MarBIN Workshop:

The 2008 SCAR-MarBIN workshop was held in Valencia (Spain) in conjunction with the CAML workshop and the World Conference on Marine Biodiversity. The workshop welcomed 21 participants from 12 countries.

The main topics discussed at the workshop included: SCAR-MarBIN Progress Report, progress assessment, biodiversity data, oceanographical and environmental data, cooperation with CAML, future funding & collaboration, technical developments and integration, products & fitness for use.

Actions points from the workshop include: developing a new data portal (V2.0), completing the first Register of Antarctic Marine Species (RAMS), giving access to over 100 interoperable databases, and reaching a million occurrence records.
More information can be found on the website: www.scarmarbin.be.

A brochure is available for download: www.scarmarbin.be/documents/brochure.pdf

A Note From the Chair of the Committee for Environmental Protection

Dr Neil Gilbert (n.gilbert@antarcticanz.govt.nz) - Antarctica New Zealand

The Committee for Environmental Protection (CEP) was established by the Protocol on Environmental Protection to the Antarctic Treaty (the Protocol). There are currently 33 Member countries to the CEP, which meets annually. SCAR participates in CEP meetings as an Observer. The Committee is currently preparing for its 12th meeting which will be held in Baltimore, USA (6 – 9 April 2009).

The CEP's role is to advise the Antarctic Treaty Parties on the effectiveness of the implementation of the Protocol, and other environmental management issues in Antarctica, including, for example, the state of the Antarctic environment.

Over the last few years the CEP has attempted to refine its agenda and give increased focus to matters posing the highest risk to the Antarctic environment. Issues currently being addressed include the management implications of a changing Antarctic climate, the management of non-native species, and human impacts, in particular through tourism activities.

In addressing such matters the CEP cannot work in isolation and it is essential that the Committee engages with the appropriate experts and expert bodies. Key amongst the CEP's partners is SCAR. The CEP's advice to the Antarctic Treaty Parties must be based on the best scientific advice available and the Committee is increasingly turning to SCAR as the primary provider of advice and information.

There has never been a more important time to study the Polar Regions for reasons of global importance. But the knowledge being gathered through SCAR's programmes, such as EBA, is also of significant value in informing the wise management of the Antarctic continent and the Southern Ocean. In that regard I would urge that the Committee for Environmental Protection, as well as the Antarctic Treaty Parties, are regarded as a primary customer of the knowledge being gathered through SCAR.

I warmly welcome the growing relationship between SCAR and CEP and anticipate that this will become increasingly more important if we are to retain the highest standards of environmental protection and the value of Antarctica as a natural reserve devoted to peace and science.

For more information on the Committee for Environmental Protection go to: www.cep.aq

Prince Edward Island Survey 2008

Justine Shaw (jshaw@sun.ac.za) - Centre for Invasion Biology, Department of Botany & Zoology, University of Stellenbosch, South Africa

The trip

A team of ten of researchers (mostly South African) visited Prince Edward Island for a week in December 2008. Prince Edward covers 44 km^2 (46° 38´S, 37°57´ E), and is approximately 20 km NE of the much larger Marion Island. The motivation for visiting the island was to re-survey surface nesting seabird populations, following a 2001 survey. A two person team, Prof. Steven Chown and postdoctoral researcher Justine Shaw, from the Centre for Invasion Biology, Department of Botany & Zoology Stellenbosch University, South Africa were invited to join the trip to undertake invertebrates and plant surveys, and assess the current status of aliens on the island.

Quarantine

Prince Edward Island had not been visited for over five years and historically has low human visitation. The island has never supported cats or mice (unlike neighboring Marion Island), and only three alien plant species have established. Therefore strict quarantine precautions were taken to ensure that no alien propagules were introduced. To this end, new field gear; tents, outer clothing, sleeping bags, mattresses, boots, socks and backpacks was used. No fresh fruit, vegetable, egg or meat products were taken to the island, all grey water was sieved and solid human wastes were returned to South Africa for disposal.

Prince Edward Island Camp
Photo Courtesy of Justine Shaw

Aliens in the terrestrial environment

Macro-invertebrate and springtail densities were assessed to compare with previous work for both Prince Edward and Marion Islands. All six of the weevil species were collected to assess body -size changes on Marion and Prince Edward Islands associated with climate change and mouse predation. Plant, soil and caterpillar (*Pringleophaga sp.*) samples were collected to investigate nutrient cycling in this mouse–free terrestrial system. No new alien invertebrates were found and it is clear that many invasive alien invertebrates common on Marion Island have not colonised Prince Edward Island.

Vegetation surveys aimed to identify any change in the distribution of the alien plant species, and document any new alien plants. *Sagina procumbens* and *Cerastium fontanum* have expanded their distribution, and *Poa annua* remains present. Leaf samples of several species were collected to examine the genetic relationships of two indigenous species and an alien grass *Poa annua* across sub-Antarctic islands. No new alien vascular plant species were found.

We enjoyed noticing the abundance of spiders and caterpillars crawling through our tents and over our shoes, and the noise of burrowing birds calling all night. This does not occur on Marion Island due to the legacy of cats and mice.

Upcoming Meetings

2009

Gordon Research Conference in Polar Marine Science
15-20 March 2009
Lucca, Italy
www.grc.org/programs.aspx?year=2009&program=polar

Antarctic Sea Ice Workshop-IPY Cruises
23-24 March 2009, Il Ciocco, Lucca, Italy
Steve Ackley: sackley@pol.net

4th Malaysian International Seminar on Antarctica
1-3 April 2009, Petaling Jaya, Malaysia
www.myantarctica.com.my/misa4/misa4.html

Monitoring Climate Change Impacts - Establishing a Southern Ocean Sentinel Program Workshop
20-24 April 2009, Hobart, Tasmania, Australia
www.aad.gov.au/sentinel

Oceans Past II: Multidisciplinary Perspectives on the History and Future of Marine Animal Populations
26 - 28 May 2009, University of British Columbia, Vancouver, Canada
www.scar.org/events/Oceans-Past_circular.pdf

International Conference on Biodiversity Informatics
1-3 June 2009, London, England
www.e-biosphere09.org/

3rd GLOBEC Open Science Meeting
22-26 June 2009, Victoria, British Columbia, Canada
www.confmanager.com/main.cfm?cid=1345&nid=9839

Xth SCAR International Biology Symposium (see article to right)
27 - 31 July 2009
Sapporo, Japan
www.scarbiologysymposium2009.jp/

OceanObs'09
21 to 25 September 2009, Venice, Italy
www.oceanobs09.net/

2010

IPY Oslo Science Conference
8 - 12 June 2010, Oslo, Norway
www.ipy-osc.no/

World Seabirds Conference
September 2010, Victoria, BC, Canada

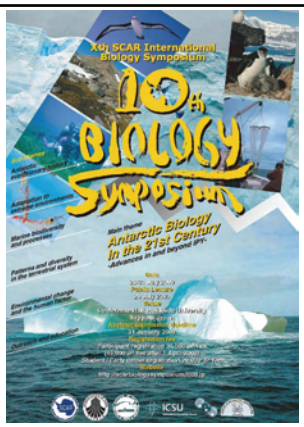
Antarctic Climate Change and the Environment (ACCE) report

Many EBA participants have finished contributing and editing the final version of the SCAR 'Antarctic Climate Change and the Environment' (ACCE) report which is designed to be the Antarctic counterpoint to the much cited Arctic Climate Impact Assessment.

This synthesises most up to date knowledge of the interactions between organisms and their environment on geological to experimental timescales, and assesses how the Antarctic marine and terrestrial biota will be impacted by climate change over the next 100+ years. The ACCE report will be published by SCAR in May/June 2009 with a PDF being made available without charge on the Internet. Hardcopy versions will be printed so that they can be placed in libraries. This benchmark publication from the SCAR community will be of immediate interest to all polar scientists.

SCAR Biology Symposium

Mitsuo Fukuchi (fukuchi@nipr.ac.jp)



The Xth SCAR Biology Symposium is being held in the Asia sector for the first time since the first symposium in Paris 1962. The symposium is being held at Hokkaido University in Sapporo city on July 26(Sun)-31(Fri), 2009. Sapporo is the capital city of Hokkaido which is the big northern island of Japan.

The main theme of the symposium is **"Antarctic Biology in the 21st Century-Advances in and beyond IPY"** and the six sub-themes are;

1: Antarctic evolutionary history
2: Adaptation to extreme environments
3: Marine biodiversity and processes
4: Patterns and diversity in the terrestrial system
5: Environmental change and the human factor
6: Outreach and education

Progress and achievement of EBA are closely related to these themes and EBA colleagues are strongly invited to present new findings since the previous symposium at Curitiba, Brazil, 2005.

We looking forward to seeing all of you in the beautiful city of Sapporo in July. It is a beautiful time of year in Hokkaido.

Mitsuo Fukuchi
Chair of Local Organizing Committee of the symposium

Conservation & Management in the Southern Islands

Marc Lebouvier (marc.lebouvier@univ-rennes1.fr)

In this note I would like to give a brief insight into the national conservation and management frameworks of the sub-Antarctic islands and their place in an international context. Several presentations dealt with management and conservation in these territories during the first International Forum on the sub-Antarctic held in Hobart, Tasmania on 6-7 July 2006 (see reference below). Situations are of course very diverse, with many successive legislation and implementation measures, both for terrestrial and marine environments . This table shows the national status (which is frequently the highest level of protection available under national legislation) and information about three international conventions for several islands.

Some sources
The sub-Antarctic. Papers and proceedings of the Royal Society of Tasmania, volume 141, 2007, P.M. Selkirk, P.G. Quilty & M. Davies (Eds.)
http://www.ramsar.org/
http://whc.unesco.org/
http://www.ccamlr.org/

	National Status	RAMSAR Convention (wetlands) Designation date	World Heritage Convention (Unesco)	Surrounding seas within the area of application of the CCAMLR
Heard Island McDonald Island	HIMI Marine Reserve (October 2002) - Environment Protection and Biodiversity Conservation Act 1999		listed in 1997	yes
Macquarie Island	Nature Reserve (1978)		listed in 1997	
South Georgia	Plan for Progress Managing the Environment 2006-2010			yes
Crozet Archipelago	Nature Reserve (2006)	2008		yes
Kerguelen Islands	Nature Reserve (2006)	2008		yes
Amsterdam and Saint-Paul Islands	Nature Reserve (2006)	2008		
Snares Islands	National Nature Reserve		listed in 1998	
Auckland Islands	National Nature Reserve		listed in 1998	
Campbell Island	National Nature Reserve		listed in 1998	
Antipodes Islands	National Nature Reserve		listed in 1998	
Bounty Islands	National Nature Reserve		listed in 1998	
Prince Edward Islands (Marion and Prince Edward Islands)	Special Nature Reserve	2007	tentative list in 2004	yes
Gough and Inaccessible Islands	Wildlife Reserve (1996)		listed in 1995 (extension in 2004)	

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The limnology and biology of the Dufek Massif, Transantarctic Mountains 82° South

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Abstract

Very little is known about the higher latitude inland biology of continental Antarctica. In this paper we describe the limnology and biology of the Dufek Massif, using a range of observational, microscopic and molecular methods. Here two dry valleys are home to some of the southernmost biota on Earth. Cyanobacteria were the dominant life forms, being found in lakes and ponds, in hypersaline brines, summer melt water, relict pond beds and in exposed terrestrial habitats. Their species diversity was the lowest yet observed in Antarctic lakes. Green algae, cercozoa and bacteria were present, but diatoms were absent except for a single valve; likely windblown. Mosses were absent and only one lichen specimen was found. The Metazoa included three microbivorous tardigrades (*Acutuncus antarcticus*, *Diphascon sanae* and *Echiniscus* (cf) *pseudowendti*) and bdelloid rotifer species, but no arthropods or nematodes. These simple faunal and floral communities are missing most of the elements normally present at lower latitudes in the Antarctic which is probably a result of the very harsh environmental conditions in the area.

Keywords: Antarctic; Cyanobacteria; Biogeography; Endemism; Refugia

1. Introduction

The evolutionary history and geographical isolation of the Antarctic continent have produced a unique environment, inhabited by species adapted to its extreme conditions. However, very little is known about the higher latitude inland limnology and biology of continental Antarctica and the few data that do exist are largely based on opportunistic non-specialist collections made during geological field studies.

The southernmost aquatic systems studied to date are in the Brown Hills and Darwin Glacier region (~80°S), about 300 km south of McMurdo Sound (Vincent and Howard-Williams, 1994). Proglacial lakes are also known to be present as far south as 85°S in the Patuxent Range, and in the Mt. Heekin area of the Transantarctic Mountains.

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In the International Geophysical Year (1957) geologists discovered the Davis Valley and 'Forlidas Valley' (the name is unofficial) – unique, over-deepened dry valleys occupying an area of 53 km^2 in the northern Dufek Massif (Behrendt et al., 1974) (Figs. 1 and 2). Although less than 1% of the area of the McMurdo Dry Valleys, Davis Valley and Forlidas Valley are nevertheless the largest ice-free valley system found south of 80°S in the sector between 90°W and 90°E. They contain a near-unprecedented geomorphological record of glacial history of the ice sheet (Boyer, 1979), including evidence of up to seven distinct phases of glaciation (Hodgson et al., unpublished data). The area also contains a number of proglacial lakes and ponds and one land-locked pond, Forlidas Pond; the remnant of a once much larger proglacial lake (Fig. 2). The latter, to our knowledge, is the southernmost example of this type of water body. Despite the cold and dry climate of this region of continental Antarctica the presence of these lakes and ponds showed that liquid water is available which could potentially support a biota. More remarkable were reports from the International Geophysical Year of a 'primitive leafy-type water plant' (Neuburg et al., 1959) and a 'strange pinkish plant that is somewhat leafy' (Behrendt, 1998). This is a reference to the abundant cyanobacterial mats found there, which grow as foliose clumps and would resemble plants to a non biologist.

After nearly 50 years since these first observations we revisited the Davis Valley to describe and measure the limnology of the lakes and ponds and carry out the first biological inventory. The area is particularly interesting for biological studies as a result of the remarkable lack of human impacts, being only briefly visited as part of a traverse in the International Geophysical Year (1957), by the US Geological Survey in 1978–1979 and for 9 days by the authors in December 2003. The primary research objective was to determine whether, compared with lower latitude and coastal Antarctic studies (e.g. Convey and Stevens, 2007; Convey et al., 2008), the terrestrial and freshwater biota present at these high continental latitudes are post-glacial colonists or long-term survivors.

2. Site description

The Dufek Massif (Fig. 1) is a range of peaks in the Pensacola Mountains (part of the Transantarctic Mountain range), centred at 82°24′ S 52°12′ W. It is situated approximately mid-way between the Support Force Glacier and the Foundation Ice Stream, two of the major glaciers draining northwards from the Polar Plateau into the Ronne-Filchner Ice Shelf. Approximately 60 km to the southeast is the Forrestal Range (also part of the Pensacola Mountains), which is separated from the Dufek Massif by the Sallee Snowfield. The Ford Ice Piedmont separates the Dufek Massif from the Ronne and Filchner Ice Shelves, about 50 km to the northwest and 70 km to the northeast respectively. The nearest significant mountain chains are the Ellsworth Mountains 800 km to the west–north–west and the Shackleton Range 400 km to the north-east, both of which do not form part of the Transantarctic Mountains.

The total area of the Dufek Massif is 11,668 km^2 and its highest point is England Peak (2150 m). Its geology consists of a middle Jurassic differentiated stratiform mafic igneous complex overlain in the lower parts of the valleys by a glacial drape sorted into polygons by freeze thaw processes. The geology has been described by Behrendt et al. (1974), Ford (1976, 1990) and Ford et al. (1978) and remotely by Ferris et al. (1998). Cosmogenic isotope surface exposure age dating and geomorphological data point to a complex glaciological history with the repeated exposure of ice free surfaces for at least the last 1.6 million years (Hodgson et al., unpublished data). Meteorological studies are limited, but mean annual temperatures inferred from nearby ice boreholes lie between −24.96 °C, 32 km due north of Forlidas Pond on the Ford Ice Piedmont measured in December 1957 (Aughenbaugh et al., 1958), and −9 °C measured in December 1978 in the Enchanted Valley, 26 km to the south (Boyer, pers. comm.). Near surface winds in winter are predominantly from the west–north–west with modeled mean winter velocities of c. 10 m s^{-1} (van Lipzig et al., 2004). Many geomorphological features related to wind erosion such as ventifacts and tafoni are present. Regionally, it has been identified as an ablation area comprising two 'ablation types' (van den Broeke et al., 2006). Type 1 includes erosion-driven ablation areas, caused by 1-D and/or 2-D divergence in the katabatic wind field where solid precipitation and sublimation are small but where divergence in the snowdrift transport can be considerable. Type 2 dominates in the Davis and Forlidas Valleys and includes sublimation-driven ablation areas occurring at the foot of steep topographic barriers, where temperature and wind speed are high and relative humidity low, with individual glacier valleys serving as gates for air drainage from the plateau to the Ronne-Filchner Ice Shelf. Strongest sublimation rates occur on these localized glaciers in the Transantarctic Mountains, where widespread blue ice areas are present (van den Broeke et al., 2006).

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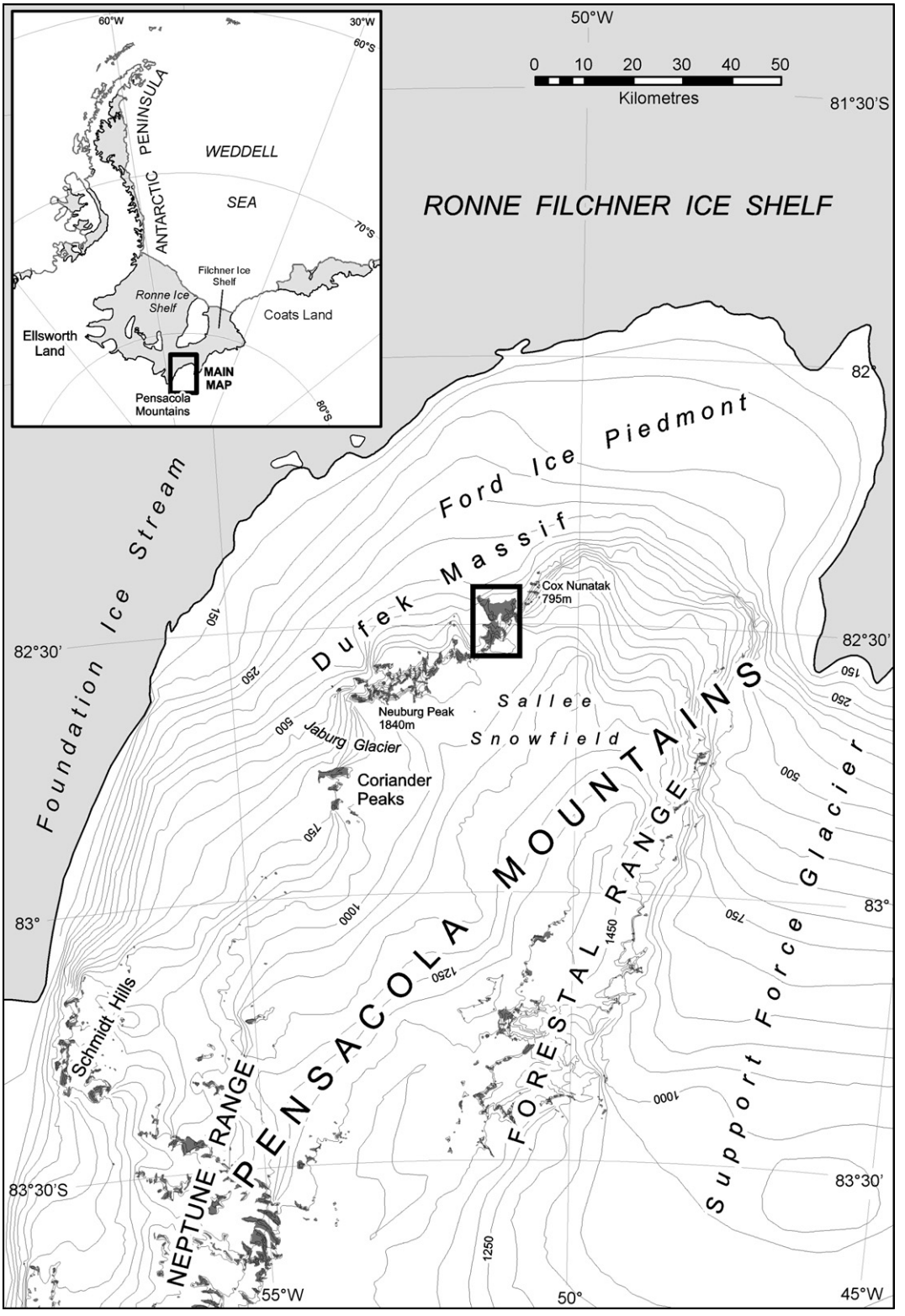

Fig. 1. Location map.

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Combined, the Davis and Forlidas Valleys are approximately 7 km north to south and 7 km west to east (Figs. 2 and 3). Their northern extent in the Davis Valley is defined by the blue ice lobes that form part of the southern margin of the Ford Ice Piedmont (Fig. 4), and southern limit rises to escarpments breached by outlet glaciers, the largest of which is the Edge Glacier which extends approximately 4 km into the Davis

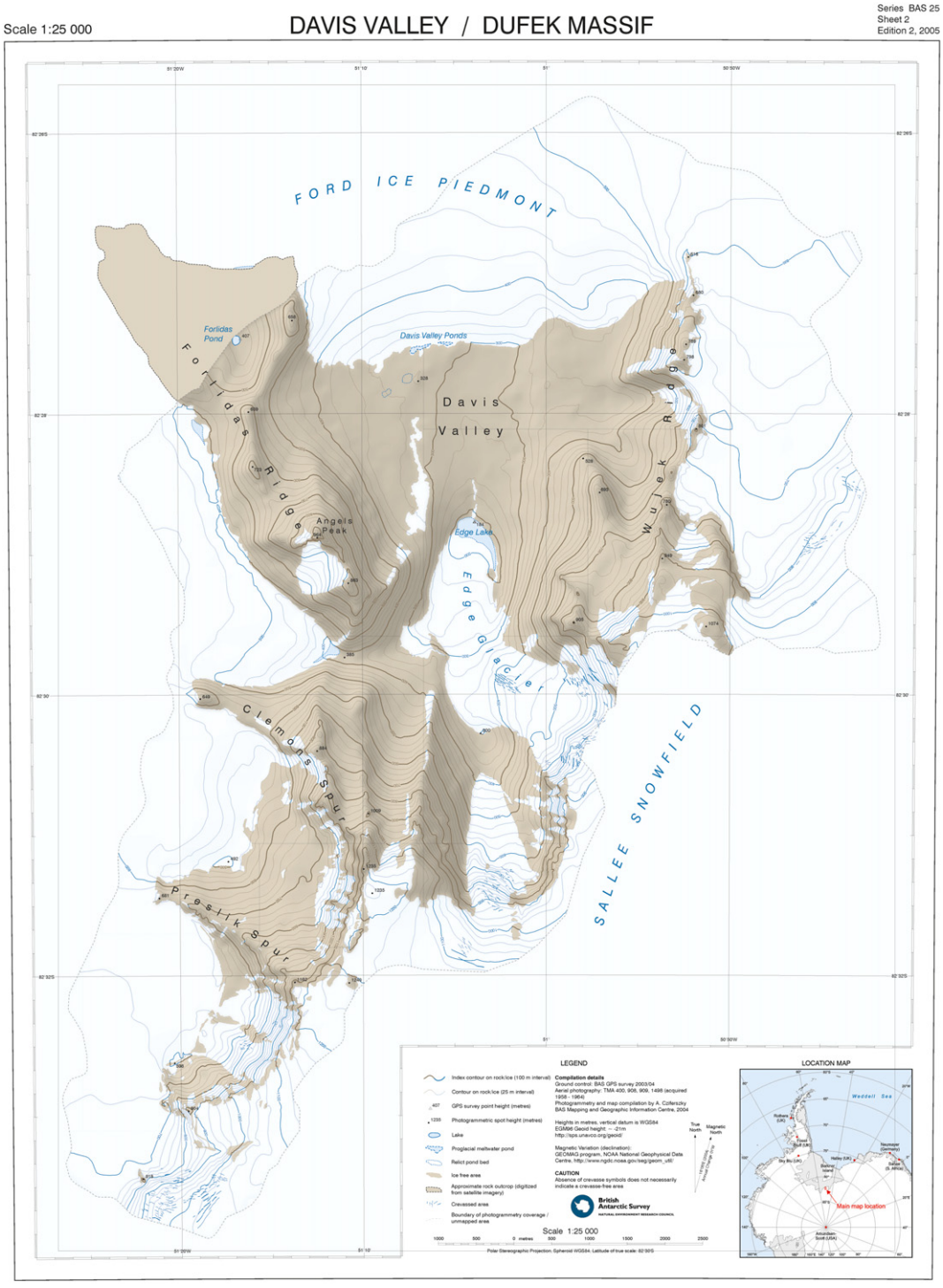

Fig. 2. Topographic map of the study area.

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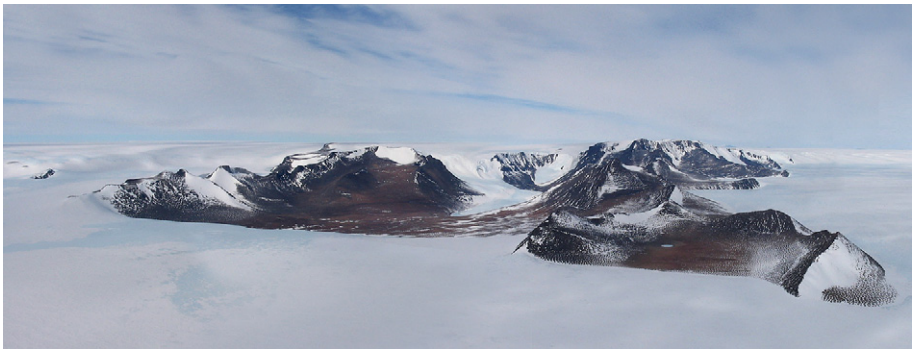

Fig. 3. Aerial panorama of the Davis and Forlidas Valleys looking south.

Valley from the Sallee Snowfield (Fig. 2). The western and eastern margins are enclosed by Forlidas Ridge and Wujek Ridge (Figs. 2 and 3).

Both valleys contain frozen and liquid water bodies. In Forlidas Valley there is Forlidas Pond (51°16′48″W, 82°27′28″S), a 90.3 m diameter, shallow pond (1.83 m deep) with a perennially frozen water column and evidence of an occasional freshwater moat. It is an isolated remnant of a formerly much more extensive proglacial lake, which had mid-late Holocene water levels up to 17.7 m above present delineated by an upper limit of salt efflorescence, an absence of well-developed frost-sorted polygons, and a series of lake terraces at 11.6 m, 8.61 m, 4.16 m and 1.25 m above the present water level (Fig. 5 and Hodgson et al., unpublished data). An ephemeral frozen melt water pond also occurs where the valley meets the Ford Ice Piedmont. A series of melt water ponds also occurs along the blue-ice margin of the northern Davis Valley at 51° 05.5′W, 82° 27.5′ S and 51° 07′ W, 82° 27.55′ S (Fig. 6), whilst inland of this a number of relict pond beds mark the position of former proglacial ponds likely formed during periods of ice advance into the valley. Edge Lake (Fig. 2), a perennially frozen proglacial lake at the terminus of the Edge Glacier is surrounded by a series of 4–5 depositional proglacial lake ice-push shorelines cut into the valley side, particularly near the eastern side of the terminus of the Edge Glacier, indicating higher lake ice levels in the past. The surface of the lake has an uneven, slightly domed, topography suggesting that it has accumulated from successive surface melt water refreezing events, but experiences enhanced ablation at the lake margins. Seasonal melt water streams were observed on the eastern margin of the glacier during the field sampling campaign.

Incised dry stream channels and water erosion features are evident within the ice-free area. Some are fed by seasonal supraglacial melt water, but others appear to be relict features. The presence of liquid water at or near the surface of all the water bodies, and even the small glacial melt streams at the margin of the Edge Glacier, illustrates the ability of the relatively large areas of bare rock and soil to absorb solar radiation and emit heat causing local ice and snow melt.

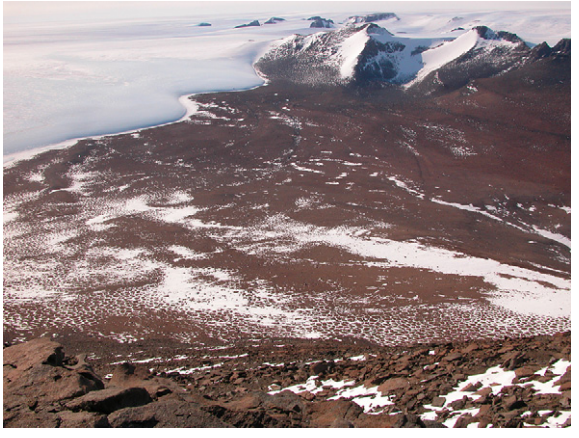

Fig. 4. Davis Valley looking east from Forlidas Ridge showing the blue ice lobes of the Ford Ice Piedmont.

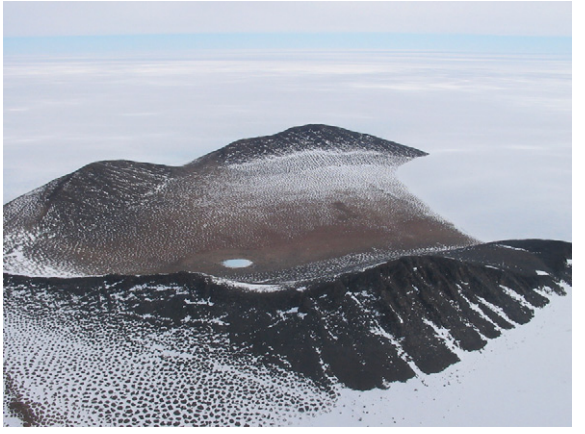

Fig. 5. Forlidas Valley and Forlidas Pond looking west northwest from the air. The area of the valley floor without frost-sorted polygons marks the upper Holocene water level, 17.7 m above the present day pond.

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Fig. 6. Davis Valley Ponds at the foot of the blue ice lobes of the Ford Ice Piedmont.

Soils are not well-developed in the area and generally lack a significant organic component. Parker et al. (1982) collected a soil sample (S7) that was light brown in colour, resulting from gravel weathering predominantly to muscovite. The soil comprised sand (81%) with silt (14%) and clay (5%); a composition different from other sites in the Pensacola Mountains where the clay proportions of six samples ranged from 0.4% to 1.6%. The soil sample from the Davis Valley had a pH of 6.4 (Parker et al., 1982). Nitrate was the primary nitrogen ion and orthophosphate-P concentration was below the detection limit of 0.01 $\mu g\, g^{-1}$. Microbial analyses of soil cultures showed that one pseudomycelium-forming yeast and measurable numbers of viable, aerobic heterotrophic microorganisms were present including Gram-negative rods, but these were not identified further (Parker et al., 1977, 1982).

On the grounds that the area contains some of the most southerly freshwater ponds known in Antarctica that contain plant life which would be threatened by possible contamination by human activity (Behrendt, 1998), Forlidas Pond and Davis Valley Ponds were designated as a Specially Protected Area (no. 23) and then an Antarctic Specially Protected Area (ASPA No. 119). ASPA No. 119 lies within Specially Reserved Area No. 1, proposed by the USA and adopted at the Antarctic Treaty Consultative Meeting XVI (held in Bonn, 1991; http://cep.ats.aq/cep/apa/aspa/sites/aspa119/summary.html).

3. Methods

3.1. Surveying and environmental measurements

Topographic maps were compiled by the Mapping and Geographical Information Centre (BAS) at 1:50,000 scale using aerial photographs from the United States Geological Survey (Lassiter Station 1–16 and 1–17, 01.02.1958), GPS-surveyed ground control and differential geodetic GPS survey transects using a Trimble 5700 base station and a Magellan ProMark 10CM rover unit. Altitudes were referenced to the WGS84 reference ellipsoid, and included accurate photogrammetric height measurements of key landforms. Gemini Tinytag Plus data loggers were deployed to measure temperature and relative humidity at the sampling sites from 3 to 15 December 2003. Loggers were placed over snow and rock to measure the influence of advected radiation, with the sensors oriented to shield them from direct sunlight and data recorded at 30 min intervals. Lake water conductivity, temperature and oxygen saturation were measured using a SOLOMAT WP4007 water quality meter and 803PS Sonde. Water chemistry analyses followed the protocols described in Hodgson et al. (2009). Briefly, sodium, potassium, calcium, magnesium, iron, aluminium, manganese and silicon were determined by ICP-OES. Anions nitrate, chloride and sulphate were determined from direct analysis of aqueous solutions by Ion Chromatography. Total dissolved nitrogen was determined on a Shimadzu TNM-1 analyser equipped with a thermal conductivity detector DOC and TOC were determined by Shimadzu TOC-Vcph analyser with a detection limit of 0.5 mg/l. Nutrients phosphate and ammonium were determined by colorimetry.

3.2. Biological sampling

The biological sampling programme is summarised in Table 1. To minimise contamination we followed the protocols outlined in the Management Plan for Antarctic Specially Protected Area No. 119. All sampling equipment was scrubbed with Virkon multi-purpose disinfectant before use, and subsamples were collected in sterile WhirlPak bags or acid-washed bottles. Water samples were collected directly into acid-washed bottles (surface waters) or via a UWITEC water sampler (brine layer). Biological material was collected manually from lake benthic, littoral and catchment areas at each of the study sites. Samples for microscopic investigation were preserved in Lugol's iodine solution or ethanol and those for molecular analysis were frozen.

The presence of mosses and lichens was assessed. Due to the extreme rarity of lichens in the area (and its protected status) we did not remove samples, but instead photographed them for later taxonomic study.

A combination of microscopic and molecular methods was used to identify the microbiota.

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Table 1
Biological sampling program in the Davis and Forlidas Valleys: groups of taxa identified and the methods used.

Description	Method	Number of samples	Number of taxa	Taxa
Bryophyta	Observational survey	0	0	n/a
Lichens	Observational survey	1	1	*Lecidea cancriformis* Dodge & Baker
Bacillariophyceae/Diatoms	Survey under light microscope	2	1	*Pinnularia microstauron* (Ehr.) Cl.[d]
Cyanobacteria	Clone library, DGGE + band sequencing, isolation of strains + sequencing (microscopy)	3	6	Sample TM1: 16ST63, 16ST14, 16ST49 Sample TM2: 16ST63, 16ST14, 16ST44, 16ST49, 16ST80 Sample TM3: 16ST44, 16ST49, 16ST80, 16ST07
Chlorophyta/Green algae	DGGE + band sequencing	2	1	*Urospora* sp.
Rhizaria/Cercozoa	DGGE + band sequencing	2	2	Heteromitidae, *Paulinella* sp.
Bacteria	DGGE + band sequencing	2	32	Cyanobacteria:Nostocales, Oscillatoriales, Chroococcales, Gloeobacteriales[b] Bacteroidetes: Sphingobacteriales, Flavobacteriales Firmicutes: Clostridiales Gammaproteobacteria: Pseudomonadales, Psychrobacter
Bacteria	Isolation of strains + sequencing	1	330 isolates	*Firmicutes* 33%, *Bacteroidetes* 23%, *Alphaproteobacteria* 25%, *Actinobacteria* 9%, *Betaproteobacteria*. 8%, *Gammaproteobacteria* 1.5%, Deinococci 0.3%
Arthropods	Tullgren extractions	50	0	None obtained
Invertebrates	Baermann extractions	130	3	See Tardigrades (below)
Tardigrades	Light microscope	14	3	*Echiniscus* (cf) *pseudowendti* Dastych,
	(Molecular[c])	20	1	1984 (Heterotardigrada), *Acutuncus antarcticus* (Richters, 1904)
				Diphascon sanae Dastych, Ryan and Watkins, 1990 (Eutardigrada)
Rotifers	Baermann extractions and light microscope	130	present	Bdelloid rotifers
Soil bacteria and algae	Cultured (Parker et al., 1982)[a]	1	3	Cyanobacteria: *Oscillatoria* sp. Algae: *Trebouxia* sp., *Heterocous* sp. (viable yeasts present)
Avifauna	Observation	n/a	1	Snow petrel (*Pagadroma nivea*)

[a] Previously published.
[b] Tentative identification based on about 100 bases.
[c] Analyses carried out on morphologically congruent samples from the Shackleton range.
[d] Not considered as evidence of an extant community.

Microscopic methods included analyses of water samples in UWITEC plankton counting chambers, and analyses of natural samples and cultures. Representative samples obtained for microbial analyses included the hypersaline brine at the bottom of the Forlidas Pond (sample TM1), a cyanobacterial mat that was actively growing in the littoral zone (air bubbles on the mat surface and trapped under ice were taken as evidence of recent photosynthetic activity) situated under 15 cm of ice and 15 cm of water (TM2), and a sample of a red-orange foliose clump of terrestrial cyanobacterial mat located 20 m from the shoreline and probably corresponding to the "plant-like" organisms reported previously (Neuburg et al., 1959; Behrendt, 1998) (TM3).

Cyanobacteria were analysed by a combination of morphological and molecular methods, as described in detail by Fernández-Carazo et al. (unpublished data). Briefly, they involved the observation of cultures by microscopy with reference to the taxonomic works of Komárek and Anagnostidis (2005) and the diacritical morphological traits described by Taton et al. (2006a). DNA extraction methods were slightly modified from Taton et al. (2003). Following this, DGGE, construction of clone libraries and isolation and characterisation of the strains were carried out (Taton et al., 2003, 2006b). Sequencing was performed by GIGA (http://www.giga.ulg.ac.be/) (Liège, Belgium) using an ABI 3730 xls DNA analyser (Applied Biosystems, Foster City, USA). A distance tree was constructed with the software

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package TREECON for Windows 1.3b (Van de Peer and De Wachter, 1997) by the neighbor-joining method (Saitou and Nei, 1987) using 379 positions covered by most sequences. The formula of Jukes and Cantor (1969) was used to correct for multiple mutations. The tree comprised DGGE bands, clones and strains' sequences from Forlidas Pond samples as well as their three most similar strain sequences, and five uncultured sequences selected using Seqmatch from RDP (http://rdp.cme.msu.edu). A bootstrap analysis was performed involving the construction of 500 resampled trees. The OTUs were calculated using DOTUR (Schloss and Handelsman, 2004), with a threshold at 97.5%.16S rRNA similarity to define OTUs. In the case of OTU 16ST80, the sequence of sdG4 is exactly at the limit of similarity, and it was included in this OTU.

In order to study the cultivable bacterial diversity, isolates of the littoral sample (TM2) were grown on a selection of heterotrophic media (Peeters et al., unpublished data). The isolates were screened for duplicates and grouped by rep-PCR using primer (GTG)5 as described in Gevers et al. (2001). Representative isolates were identified by partial 16S rRNA gene sequencing using primer BKL 1 (Coenye et al., 1999). A fragment of approx. 400 to 450 bp from the 5′ end was obtained and compared with the EMBL database using FASTA (http://www.ebi.ac.uk/Tools/fasta33) for preliminary identification.

To study the uncultivable diversity of bacteria, green algae and cercozoa, we first optimized the protocols to extract DNA from environmental samples by removing extracellular DNA (Corinaldesi et al., 2005) prior to bead-beating extraction. For the Denaturing Gradient Gel Electrophoresis (DGGE) analysis of the bacteria, we followed the protocols as described in Van der Gucht et al. (2001). For the green algae, we used a nested PCR approach using the primer combinations Euk1A-CHLO02r and Euk1A–Euk516r–GC (Díez et al., 2004). These primers are known to also detect cercozoa (Zhu et al., 2005), hence these taxa were also included in the DGGE analysis. Excised bands were sequenced and identified after re-extraction and amplification. A nucleotide BLAST search (Altschul et al., 1997) was performed in order to obtain sequences that were most similar.

Samples for diatom analysis were prepared following a slightly modified protocol from Renberg (1990) and embedded in Naphrax®. The slides were screened for the presence of frustules at 1000× magnification using a Zeiss Axioplan II microscope.

Fauna were extracted using Baermann and Tullgren extractions on return of the collected substrata to the BAS Rothera Research Station, having been kept under field conditions in the intervening ~two week period. Tardigrades from the Baermann funnel extractions were permanently mounted on microscopes slides (using de Faures medium) for identification. Individual tardigrades were grouped into morphotypes under 400× magnification. A representative of each morphotype was mounted and examined under high power (1000×) magnification for detailed taxonomic identification. It was not possible to extract tardigrade DNA from ethanol preserved samples from the Dufek Massif, so our specimens were compared with DNA from morphologically congruent dried meiofaunal samples collected from Lake Lundström, in the adjacent Shackleton Range (400 km distant). Meiofauna were separated from the substrate by homogenising and centrifugation of samples using an Optiprep™ gradient solution (see Sands et al., 2008 for detailed methods). Specimens were lifted into individual tubes with 5 μL double distilled H_2O and kept stored frozen (−80 °C). DNA was released from individual tardigrades by disrupting the tissue by a series of freeze thaws followed by a 15 min incubation at 95 °C in a 5% chelex solution. Three genes were amplified, a fragment of mitochondrial cytochrome c oxidase (CO1), the near complete small ribosomal sub unit (18S), and a fragment of the *Wingless* gene (Wnt). Details of the above protocol, including primers and amplification strategy, and results are given in Sands et al. (2008).

4. Results and discussion

4.1. Environmental measurements and observations

Microclimate temperatures over snow from 3 to 15 December 2003 ranged from a maximum of +12.8 °C to a minimum of −14.5 °C, with a mean over the period of −0.56 °C. Microclimate temperatures over rock ranged from a maximum of +16.0 °C to a minimum of −8.6 °C, with a mean over the period of +0.93 °C (Fig. 7). Relative humidity recorded over snow ranged from a maximum of 80.4% to a minimum of 10.8%, with a mean over the period of 42.6%. Over rock surfaces, relative humidity ranged from a maximum of 80.9% to a minimum of 5.6%, with a mean over the period of 38.7% (Fig. 7).

Being in an ablation area (van den Broeke et al., 2006) evaporation and sublimation dominate over precipitation. As a result Forlidas Pond has evaporated down to a small remnant of a once much larger proglacial lake (Fig. 5). The pond was frozen almost

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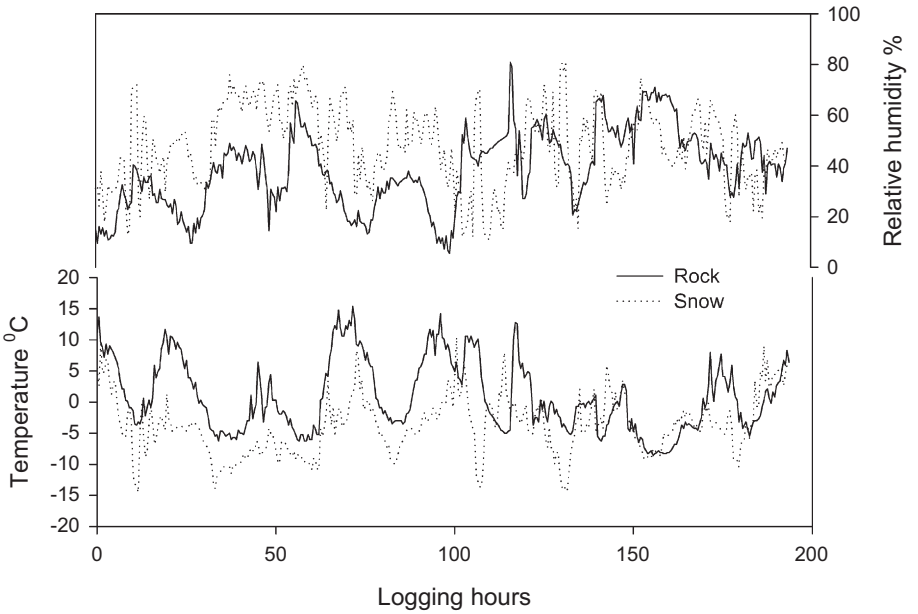

Fig 7. Microclimate temperature (°C) and relative humidity (%) data for the Dufek Massif. Logging was at 30 min intervals from 12.52 p.m. on the 3 Dec 2003 to 17.22 p.m. on 11 Dec 2003. Sensors were oriented to partially shield them from direct sunlight but were not located within a Stevenson screen.

completely to its base, with a thin layer of hypersaline slush at the lake bottom. The depth of the pond was 1.83 and the thickness of the ice between 1.63 and 1.83 m. The conductivity of the hypersaline slush was 142.02 mS cm^{-1}, approximately four times greater than seawater. The ionic order of the brine layer was Cl–Na–Mg–SO_4–Ca-K and its temperature was −7.67 °C (Table 2). At the margins of the pond, liquid water was present in a moat area under 10–15 cm of ice. At Forlidas Pond this moat water had a freshwater ion sum of 178 mg L^{-1}compared with the 111,942 mg L^{-1} of the brine layer (Table 2). The Davis Valley Ponds also had shallow freshwater littoral moats that, at the time of sampling, were either locally ice free, frozen with liquid water present under ice, or frozen to the bed. The surface morphology of the pond ice suggested that that these moats could have been 1–2 m laterally more extensive during warmer years.

4.2. Flora

Visible biota was limited within the study area, and macroscopic vegetation appeared to be restricted to cyanobacterial mats, found both in lakes and terrestrial habitats, and a very sparse occurrence of small (∼mm scale) yellow and black crustose lichens deep within crevices on larger boulders (Fig. 8), as previously observed by Neuburg et al. (1959). Often, only the black apothecia were visible. Through analyses of the photographs the species has been identified as *Lecidea cancriformis* Dodge & Baker; one of a few lichens which occurs in the severest environments of continental Antarctica, especially on far inland nunataks as

Table 2
Water chemistry of Forlidas Pond.

Parameter	Brine layer	Freshwater moat
Sample depth (m)	1.63	0.2
Conductivity (mS cm^{-1})	142.37	2.22
Temperature (°C)	−7.7	0.7
pH	7.3	8.15
Al (mg L^{-1})	0.834	<0.002
Fe (mg L^{-1})	0.363	0.004
Mg (mg L^{-1})	10,500	13.9
Ca (mg L^{-1})	1240	11.4
K (mg L^{-1})	908	1.36
Na (mg L^{-1})	31,000	45
Si (mg L^{-1})	3.1	0.222
Cl (mg L^{-1})	65,700	88.6
SO_4–S (mg L^{-1})	2590	17.5
Ratio SO_4–S/Mg	0.25	1.26
Ratio Mg/Ca	8.48	1.22
Ion sum (mg L^{-1})	111,942	178
TN (mg L^{-1})	3800	4.3
TOC (mg L^{-1})	53	0.97
DOC (mg L^{-1})	55.3	1.04
NO_3–N (mg L^{-1})	3600	4.42
NH_4–N (mg L^{-1})	2.71	0.043
PO_4–P (mg L^{-1})	0.078	<0.005

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far as 86°S, and at high altitudes (Ovstedal and Smith, 2001, p. 220, plate 48). The British Antarctic Survey Plant Database also reports *Blastenia succinea* Dodge & Baker and *Xanthoria elegans* (Link.) Th. Fr. in samples from elsewhere in the Dufek Massif; although these have not been independently verified by us. Previous anecdotal reports of the possible occurrence of mosses within the area could not be substantiated, and it is probable that the rich cyanobacterial mat growth was previously mistaken for bryophytes by non-specialists. The cyanobacterial community is the most abundant biota and is present in at least three distinct environments:

(1) In the permanent water bodies; particularly in the moat of Forlidas Pond, at the bottom and littoral zones of the Davis Valley Ponds, and in the seasonally wetted perimeter of Edge Lake. These habitats were extensively covered by red-brown cyanobacterial mats (Fig. 9a). These were actively photosynthesizing, as evidenced by gas bubbles trapped against the lower ice surfaces, and bubbles incorporated into the ice. Because perennially ice covered lakes have elevated concentrations of dissolved O_2 gas, the microbial mats growing on the bottom can become buoyant and start to float off the bottom as 'lift-off' mats (cf. Doran et al., 2004, p. 480), or become incorporated into the base of the lake ice when it makes contact with the bed. In Forlidas Pond and the Davis Valley Ponds lift off mats frozen into the base of the lake ice eventually migrate up through the ice profile (cf. Adams et al., 1998). In the Davis Valley, this appeared to take place over several years with each summer marked by the development of a 2–3 cm melt-cavity formed by the upward progression of the clump thorough the lake ice due to preferential heating of its upper surface (Fig. 9b). These clumps eventually break out at the surface and are dispersed by wind onto the shoreline, or further afield. In the littoral zone of Forlidas Pond, melting and refreezing of the moat has resulted in cyanobacterial mats being incorporated under shoreline boulders. Fossil examples of this type of mat were also found buried under boulders between the present and previous (higher) shorelines. Cyanobacteria were also present in the hypersaline brine of Forlidas Pond as single cells and as small flakes. A strain corresponding to the morphology of *Leptolyngbya antarctica* was isolated from the saline slush of TM1 (Fernández-Carazo et al., unpublished data).

(2) In exposed terrestrial locations, particularly at the edge of larger rocks and within the boundary crevices of frost sorted polygons. These were generally very foliose in form, mid brown in colour, and best developed at the edge of larger rocks accumulated to depths of at least 10–15 cm (Fig. 9c and d). Nearly all clumps were completely dry on discovery, although those near to melting snow were damp and some had lower thalli that were often deep green in colour. Particularly good examples of this growth form were found in the mid valley floor of Forlidas Valley and in Davis Valley (near a large snow gully where it meets the second major terrace above Edge Lake).

(3) In a series of dry pond beds, two of up to 50 m diameter are present in the Davis Valley (Fig. 2), and have extensive areas of almost continuous cyanobacterial mat on the former pond floors (Fig. 9e). These pond beds and gullies occupy depressions and therefore may accumulate snow in winter, permitting the cyanobacteria to take advantage of the wet and protected environment within the snow patches (Cockell et al., 2002).

Analyses of the cyanobacterial molecular diversity in and around Forlidas Pond showed that the richness obtained in the current study was lower (3–5 OTUs per sample) than in Antarctic coastal lakes (4–12 OTUs per sample) (Fernández-Carazo et al., unpublished data). The spatial distribution of the cyanobacterial OTU's showed that TM1 (from the hypersaline brine at the bottom of the pond) and TM2 (mats in the littoral zone) shared three OTUs, 16ST63, 16ST14 and 16ST49 (Table 1, Fig. 10). In addition,

Fig. 8. Lichen *Lecidea cancriformis* in rock crevice (1 cm diameter lip balm container for scale).

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TM2 shared OTUs 16ST44, 16ST49 and 16ST80 with the terrestrial mat (TM3). These data support the idea that the cyanobacterial diversity is not limited to specific aquatic or terrestrial habitats, but that aquatic species are able to colonise the surrounding terrestrial niches, and *vice versa* (cf. Gordon et al., 2000). On the basis of the geomorphological evidence around Forlidas Pond of the former presence of a larger proglacial lake that evaporated, it is possible that the terrestrial cyanobacteria were once aquatic, but still survive on the now dry lake bed. Another hypothesis could be that the foliose clumps were made by submersed aquatic cyanobacteria that died subsequently, but whose undegraded DNA has contributed to the 16S rRNA survey for TM3.

Microscopic analyses revealed that no diatom communities were present. One solitary valve of the diatom *Pinnularia microstauron* (Ehr.) Cl. was detected but, as this is a common windblown subaerial diatom, we do not consider it as evidence of an extant

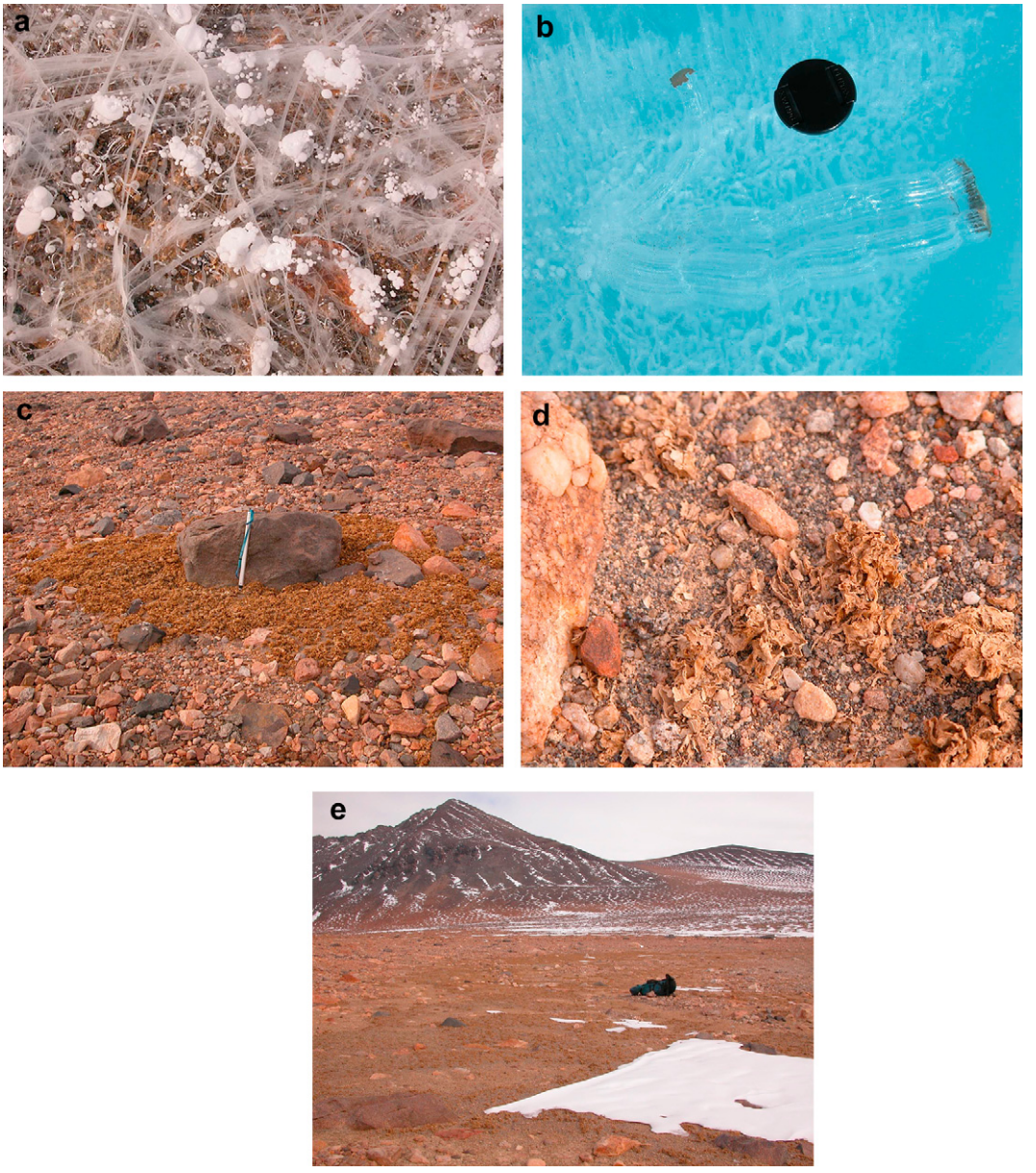

Fig. 9. Main cyanobacterial habitats (a) cyanobacterial mat under ice, Forlidas Pond moat (b) cyanobacterial mat melting up through the ice of the Davis Valley in annual increments of 2–3 cm. The ice is more than 2 m deep. Lens cap for scale (c) cyanobacteria around a boulder in the Davis Valley. Ice axe for scale (d) detail of terrestrial cyanobacteria (e) cyanobacteria in relict proglacial pond beds, Davis Valley.

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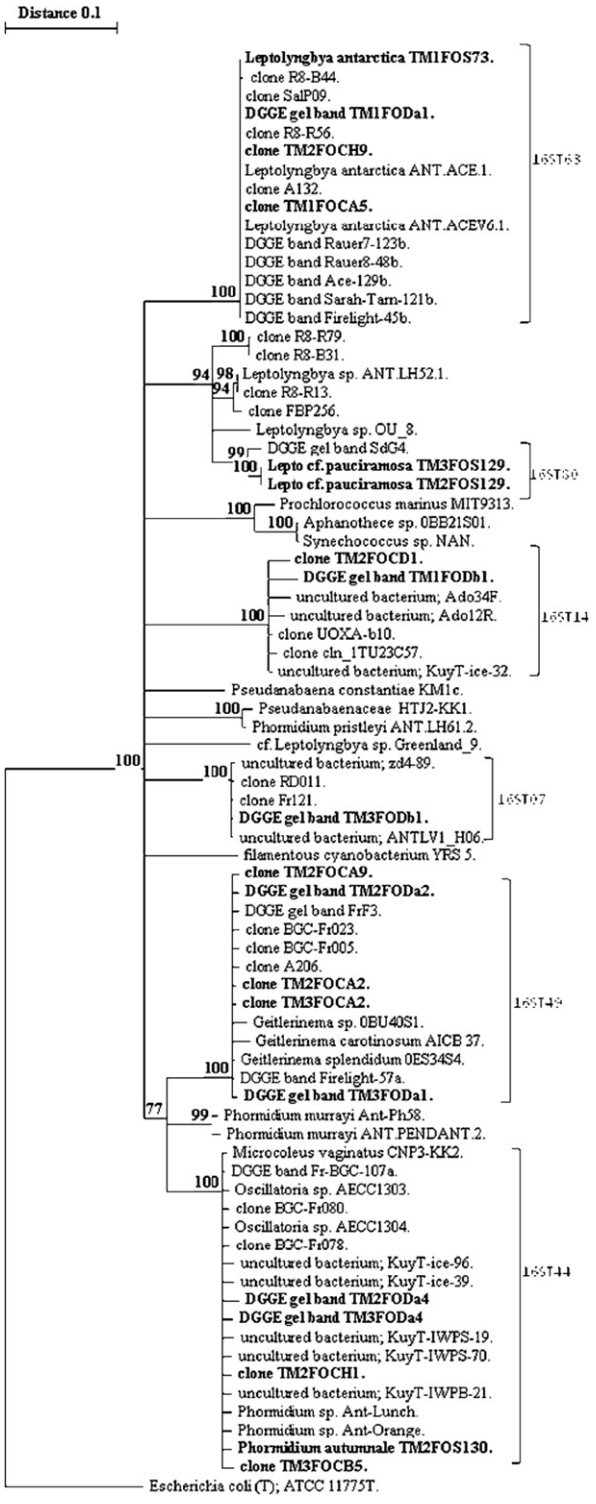

Fig. 10. Distance tree based on cyanobacterial partial 16S rRNA sequences (*E. coli* positions 380–757) constructed by the Neighbor-joining method (Saitou and Nei, 1987). A bootstrap analysis was performed that involved construction of 500 resampled trees (values indicated at the node), and the branches with less than 70% bootstrap support are drawn as unresolved. The tree comprised the sequences of 7 DGGE bands, 8 clones and 4 strains from the Forlidas Pond samples (in bold italics) and their 3 most similar strain sequences and 5 uncultured sequences from rdpII (http://rdp.cme.msu.edu). The sequences found in Antarctic samples are in bold. *E. coli* sequence is the outgroup. The OTU numbers are indicated on the right. The evolutionary distance between two sequences is obtained by adding the lengths of the horizontal branches connecting them and using the scale bar (0.1 mutation per position).

community. A study of ponds in the Darwin Glacier region (79.7°S) reported a similar absence of diatoms (Vincent and Howard-Williams, 1994). Analysis of the DGGE bands in the environmental samples revealed only a single green algal sequence (*Urospora* sp.; E30) and two cercozoans (a Heteromitidae; E12_5 and a *Paulinella* sp.; E23). Bacteria identified by DGGE bands included Cyanobacteria (*Nostocales, Oscillatoriales, Chroococcales, Gloeobacteriales*), Bacteroidetes (*Sphingobacteriales* and *Flavobacteriales*), *Firmicutes* (*Clostridiales*) and Gammaproteobacteria (*Pseudomonadales*; *Psychrobacter*) (Tables 1 and 3). Preliminary data based on partial 16S rRNA gene sequence analysis of the isolated bacteria in cultures revealed a large diversity. Of more than 330 isolates sequenced, 33% belonged to the *Firmicutes* (low %GC Gram-positive bacteria), 23% were *Bacteroidetes,* 25% were *Alphaproteobacteria*, 9% were *Actinobacteria* (high %GC Gram-positive bacteria) and 8% were *Betaproteobacteria*. Gammaproteobacteria (1.5%) and Deinococci (0.3%) were present in smaller numbers.

Viable yeast species have previously been recorded in the soil, along with the cyanobacterium *Oscillatoria* sp., and the algae *Trebouxia* sp. and *Heterococcus* sp. (Parker et al., 1982). Chasmoendolithic cyanobacteria have been recorded in rocks on the west spur of Walker Peak at about 1070 m in the Dufek Massif (Friedman, 1977), although we found no evidence of their presence within the dry valley areas included in our survey, suggesting that endolithic organisms are not widespread here.

4.3. Fauna

The invertebrate fauna within the area was equally impoverished, with both the diversity and abundance of organisms being extremely limited compared with lower latitude and coastal Antarctic sites. A total of 50 Tullgren extractions of terrestrial cyanobacterial mat and soil substrates generated no arthropods. Although a negative result, this strongly suggests that these groups are absent from the area as there are no other obvious habitats which might be expected to harbour them. A total of 130 Baermann extractions revealed three species of tardigrade

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from two Classes: *Echiniscus* (cf) *pseudowendti* Dastych, 1984 (Heterotardigrada), *Acutuncus antarcticus* (Richters, 1904) and *Diphascon sanae* Dastych, Ryan and Watkins, 1990 (Eutardigrada) and a few unidentified bdelloid rotifers (Table 1). Tardigrades were commonly found (c. 40–60 per cc) while rotifers were rarer (c. 5–15 per cc); although not a quantitative extraction technique, these values are low in comparison with those obtained from similar extractions from other Antarctic locations. Surprisingly, the most productive sites for these organisms were not the aquatic environments of the permanent lakes, but the former pond beds in the Davis Valley. *Acutuncus antarcticus* is an Antarctic species that occurs in semi-permanent damp/wet habitats throughout the Antarctic continent and sub-Antarctic islands, but has not been reported from any of the close neighbor continents. *Echiniscus* (cf) *pseudowendti* and *Diphascon sanae* found in samples from Forlidas Pond are also endemic to the Antarctic, with restricted distributions. For example, *Echiniscus* (cf) *pseudowendti* has been found in the maritime regions of the Antarctic Peninsula, Dronning Maud Land (Heimefrontfjella) and Enderby Land (Thala Hills), and *Diphascon sanae* from Dronning Maud Land (Robertskollen), Enderby Land (Prince Charles Mountains and Mawson Station) and Ellsworth Land (for a discussion of the molecular data see Sands et al., 2008).

Avifauna was sparse. A single snow petrel (*Pagadroma nivea*) was noted flying around one of the peaks above Davis Valley.

4.4. Biological interactions with the physical environment

As "dry valley" ecosystems, the Davis and Forlidas Valleys share environmental features with the Dry Valleys of Victoria Land. However, they are 2100 km distant from them and 570 km further south (82°S vs. 77°S). The climate of the Dufek Massif is that of an Antarctic cold desert, experiencing limited precipitation and rapid ablation. This restricts the potential for metabolic activity and prevents growth for much of the year, possibly even requiring dormancy on multi-year timescales. However, summer microclimate temperatures measured during the field campaign were relatively high, ranging from −14.5 °C to +12.8 °C over snow and −8.6 °C to +16.0 °C over rock during our visit. The valleys also have many features related to wind erosion but some of these may be relatively ancient, as they occur mostly on rock surfaces above the glacial drift limits, whilst the foliose terrestrial cyanobacterial growth forms remain intact on the valley floor.

Table 3
Bacteria (uncultured diversity).

	TM1	TM2	Total
Unsequenced or unidentified DGGE bands	16	11	21
Bacteroidetes	3	2	3
Cyanobacteria	2	3	5
Firmicutes	0	1	1
Gammaproteobacteria	1	1	2
Total	22	18	32

With these relatively harsh conditions and physical isolation, it is perhaps not surprising that the valleys appear to lack many of the components typical of the Victoria Land ecosystems (see Adams et al., 2006), including nematodes, arthropods and mosses, and that there is an extremely sparse development of lichens. Cyanobacteria are the dominant phototrophs, in common with other aquatic and terrestrial polar ecosystems (cf. Vincent, 2000), and benefit from a range of biochemical adaptations for survival in shallow water and terrestrial habitats (Hodgson et al., 2004). In the Dufek Massif their greatest biomass was found in the microbial mats that form in the benthic and littoral zones of lakes and ponds and in terrestrial habitats.

The abundance and macroscopic growth-form of the terrestrial cyanobacteria despite apparent limited water availability is something of a paradox. At the time of our visit there was very little snow within either valley bottom. However, water is required for the mats to be metabolically active and is likely derived from within snow patches or from snow melt focussed into depressions and in the lee of boulders, with metabolically active periods being short and unpredictable. Alternatively, later in the season there may be periods of increased supraglacial melt water flowing off the local ice sheet and outlet glaciers, which could potentially provide a source. Although there was no implication of this process occurring during our visit, we located deep footprints from a previous visit (i.e. 20–45 years old), which indicated that some ground was waterlogged at that time.

The most productive terrestrial habitats appeared to be the dried areas of mat on the beds of the relict proglacial ponds (Fig. 9e) and in cracks, crevices, the lee side of boulders (Fig. 9c), and in the shallower parts of the ponds (Fig. 9a). This is a likely function of these environments either containing seasonally liquid water or accumulating snow cover in winter which persists into spring. Previous studies suggest that such under snow habitats can be biologically very active in Antarctica (Cockell et al., 2002), and they also have the advantage of protecting the biota from exposure to

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wind abrasion. Extractions from samples taken from within these areas were found to yield the greatest numbers of rotifers and tardigrades.

Mats collected within the freshwater ponds did not generate larger numbers or diversity of invertebrates. Rotifers, at least, were present in clumps of mat that had travelled upwards through the pond ice – it is plausible that they have been present throughout the upwards journey, although colonisation while on the ice surface is also possible.

4.5. Species diversity and endemism

The very limited species diversity in the Forlidas and Davis Valleys supports the hypothesis that, in the more extreme and remote regions of continental Antarctica, species assemblages present are characterised by a low biodiversity, taxa tolerant of extreme cold and dry conditions, and a certain degree of endemism. For Antarctic microbial communities, molecular studies have shown that their composition includes both cosmopolitan OTUs, and also a greater number of Antarctic endemic species than has been estimated by traditional morphological methods (Taton et al., 2006a; DeWever et al., 2009). Our molecular data for cyanobacteria in Forlidas Valley show a depleted diversity, with only 3–5 OTUs per sample compared with the greater number of OTUs found in other regions of Antarctica (Taton et al., 2006a). This is likely a product of geographical isolation (cf. Vyverman et al., 2007; Verleyen et al., 2010) combined with multiple environmental stressors such as salinity and seasonal desiccation, and UV radiation (Bowman et al., 2000; Taton et al., 2006a). However some of the cyanobacteria, such as OTU 16ST63 from the brine of Forlidas Pond, are related to sequences from other hypersaline Antarctic lakes such as Rauer 8 in the Rauer Islands (for location see Hodgson et al., 2001) and Ace Lake in the Vestfold Hills (16ST23 in Taton et al., 2006a), but are also present in desert crusts on sand dunes in Israel (AM398947) and in saline lakes in Chile (EF633019). OTU 16ST07 only includes sequences from different sampling locations on the Antarctic continent and sequences from Tibetan glaciers, which could indicate that this OTU has particular adaptations to glacial conditions (Fig. 10). The six cyanobacterial OTUs are all found outside Antarctica, but in addition, they are all distributed in more than one location within the continent (Fig. 10). This is in agreement with previous studies where the cosmopolitan OTUs have been found to be more widespread on the continent than endemics. For example, Taton et al. (2003, 2006a, b) studied the molecular diversity in benthic cyanobacteria in lakes from different and geographically separated Antarctic biotopes, including Lake Fryxell (McMurdo Dry Valleys) and coastal lakes in the Prydz Bay region (East Antarctica). In addition, two melt water samples from Livingston Island (Antarctic Peninsula) have recently been studied (unpubl. data). Using clone libraries based on 16S rRNA sequences, 70% of OTUs in these studies were only found in Antarctica. This suggests a rather high degree of endemism, though the influence of geographic gaps in the database might be biasing the data. However, within these studies, a higher proportion of the cosmopolitan genotypes were found in multiple Antarctic regions (47%, compared to 16% for the apparently 'endemic' sequences). These cosmopolitan genotypes are likely to possess resistance capacities (Taton et al., 2006b) that will also be beneficial during dispersal to and between different Antarctic regions (Zakhia et al., 2007).

At Forlidas Pond the three OTUs found in the hypersaline brine (TM1) were also present in the littoral zone (TM2), three of the OTUs from the littoral zone were also found in the terrestrial sample (TM3) and one OTU (16ST49) was present in all three samples, suggesting that although the diversity is low some OTU's have a wide environmental tolerance (Fernández-Carazo et al. unpublished data). Contrary to some of the observations in Wright and Burton (1981), who reviewed the biology of Antarctic saline lakes, the cold brine in Forlidas Pond does not appear to be incompatible with cyanobacterial growth, although salinity and freezing conditions are likely to limit metabolic processes as they do in some of the more saline ponds on the McMurdo Ice Shelf (cf. Vincent, 2000). The dominance of the cyanobacteria over the green algae agrees with other studies which have suggested that continental cyanobacteria are more resistant to freezing and desiccation regimes than sub-Antarctic taxa, and are more abundant than green algae whose membranes are poorly adapted to freeze-thaw processes (Šabacká and Elster, 1996).

Of the cultivated bacterial isolates characterised from sample TM2, 13.5% had less than 97% similarity to known sequences in the EMBL database, indicating that these taxa represent organisms that have not been reported previously and are potentially new to science. This observation is in line with previous reports (Brambilla et al., 2001; Van Trappen et al., 2002) and is not unexpected in view of the limited amount of studies using cultivation to study Antarctic bacterial diversity, and the estimate that only a small fraction of bacterial species have so far been described (Schloss and Handelsman, 2004).

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The degree of endemism of all microbial groups in Antarctica is still debated (see Vyverman et al., 2010; this volume). A consistent problem faced by researchers interested in the possibility of microbial endemism is the paucity of Antarctic data relating to microbial diversity and distribution (Wynn-Williams, 1996). As a broad generalisation, microbiota are thought not to face the same dispersal limitations as do many larger organisms or their propagules. This has led to the development of the 'global ubiquity hypothesis' (Finlay, 2002), whereby their small size means that they can easily enter and remain in the air column and thereby reach all parts of the planet. This has also been rationalised in terms of 'everything is everywhere, and the environment selects', in other words that dispersal is not a limiting factor on species distribution, which is rather controlled by possession of appropriate adaptations to allow survival, development and reproduction under the conditions imposed by the 'recipient' environment. This is consistent with previous descriptions of a largely cosmopolitan microbial flora, for example as applied to classical morphological studies of the eukaryotic algae (Broady, 1996) and many of the diatom studies cited in Jones (1996).

However, some authors suggest that microbial endemism is still possible because of the long isolation of Antarctica from other parts of the world, the fact that dispersal processes which favour local species are more efficient than long distance dispersal processes and that there has probably been strong environmental selection for adaptive strategies (Vincent, 1988; Franzmann, 1996). The application of molecular biological techniques of identification has led recently to an increase in records of microbial diversity through sequence data (e.g. Adams et al., 2006), although an inherent weakness of these approaches, as with more classical culture techniques, is that sequence presence and detection does not automatically prove biological activity or functional significance within the ecosystem. In the absence of baseline microbial diversity data against which to compare, assessment of endemism, or indeed of post colonisation adaptation, remains difficult. However, Lawley et al. (2004) argued that there was circumstantial support for Antarctic endemism in diverse microbial groups based on very limited overlap in OTU composition between different locations in a comparative study based on the same soil habitat, and Boenigk et al. (2006) argued that there is evidence for considerable ecophysiological specialization within Antarctic strains of certain microbial taxa, only possible if these have been isolated for long periods of evolutionary time.

Few studies have attempted to assess directly the mechanism of dispersal employed by microbial communities in Antarctica, although representatives of the cyanobacteria have been recorded in simple aerobiological trapping studies at locations on the Antarctic Peninsula (Hughes et al., 2004; Pearce and Galand, 2008). However, the mechanisms of dispersal in Antarctica are unlikely to be different to those commonly recorded elsewhere, with the major difference for terrestrial biota relating to the paucity and isolation of suitable establishment sites. Thus, the major routes of dispersal are likely to be through transport in the air column, incidental attachment to other biota, transport in freshwater flows (on a local scale within Antarctica) and, more recently, human transportation (Frenot et al., 2005; Hughes et al., 2006; Convey, 2008).

In the Dufek Massif, we found no evidence of local endemic organisms isolated there for long periods of evolutionary time. This is consistent with the glaciological history which suggests the repeated exposure of ice free surfaces for about 1.6 million years (Hodgson et al., unpublished data) which is insufficient time for in-situ speciation, which for bacterial 16S rRNA genes, is c. 50 million years for 1% divergence. Instead, there are some groups of taxa present that are endemic to the Antarctic continent (tardigrades, a lichen and possibly some bacteria), and others which are cosmopolitan (for example none of the green algal, cercozoan or bacterial DGGE-bands are unique to Antarctica). For the cyanobacteria, only cosmopolitan cyanobacterial OTUs were found, and among the bacterial strains obtained in cultivation about 13.5% represent potentially new species some of which may be endemic. These results imply that the Dufek Massif has not functioned as a biological refuge over long timescales (cf. Convey et al., 2008, 2009). Instead it has been colonised in the Quaternary by a combination of Antarctic endemic and cosmopolitan taxa whose distribution, dispersal and establishment has been dependent upon life cycle characteristics (e.g., formation of resting spores and resistance to the extreme environmental conditions).

5. Conclusions

There has been recent recognition that levels of endemism and/or molecular evolutionary differentiation are considerably greater than previously appreciated across most of the groups of terrestrial biota (with the exception of bryophytes) that currently dominate Antarctic terrestrial communities (Convey and Stevens, 2007; Peat et al., 2007; Convey et al., 2008; Pugh and Convey, 2008). These are interpreted as supporting long-term terrestrial biological presence in Antarctica

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through glacial cycles and even in some cases back to the breakup of Gondwana. Furthermore, this includes considerable regionalisation within Antarctica itself (Chown and Convey, 2007; Pugh and Convey, 2008). Although we found no evidence in this study of regionalisation within the Dufek Massif, we did find a mix of Antarctic endemic and cosmopolitan species that have colonised ice free land there during the Quaternary period. We also found that the Dufek Massif contains some of the most reduced metazoan terrestrial and freshwater ecosystems known from Antarctica with autotrophs limited to cyanobacteria, plus a few green algae and lichens. This pristine and low diversity flora and fauna, only 800 km from the South Pole reinforces the importance of the area's designation as an ASPA, and highlights a particular vulnerability to human impacts.

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RESEARCH ARTICLE

Low cyanobacterial diversity in biotopes of the Transantarctic Mountains and Shackleton Range (80–82°S), Antarctica

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Transantarctic Mountains; Forlidas Pond; Lundström Lake; cyanobacterial biodiversity; Antarctica; endemism.

Abstract

The evolutionary history and geographical isolation of the Antarctic continent have produced a unique environment rich in endemic organisms. In many regions of Antarctica, cyanobacteria are the dominant phototrophs in both aquatic and terrestrial ecosystems. We have used microscopic and molecular approaches to examine the cyanobacterial diversity of biotopes at two inland continental Antarctic sites (80–82°S). These are among the most southerly locations where freshwater-related ecosystems are present. The results showed a low cyanobacterial diversity, with only 3–7 operational taxonomic units (OTUs) per sample obtained by a combination of strain isolations, clone libraries and denaturing gradient gel electrophoresis based on 16S rRNA genes. One OTU was potentially endemic to Antarctica and is present in several regions of the continent. Four OTUs were shared by the samples from Forlidas Pond and the surrounding terrestrial mats. Only one OTU, but no internal transcribed spacer (ITS) sequences, was common to Forlidas Pond and Lundström Lake. The ITS sequences were shown to further discriminate different genotypes within the OTUs. ITS sequences from Antarctic locations appear to be more closely related to each other than to non-Antarctic sequences. Future research in inland continental Antarctica will shed more light on the geographical distribution and evolutionary isolation of cyanobacteria in these extreme habitats.

Introduction

Cyanobacteria are the dominant phototrophs in both aquatic and terrestrial ecosystems in many regions of Antarctica. They develop considerable biomass in microbial mats (laminated biofilms) that typically form on the benthos and littoral zones of lakes and ponds, and on the surface of areas of soil where seasonal snow patches accumulate or summer water availability is reliable (Vincent, 1988).

Despite the prominent role of cyanobacteria in Antarctica, it is still unclear whether geographic isolation is a structuring factor in the biogeography of microorganisms in this region. Several studies elsewhere have shown that biogeographical distribution patterns do occur in free-living microorganisms, although separating the respective roles of contemporary environmental conditions and historical contingencies is often difficult (see Martiny *et al.*, 2006 for a review). Recently, Bahl *et al.* (2010) found that the global distribution of desert cyanobacteria is the result of an ancient evolutionary legacy, rather than contemporary dispersal, highlighting the importance of temporal scales in biogeographical studies. In Antarctica, endemism has been reported in several taxa, for example in terrestrial metazoa and lichens (see Convey & Stevens 2007; Convey *et al.*, 2008 for a review). Similarly, the chironomid midges (*Diptera*) appear to be restricted to tectonically distinct parts of the Antarctic Peninsula and the Scotia Arc (Allegrucci *et al.*, 2006), and possibly all nematode species present in Antarctica are endemic to the continent (and to smaller regions within it) (Andrássy, 1998; Maslen & Convey, 2006). Finally, the review of Vyverman *et al.* (2010) identified a high degree of endemism in the diatom flora. However, although endemism has been commonly recognized in higher eukaryotic taxa, prokaryotes are commonly assumed to be widely dispersed by effective mechanisms that should reduce the occurrence of endemicity (Baas Becking, 1934; Finlay, 2002).

One of the challenges in determining the presence of endemic organisms is applying an appropriate level of taxonomic resolution. This is because different resolution molecular markers can influence the interpretation of

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the biogeographic distribution of particular taxa. For example, Cho & Tiedje (2000) demonstrated that the use of different markers [16S rRNA gene, internal transcribed spacer (ITS) and genomic fingerprints] on fluorescent strains of *Pseudomonas* showed no endemicity on the basis of 16S rRNA gene, but a high level of endemicity was revealed by the genomic fingerprints.

Previous molecular studies of Antarctic cyanobacteria have shown that they include both cosmopolitan and potentially endemic operational taxonomic units (OTUs) (Taton *et al.*, 2003, 2006a, b). The OTUs were defined as groups of 16S rRNA gene sequences that shared more than 97.5% similarity (Taton *et al.*, 2006a). The observed divergence of Antarctic endemic OTUs implies an isolation of at least 125 My, as the mutation rate of 16S rRNA genes has been estimated as 1% every 50 million years (Ma) (Ochman & Prager, 1987). The most comprehensive studies on cyanobacterial endemism in Antarctica, to date, have worked on samples from coastal regions of east Antarctica (Taton *et al.*, 2006a, b) and the McMurdo Dry Valleys (Taton *et al.*, 2003). These studies led to two conclusions: (1) the molecular approach suggested that potential Antarctic endemic OTUs (57%), including a previously undiscovered diversity (39%), were more abundant than previously estimated using morphological approaches (35%) and (2) cosmopolitan OTUs were more widespread over the continent than potential endemics. However, since 2006, the databases have grown and the proportion of these potential Antarctic endemic OTUs has declined to 28.5%.

As previous studies have mostly focused on near-coastal locations, little is known about the relative abundances of cosmopolitan vs. endemic cyanobacteria in the more southerly, inland, extreme and isolated freshwater-related ecosystems of Antarctica. To address this, we examined the morphological and molecular diversity of cyanobacteria inhabiting Forlidas Pond in the Transantarctic Mountains (TAM) and Lundström Lake in the Shackleton Range (80–82°S). This involved a polyphasic study of the diversity and distribution of the cyanobacteria in four samples, including an analysis of the ITS region to improve the precision of groupings based on 16S rRNA genes (Taton *et al.*, 2006a). The results were compared with sequence databases, in order to better understand the distribution patterns of the cyanobacterial genotypes identified. This work is the first report of the cyanobacterial diversity, studied using a polyphasic approach, in the TAM and the Shackleton Range.

Materials and methods

Sampling and site description

The TAM are one of the world's longest continental rift flank uplifts. They divide the continent into two unequal (West and East) elements. The mountains rise from the sea level to elevations of *c.* 4000 m. They constitute one of the harshest ecological areas of Antarctica (Turner *et al.*, 2009; Hodgson *et al.*, 2010).

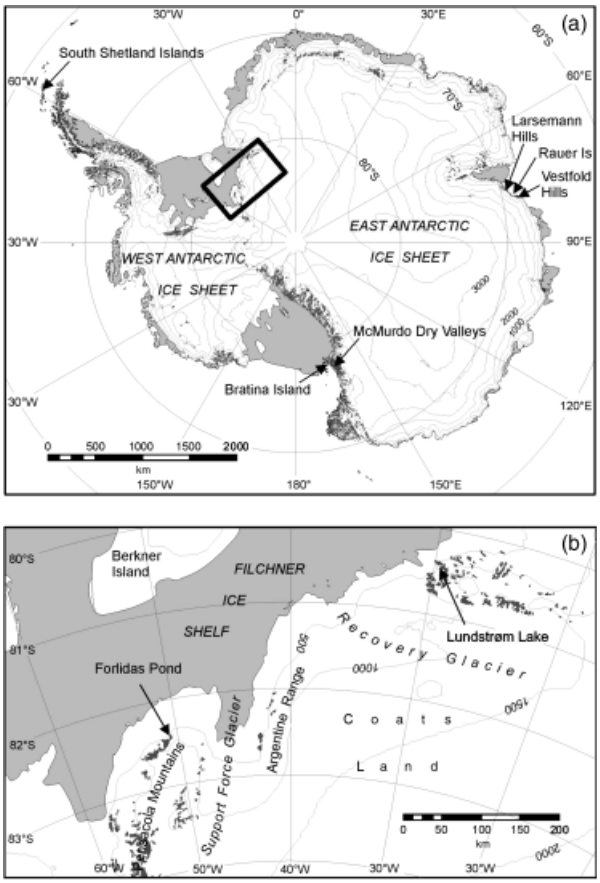

Fig. 1. Location map of the sampled water bodies. (a) The sampled area is marked with a black rectangle. (b) Lakes are indicated by arrows.

Our first study site, 'Forlidas Pond' (51°16′48″W, 82°27′28″S, within the Antarctic Specially Protected Area 119), is a 90 m diameter frozen freshwater lake overlying a 20 cm thick layer of hypersaline brine located in an ice-free 'dry' valley situated at the north-eastern end of the Dufek Massif (see Hodgson *et al.*, 2010 for details). Our second study site is Lundström Lake (29°26′56″W, 80°26′30″S) (Fig. 1 and Supporting Information, Fig. S1a and b), located in the Haskard Highlands of the Shackleton Range, 420 km north-east of Forlidas Pond. Lundström Lake is a shallow, perennially ice-covered lake, 400 m long and 300 m wide and, like Forlidas Pond, is the remnant of a much larger proglacial lake that last filled the valley in the mid-Holocene (Höfle & Buggisch, 1995). The lake, at 640 m above sea level, is frozen down to the bed at 3.14 m ice depth. The conductivity in the interstitial water in the ice at the bed was higher (2.9 mS cm^{-1}) than the littoral surface water

(0.227 mS cm^{-1}), suggesting a limited accumulation of catchment-derived salts in the lake basin (Table S1).

Four samples were collected during the 2003/2004 Antarctic summer at the two study sites. Three samples were analyzed from Forlidas Pond (TM1 and TM2, from the hypersaline brine and the freshwater littoral zone, respectively) and its catchment (TM3) (see Hodgson *et al.*, 2010 for details). The TM3 sample was selected as an example of the cyanobacterial mats that are frequently observed in the dry valleys of the Dufek Massif (Hodgson *et al.*, 2010). A fourth microbial mat sample (TM4) was taken from the littoral zone of Lundström Lake.

Samples of cyanobacteria in the benthic brine layer of Forlidas Pond were obtained using a UWITEC water sampler (Hodgson *et al.*, 2010) and those from the littoral and terrestrial zones of both lakes were sampled manually into sterile bags using tweezers. All samples were stored in sterile Whirlpak bags, frozen in the field and transferred frozen to the UK via the British Antarctic Survey station at Rothera Point. Subsamples were then sent to the University of Liège under dry ice.

Morphological characterization and cultures

The major cyanobacterial taxa were examined in both the environmental samples and cultures using a Leica DM LB2 microscope (Leica Microsystems GmbH, Wetzlar, Germany). The taxonomic work of Komárek & Anagnostidis (2005) was used as a reference, and the diacritical morphological traits used for botanical species identification followed Taton *et al.* (2006b). Cells were measured using the AXIOVISION software (Carl Zeiss MicroImaging GmbH, Germany), and the results were expressed as the mean measurements of at least five cells per morphotype.

Environmental samples were grown in several media to isolate strains. In addition to BG11 (Rippka *et al.*, 1981), we used media with different salinities (0.068–33 g L^{-1} NaCl) because the TM1 sample was collected from a hypersaline brine. Thus, media $BG11_0$, 2, 3 (nitrogen-free media) and 2NP, 3NP, ASNIII/2 and ASNIII media (nitrogen-containing media) were used (Rippka *et al.*, 1979; Taton *et al.*, 2006b). Cycloheximide (50 mg L^{-1}) was used when inoculating the environmental samples on culture plates for the first time, to avoid eukaryotic contaminants (Taton *et al.*, 2006b). The temperatures of incubation used were 12 and 22 °C, to allow the growth of psychrophilic and/or psychrotolerant cyanobacteria, respectively (Morita, 1975).

DNA extraction and purification

The DNA extraction method was modified from Taton *et al.* (2003). A 0.5-g mat sample was thawed and then frozen overnight and thawed again, and then rinsed two times with 1 mL of phosphate-buffered saline (0.08 M Na_2HPO_4, 0.03 M NaH_2PO_4, 1.50 M NaCl, pH 7.2). Glass beads (0.25 g) (diameter 0.17–0.18 mm; Braun Biotech) were added and pressed with a pestle (Eppendorf, Hamburg, Germany) several times in a 2-mL Eppendorf tube. Next, 250 μL of SNT solution was added (500 mM Tris-HCl pH 8, 100 mM NaCl, 25% saccharose) supplemented with the addition of 130 μL of fresh lysozyme (50 mg mL^{-1}). Cells were further disrupted using the pestle, followed by the addition of 250 μL of SNT solution. The suspension was then incubated for 1 h at 37 °C with mixing every 15 min. After this incubation, 0.5 mL of solution II (Tris base 500 mM, EDTA 500 mM, SDS 1%, phenol 6%) was added and the suspension was vortexed continuously for 30 min. The suspension was then placed on ice for 1 h and shaken briefly by vortex every 10 min. After this incubation, the suspension was centrifuged for 10 min at 720 ***g*** (Centrifuge 5424, Eppendorf) and 1 mL of the aqueous phase was mixed with an equal volume of phenol, after which it was centrifuged for 5 min at 13 600 ***g***. The supernatant was then transferred into new tubes, extracted with equal volumes of phenol–chloroform–isoamyl alcohol (25 : 24 : 1) and re-extracted with equal volumes of chloroform–isoamyl alcohol (24 : 1). Then, a Na acetate–ethanol precipitation was performed and the dried pellet was resuspended in 200 μL of TE-4 buffer (10 mM Tris-HCl pH 8, 0.1 mM Na_2EDTA pH 8). The environmental DNA was purified using the Wizard DNA clean-up system (Promega, Madison). The DNA was eluted by adding 100 μL of TE-4 buffer.

Cloning and screening of clone libraries

PCR amplification of the cyanobacterial 16S rRNA gene plus the ITS was performed as described by Taton *et al.* (2003), with the minor modifications that 1 μL of DNA was added and the PCR cycles were altered: amplification was carried out using an Icycler thermocycler (Bio-Rad), with one cycle of 5 min at 95 °C; 35 cycles of 45 s at 95 °C, 45 s at 54 °C and 3 min at 68 °C; and a final elongation step of 7 min at 68 °C. Three PCRs were carried out and the products were pooled. Negative controls (PCR mix with no DNA) were always included. This mix of amplicons was purified two times using Quantum Prep PCR Kleen Spin columns (Bio-Rad). Poly(A) extension was performed using the Qiagen A-addition Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Cloning of the PCR products (*c.* 1600 bp) utilized the TOPO TA cloning kit (Invitrogen BV, Breda, the Netherlands) as described by Taton *et al.* (2003), except that the screening PCR was performed with the 16S378F and 16S784R primers. The amplification conditions described above were used, except that 0.8 U of Super *Taq* polymerase (HT Biotechnology, Cambridge, UK) were used and amplification was carried out as follows: 10 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 60 °C

and 1 min at 72 °C; and a final elongation step of 7 min at 72 °C. Plasmid DNAs were extracted using a Quantum Prep Plasmid Miniprep kit (Bio-Rad) following the manufacturer's instructions. All the primers used have been described by Taton *et al.* (2003, 2006a).

Amplified ribosomal DNA analysis (ARDRA)

The direct sequencing of a number of clones with primer 16S1092R was complemented by a screening of the remaining clones with ARDRA to avoid replicates. The clones of each clone library were digested together, with at least one sequence belonging to the different OTUs detected by direct sequencing. The inserted 16S rRNA gene plus ITS was reamplified with primers 16S378F and 23S30R as described above. All steps were performed as described previously (Scheldeman *et al.*, 1999; Taton *et al.*, 2003), except for the following changes: MboI and HpaII (MBI Fermentas, Vilnius, Lithuania) were used as restriction enzymes and electrophoresis was performed at a constant voltage of $3\,V\,cm^{-1}$ for 235 min in NuSieve (3 : 1) agarose gels (Cambrex, NJ). The numbers of clones analyzed by ARDRA were 40, 25, 55 and 44 for TM1, TM2, TM3 and TM4, respectively (Table S2). Clones with different band patterns were sequenced.

Denaturing gradient gel electrophoresis (DGGE) analysis

Two successive PCR reactions were run to produce 422-bp-long fragments, as described in Taton *et al.* (2003), except that electrophoresis was performed for 999 min at 75 V and 60 °C. Two DGGE (a and b) were performed for each sample. The PCR primers used to perform the DGGE (a) and (b) targeted filamentous and unicellular cyanobacteria, respectively (Nübel *et al.*, 1997). A marker was provided by S. Cousin (U. Ghent, pers. commun.), which provided nine bands uniformly distributed along the gel. The excised DGGE bands were incubated in TE-4 buffer for 12 h at 4 °C. Each DNA solution was used as a template for PCR reamplification (primers 16S378R and 16S784R), followed by purification (Ilustra DNA and band gel purification Kit, GE Healthcare, Belgium) and sequencing. When several bands with identical or quasi-identical sequences were obtained for one sample, only one representative is shown in the tree.

Sequencing was carried out by GIGA (http://www.giga.ulg.ac.be/) (Liège, Belgium) using an ABI 3730xls DNA analyser (Applied Biosystems, Foster City). DGGE bands were sequenced using the 16S784R and/or 16S359F primers to obtain sequences of about 350 bp. Almost complete 16S rRNA gene (*Escherichia coli* positions 364–1526) plus ITS sequences were determined for clones from the clone libraries using the sequencing primers 16S1092R (Taton *et al.*, 2006a), 16S1494R (Wilmotte *et al.*, 2002) and 23S30R (Taton *et al.*, 2003). Thirty sequences were submitted to GenBank and assigned the following accession numbers for 16S rRNA genes from sample TM4 (EU852501–EU852506, HQ219056–HQ219059, HQ219060–HQ219062) and for ITS from samples TM1 to TM4 (EU852516–EU852519, EU852521–EU852532 and HQ336421).

Analysis of sequence data

Partial sequences of 16S rRNA genes from clones (*E. coli* positions 379–1542) and DGGE bands (*E. coli* positions 359–800) were used for the BLAST analyses. The 16S rRNA gene sequences from the DGGE and clone libraries from samples TM1, TM2 and TM3 were reported by Hodgson *et al.* (2010) and a BLAST analysis including these data and those obtained here from TM4 was carried out (July 17, 2010). Similarly, complete ITS sequences from the four clone libraries (TM1 to TM4 samples) were used for BLAST analysis, with the exception of some sequences that were slightly shorter. Chimeras were detected using CHIMERA CHECK in the Ribosomal Database Project (Maidak *et al.*, 2001), PINTAIL (Ashelford *et al.*, 2005) and BELLEROPHON (Huber *et al.*, 2004) and then excluded from the analysis. Aligned partial 16S rRNA gene sequences corresponding to *E. coli* sequence positions 379–806 were used to select the three most similar strain sequences and three uncultured sequences using the option SEQMATCH in RDPII (http://rdp.cme.msu.edu) (Cole *et al.*, 2009). A distance tree was constructed using the software package TREECON for Windows 1.3b (Van de Peer & De Wachter, 1997) not taking into account indels and ambiguous bases. The dissimilarity values were corrected for multiple substitutions using the method of Jukes & Cantor (1969) and were used to calculate a distance matrix. The tree was constructed using the neighbor-joining method (Saitou & Nei, 1987). A bootstrap analysis was performed that involved the construction of 1000 resampled trees.

The sequences were grouped into OTUs, following the numeration proposed by Taton (2005). A distance matrix with the Jukes and Cantor correction was calculated using the DNADIST software from the package PHYLIP 3.67 (Felsenstein, 1989). Clustering into OTUs was carried out with the DOTUR software (Schloss & Handelsman, 2005) using the average neighbor method (*E. coli* positions 405–780).

The ITS sequences of several clones were determined to assess the homogeneity of ITS within the same OTU. The ITS sequences were analyzed by a similarity search using the BLAST program and then aligned on the basis of conserved domains (Iteman *et al.*, 2000). The ITS sequences were grouped into 'ITS types', which are groups of sequences that can be meaningfully aligned (Wilmotte *et al.*, 1994), sharing at least 75% pairwise identity.

The clone library accumulation curves, coverage index and Berger–Parker index were analyzed using the software 'SPECIES DIVERSITY AND RICHNESS' (Pisces Conservation Ltd, New Milton, UK).

Results

Microscopic diversity

Six morphotypes were observed in the four samples, all belonging to the order *Oscillatoriales* (Table 1 and Table S3). Four of these were isolated in a pure culture using BG11 media. Three isolated strains, in unicyanobacterial cultures, were added to the BCCM/ULC Belgian collection of (sub)-polar cyanobacteria (http://bccm.belspo.be/about/ulc.php). The diacritical characters used for botanical species identification and their corresponding values are listed in Table 1.

No heterocystous or unicellular cyanobacteria were detected by microscopy in this study, although they have been observed in lakes located at lower latitudes (Taton *et al.*, 2006a; Verleyen *et al.*, 2010).

The isolated strains were characterized at the species level on the basis of their morphology. The two TM4 morphotypes (Fig. 2c and e) that could not be cultured, possibly because of their rareness, were detected only by microscopy in the environmental samples. The first was identified as *Phormidium crassior* (Fig. 2c). The small size of the second morphotype (Fig. 2e) only allowed assignment at the genus level as *Leptolyngbya* sp., because of its cell shape, thin sheaths and usually regularly twisted trichomes.

Molecular diversity

The complete 16S rRNA gene sequences plus ITS were obtained from the four strains isolated in this study (Tables 2 and 3). The 16S rRNA gene sequences from TM1, TM2 and TM3 analyzed in Discussion are reported from Hodgson *et al.* (2010) to allow for the comparison of the diversity found in Forlidas Pond and Lundström Lake. The analysis of the four clone libraries (TM1, TM2, TM3, TM4) characterized 264 clones (16S plus ITS) with an insert of the correct size (*c.* 1700 bp). To assign clones to taxonomic clusters, 100 clones chosen at random were partially sequenced (*E. coli* positions 364–1044) and corresponded to 1, 3, 2 and 1 OTUs for TM1, TM2, TM3 and TM4, respectively. The remaining 164 clones were studied by ARDRA (16S plus ITS) and belonged to seven different clusters based on the band patterns. At least one complete sequence from the clone libraries was determined for each OTU. In total, 12 different almost complete sequences were obtained for seven OTUs.

All TM1 sequences and ARDRA patterns were identical. It can be assumed that all the clones were identical, especially as the PCR product included the variable ITS (Taton *et al.*, 2003). For the TM2 sample, 12 different restriction patterns

Table 1. Diacritical characteristics of the cyanobacterial morphotypes in the samples, presence and correspondence with the OTUs

Morphospecies	Strain name	Isolation medium	Sheath	False branching	Cross-wall constriction	Necridic cell	Cell shape	Apical cell shape	Cell width (minimum–maximum) (μm)	Cell length (minimum–maximum) (μm)	Presence in samples*	OTU
A – *Leptolyngbya glacialis*	TM1ULC73†	BG11	+	–	+	–	c	r	1.2 (0.9–1.3)	1.7 (1.4–2.4)	TM1, TM2	16ST63
B – *Phormidium murrayi*	TM2ULC130†	BG11	–	+	–	+	c	c	4.9 (4.2–5.8)	9.5 (6.9–14.1)	TM2	16ST49
C – *Leptolyngbya* cf. *foveolarum*	TM2ULC129†	BG11	–	–	+	+	b	r	1.2 (1.1–1.3)	0.8 (0.7–1.0)	TM2, TM3	16ST80
	TM3FOS129											
D – *Phormidium crassior*	¶	–	+	–	–	+	b	r	8.3 (8.0–9.1)	NA	TM4	NA
E – *Phormidium priestleyi*	TM4LUS131	BG11	+	–	+	–	a	r	2.6 (2.2–3.0)	1.8 (1.2–2.3)	TM4	16ST10
F – *Leptolyngbya* sp.	¶	–	+	–	–	+	a, b	r	1.2	0.9	TM4	NA

*Observation of the morphotypes in the samples.
†Strains added to the BCCM/ULC Belgian collection of (sub)polar cyanobacteria.
¶, Morphospecies was detected in the environmental sample, but not isolated in culture; a, isodiametric; b, wider than long; c, longer than wide; r, rounded; c, calyptrate; NA, data were not available.

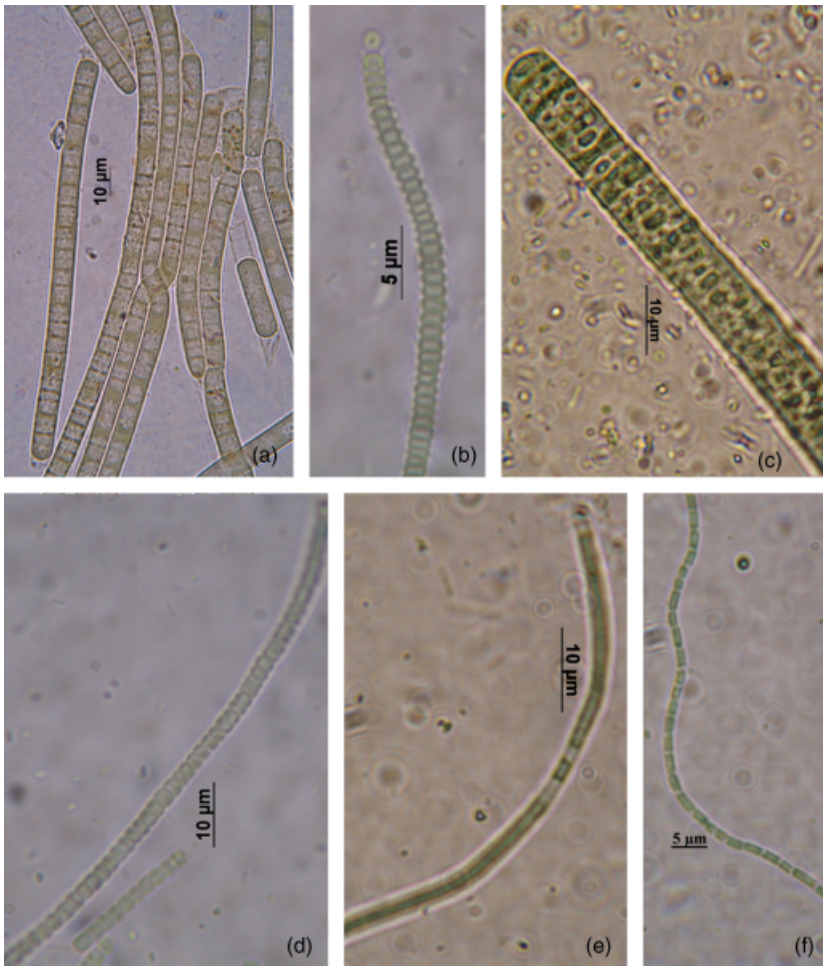

Fig. 2. Diversity of cyanobacterial morphotypes identified in environmental samples and cultures from Forlidas Pond and Lundström Lake. (a) *Phormidium murrayi* (TM2ULC130); (b) *Leptolyngbya* cf. *foveolarum* (TM2ULC129); (c) *Phormidium crassior*, TM4 environmental sample; (d) *Phormidium priestleyi* (TM4LUS131); and (e) *Leptolyngbya* sp., TM4 environmental sample; (f) *Leptolyngbya glacialis* (TM1ULC73).

were found by ARDRA. Eight were chimeras and only the clone TM2FOD1 belonged to a new genotype by comparison with the OTUs obtained by direct sequencing. For TM3, seven different ARDRA restriction patterns were obtained, but they were all chimeric. For TM4, the ARDRA analysis showed four different restriction patterns, but two of them were chimeric. Chimera analysis showed that chimerical sequences were absent from the TM1 sample, but accounted for 22% of the clones for TM2, 10% for TM3 and 3% for TM4. One of the TM4's chimeras contained a fragment derived from the OTU 16ST16, but this sequence is not shown in the distance tree (Fig. 3) as the fragment location (*E. coli* positions 811–1534) did not correspond to the positions used for the phylogenetic analysis.

The DGGE analysis of the sample TM4 revealed four OTUs (16ST02, 16ST44, 16ST53, 16ST92). In total, seven OTUs were retrieved from TM4, including clones, DGGE sequences and an isolated strain. The presence of OTUs from clone libraries, DGGE gels and isolated strains from all the samples is given in Table 4.

The clone library accumulation curves approached an asymptote (data not shown), indicating that it is unlikely that the diversity would be increased significantly if more clones were sequenced. In addition, the coverage index (Good, 1953) was 100% for TM1 and TM3 and $> 97.5\%$ for TM2 and TM4 (Table S2). The Berger–Parker index corroborated the low biodiversity, as it was > 0.94 for all the samples, except for TM2, where it was 0.43.

Distribution analysis

The 16S rRNA gene sequences from the cultures, the clone libraries and the DGGE were grouped into 12 OTUs, including the sequences from Forlidas Pond (Hodgson *et al.*, 2010) (Fig. 3). Five OTUs were only found in Forlidas Pond and its catchment (16ST07, 16ST14, 16ST49, 16ST63 and 16ST80), six were only present in Lundström Lake (16ST02, 16ST11, 16ST16, 16ST53, 16ST57 and 16ST92), while only one OTU (16ST44) was observed in both lakes.

Table 2. Analysis of the 16S rRNA gene sequences from Forlidas Pond (Hodgson *et al*., 2010) and Lundström Lake and the geographical location of OTUs

Selected sequences*	Accession number	Closest BLAST uncultured hit†	Closest BLAST strain hit†	OTUs	Origin of related sequences in Antarctica‡	Origin of related sequences outside Antarctica‡
TM4LUD1a3	HQ219059	Clone H-C30 (DQ181727) (99%)	*Synechococcus* sp. PCC 7502 (AF44808) (96%)	16ST02[A]	Fresh Pond, McMurdo Ice Shelf (AY541565); Lake Rauer7, Rauer Islands, (EU009706); Heart Lake, Larsemann Hills (DQ181726); Pup Lagoon (EU009721); and Lake Reid (DQ181712, EU009717), Larsemann Hills	–
TM3FODb1¶	EU852512	Clone ANTLV1_H06 (DQ521499) (100%)	*Leptolyngbya appalachiana* GSM-SFF-MF60 (EF429286) (93%)	16ST07[C]	Lakes Vida (DQ521499) and Fryxell (AY151728), McMurdo Dry Valleys; Lake Reid (DQ181673)	High Arctic microbial mat (FJ977124); Artic soil from ML-RS1 site (AM940881)
TM4LUS131‖	EU852504	Clone H-B07 (DQ181687) (98%)	*Phormidium priestleyi* ANT.LH66.1 (AY493581) (100%)	16ST11[C]	Lake in Larsemann Hills (AY493581); red snow, Langhovde (AB519661)	Soil sample in Svalbard, Norway (AM940913)
TM2FOCD1	EU852493	Clone LMM1-4 (EU032358) (97%)	*Phormidium priestleyi* ANT. L61.2 (AY493582) (92%)	16ST14[C]	Lake Miers, McMurdo Dry Valleys (EU032358)	Lake Tuusulanjarvi, Filand (AM259242); Bogotá river (Colombia) (EF111085); Shiga river biofilm, Japan (AB275532); Sedge Bay, Yellowstone Lake (United States) (EU340161); high Arctic microbial mat (FJ977133)
TM1FOD1b1	EU852508	Clone MIS92 (FJ977133) (99%)	cf. *Leptolyngbya* sp. Greenland (DQ431004) (89%)	16ST14[C]		
TM4LUCC30	EU852502	Clone LMM1-5 (EU032359) (98%)	*Phormidium* sp. SAG 37.90 (AM398795) (97%)	16ST16[C]	Lake Reid (DQ181681); Lake Miers (EU032359); Larsemann Hills (AY493576)	*Pseudanabaena tremula* (AF218371) isolated in Canada
TM2FOCH1	EU852489	Clone UMAB-cl-34 (FN811218) (98%)	*Microcoleus vaginatus* UBI-KK2 (EF654079) (99%)	16ST44[C]	Lake Fryxell (AY151749); Orange and Lunch Ponds, Bratina Island (AF263336, AF263335)	Douglas River, Australia (F438215); river Garonne (France) (AY456650); Canyonlands National Park (United States) (AF428550); Kansas (United States) (EU300529)
TM3FOCB5	EU852490	Clone UMAB-cl-34 (FN811218) (98%)	*Microcoleus vaginatus* UBI-KK2 (EF654079) (99%)	16ST44[C]		
TM4LUDa1¶	EU852514	DGGE gel band OTU_23 (FJ796382) (99%)	*Phormidium autumnale* CYN52 (GQ451424) (99%)	16ST44[C]		
TM2ULC130‖	EU852498	Clone AK4DE1 (GQ397048) (98%)	*Microcoleus vaginatus* UBI-KK2 (EF654079) (99%)	16ST44[C]		
TM3FODa4	HQ219058	Clone KuyT-ice-36 (EU263778) (100%)	*Phormidium* sp. Ant-Orange (AF263336) (100%)	16ST44[C]		
TM3FOCA2	EU852492	Clone A206 (DQ181671) (99%)	*Geitlerinema* sp. Sai004 (GU935348) (99%)	16ST49[C]	Lake Fryxell (AY151723, AY151768); Ace Lake,Vestfold Hills (DQ181671); Lake Firelight, Bølingen Islands (EU009679); Lake Manning, Larsemann Hills (EU009719)	Loa river, Chile (AF317510); freshwater bloom (Sweden) (AY874006); Andes Mountains (Bolivia) (EU728890); Great Sulphur Spring (United States) (FJ967937); Thermal spring (Australia) (EU106086)
TM2FOCA9	EU852500	Clone A206 (DQ181671) (99%)	*Geitlerinema* sp. Sai004 (GU935348) (99%)	16ST49[C]		
TM2FOCA2	EU852491	Clone A206 (DQ181671) (99%)	*Geitlerinema* sp. Sai004 (GU935348) (99%)	16ST49[C]		
TM4LUD2a3¶	HQ219060	Clone H0w-51 (EF632992) (98%)	*Lyngbya* sp. 0ES35S5 (DQ264199) (98%)	16ST53[C]	Lake Fryxell (AY151731, AY151724); benthic communities in King George Island (DQ533827)	Salar de Huasco (Chile) (AY151731); soil crusts of the Colorado Plateau (F428510); planktonic european cyanobacteria (DQ264199)
TM4LUCG9	EU852503	Clone RD017 (DQ181674) (100%)	*Leptolyngbya badia* CRS-1 (EF429297) (94%)	16ST57[c]	Lake Reid (DQ181675)	High mountain lake epilithic biofilm. Pyrenees (Spain) (FR667367)
TM1FOCA5	EU852494	Clone R8-R56 (DQ181691) (99%)		16ST63[C]	Ace Lake (AY493589); Lake Rauer 8 (DQ181691) and Salt Pond, McMurdo Ice Shelf (AY541528)	Salar de Huasco (EF633019); Shark Bay (Australia) (AY430152); agricultural soil (Spain) (AM503974);

Table 2. Continued.

Selected sequences*	Accession number	Closest BLAST uncultured hit†	Closest BLAST strain hit†	OTUs	Origin of related sequences in Antarctica‡	Origin of related sequences outside Antarctica‡
			Leptolyngbya antarctica ANT.ACEV6.1 (AY493589) (100%)			Bubano Basin (Italy) (AJ639892); Chaerhan Lake (Tibet) (HM127136); Lake Ferto (Hungary) (EU914882); Yellowstone National Park (United States) (AY790846)
TM1ULC73‖	EU852495	Clone R8-R56 (DQ181691) (99%)	*Leptolyngbya antarctica* ANT.ACEV6.1 (AY493589) (100%)	16ST63^{C}		
TM1FODa1	EU852507	Clone R8-R56 (DQ181691) (99%)	*Leptolyngbya antarctica* ANT.ACEV6.1 (AY493589) (100%)	16ST63^{C}		
TM2FOCH9	EU852499	Clone R8-R56 (DQ181691) (99%)	*Leptolyngbya antarctica* ANT.ACEV6.1 (AY493589) (99%)	16ST63^{C}		
TM2FOCF1	EU852517	ND	ND	16ST63^{C}		
TM2ULC129‖	EU852496	Clone GBII-52 (GQ441323) (98%)	*Leptolyngbya* sp. OU-6 (GQ162217) (98%)	16ST80^{C}	–	Intertidal beach (the Netherlands) (GQ441323)
TM3FOS129‖	EU852497	Clone GBII-52 (GQ441323) (98%)	*Leptolyngbya* sp. OU-6 (GQ162217) (98%)	16ST80^{C}		
TM4LUCF12	EU852505	Clone D1G12 16S (EU753634) (98%)	*Leptolyngbya badia CRS-1* (EF429297) (95%)	16ST92^{C}	–	Dry stromatolites, Ruidera Pools Natural Park (Spain) (EU753629); Rocky Mountain sandstone (United States) (EF522259)
TM4LUCE5	EU852501	Clone D1G12 16S (EU753634) (98%)	*Leptolyngbya badia CRS-1* (EF429297) (95%)	16ST92^{C}		
TM4LUCF10	EU852506	Clone D1G12 16S (EU753634) (98%)	*Leptolyngbya badia CRS-1* (EF429297) (95%)	16ST92^{C}		
TM4LUDb1	HQ219061	Clone D1F08 (EU753629) (99%)	*Pseudanabaena tremula* UTCC 471 (AF218371) (92%)	16ST92^{C}		
TM4LUDb3	HQ219062	Clone D1F08 (EU753629) (99%)	*Leptolyngbya* sp. CNP1-B1-4 (AY239603) (91%)	16ST92^{C}		
TM4LUDb5	HQ219057	Clone D1F08 (EU753629) (99%)	*Leptolyngbya* sp. CNP1-B1-4 (AY239603) (91%)	16ST92^{C}		
TM4LUD1b3	HQ219056	Clone D1F08 (EU753629) (99%)	*Leptolyngbya* sp. CNP1-B1-4 (AY239603) (91%)	16ST92^{C}		

*We selected more than one sequence per OTU if they came from different samples or using different molecular methods.

†BLAST analysis based on 16S rRNA gene sequences.

‡Selection of locations of related sequences from the same OTU. OTUs superscripts are A for potentially endemic to Antarctica and C for cosmopolitan.

¶Only sequenced in one direction.

‖The sequence belongs to a strain.

Table 3. Summary of ITS sequences obtained by clone library

ITS sequences	Accession numbers	ITS types	Closest BLAST hit (similarity %)
TM1FOCA5	EU852516	ITS02	Clone R8-R56 (DQ181761) (98%)
TM1ULC73*	EU852528	ITS02	
TM2FOCH9	EU852532	ITS02	
TM2FOCF1	EU852517	ITS02	
TM3FOCA2	EU852521	ITS23	Cyanobacterium clone A206 (DQ181751) (99%)
TM2FOCA9	EU852522	ITS23	
TM2ULC129*	EU852531	ITS26	*Oscillatoriales* cyanobacterium OU4 (GQ162321) (79%)
TM3FOS129*	EU852530	ITS26	
TM2FOCD1	EU852523	ITS27	Clone Qiyi-cya-OTU 0 (AB569623) (89%)
TM2FOCH1	EU852518	ITS27	
TM3FOCB5	EU852519	ITS27	
TM2ULC130*	EU852529	ITS27	
TM4LUCG9	EU852526	ITS28	Clone LMM1-21 (EU032366) (99%)
TM4LUCC30	HQ336421	ITS29	*Leptolyngbya* sp. LLi18 (DQ786166) (77%)
TM4LUCF12	EU852524	ITS29	Cyanobacterium clone LMM1-11 (EU032362) (79%)
TM4LUCF10	EU852525	ITS29	Cyanobacterium clone LMM1-11 (EU032362) (99%)
TM4LUS131*	EU852527	ITS30	Cyanobacterium clone LMM1-18 (EU032363) (89%)

*The sequence belongs to a strain.

The sequences exhibited 97–100% sequence identity with their highest matches found by BLAST analysis. Eleven out of 12 OTUs showed a cosmopolitan distribution while the remaining OTU, 16ST02 from Lundström Lake, is potentially endemic to Antarctica (Table 2).

ITS analysis

Seventeen ITS sequences were obtained from the eight OTUs found by clone library or in the isolated strains (Table 3). The 17 ITS sequences were distributed into seven ITS types (Table 5). Only ITS sequences from related organisms can be meaningfully aligned because this spacer is highly variable (Wilmotte *et al.*, 1994). In each OTU based on 16S rRNA gene, one or two ITS types were detected. The $tRNA^{Ala}$ and $tRNA^{Ile}$ were present in all the ITS types. The aligned ITS types showed 75–100% similarity (indels were taken into account). Ten ITS sequences, from OTUs 16ST44, 16ST49 and 16ST63, corresponded to ITS types (ITS02, ITS23 and 27) already reported by Taton *et al.* (2006a). The ITS types ITS02, ITS23, ITS27, ITS29 and ITS30 were divided into subgroups when they contained sequences that were $< 96\%$ similar and formed clear subgroups (Table 5 and Fig. S2).

Discussion

Methodological considerations

The analysis of the OTUs recorded in the three samples from Forlidas Pond (Hodgson *et al.*, 2010) and one sample from Lundström Lake identified 12 OTUs. Three OTUs were only detected by DGGE, two only by clone library and two only by sequencing the isolates obtained (Table 4). The sequences from the strains *Phormidium priestleyi* (TM2ULC129) and *Leptolyngbya* cf. *foveolarum* (TM4LUS131) form two OTUs not retrieved by clone libraries or DGGE (Table 4). The high sensitivity level of the molecular detection could imply that these two morphotypes were rare in the environmental samples. It is a common observation that 16S rRNA genes retrieved directly from environmental samples rarely match those of cultivated strains or are distantly related (Nübel *et al.*, 2000). This emphasizes the complementary nature and advantage of applying both approaches.

In TM1, only one of the three OTUs was detected by clone library. In TM3, two OTUs were not detected by clone library, but by the DGGE method. Similarly, a higher number of OTUs were detected by DGGE in TM4 (Table 4). Although clone libraries are generally expected to reveal a higher diversity than DGGE, this was not observed here. This could be due to the number of sequenced clones and the use of two PCRs (semi-nested PCR) with two different primers specific for cyanobacteria [16S781R(GC) (a) and (b)] in the DGGE, whereas only one PCR was used with the primers 16S378F–23S30R for the clone library. In addition, the ARDRA analyses used to screen the clone libraries could underestimate the diversity (Cho & Tiedje, 2000).

Phylogenetic resolution and biogeographical patterns

Comparison of our sequences with the ITS sequences available in GenBank revealed that, within the same OTU, different ITS sequences could be found that were too different to be aligned. For instance, the 16S rRNA gene sequence of the clone TM4LUCG9 belonged to the OTU 16ST57 and its corresponding ITS to the type ITS28. In GenBank, only three ITS sequences corresponding to the

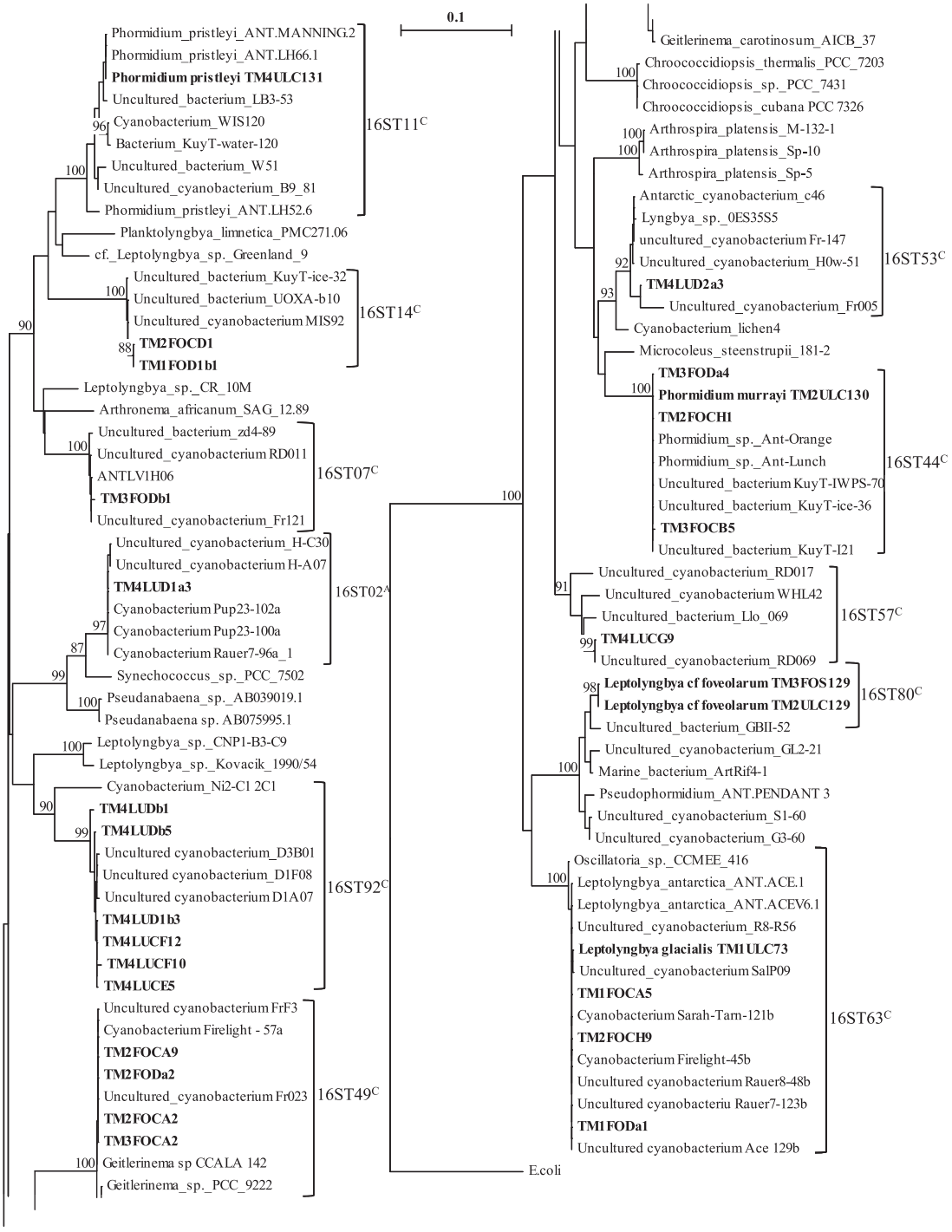

Fig. 3. Distance tree based on cyanobacterial partial 16S rRNA gene sequences (*Escherichia coli* positions 379–806) constructed using the neighbor-joining method (Saitou & Nei, 1987). A bootstrap analysis was performed that involved the construction of 1000 resampled trees (values indicated at the node). The tree comprised the sequences of 10 DGGE bands, 12 clones and five strains from the Forlidas Pond samples (from Hodgson *et al*., 2010) and Lundström Lake (both in bold) and their three most similar strain sequences and three uncultured sequences from RDPII (http://rdp.cme.msu.edu). *Escherichia coli* sequence is used as an outgroup. The OTU numbers are indicated on the right. OTU 16ST16 composed by TM4LUCC30 is not shown in the tree because the fragment of this sequence did not include the positions used for the construction of the tree. The evolutionary distance between two sequences is obtained by adding the lengths of the horizontal branches connecting them and using the scale bar (0.1 mutation per position). The superscripts in the OTUs numbering mean: 'C' for cosmopolitan distribution and 'A' for potentially endemic to Antarctica.

Table 4. Presence/absence of OTUs from clone libraries, DGGE gels and isolated strains

	16ST02	16ST07	16ST11	16ST14	16ST16	16ST44	16ST49	16ST53	16ST57	16ST63	16ST80	16ST92
TM1*				D			D			C+S+D		
TM2*				C		C+S+D	C+D			C+D	S	
TM3*		D				C+D	C+D			D	S	
TM4	D		S		C	D		D	C			C+D

*The 16S rRNA gene sequences from these samples have been obtained from Hodgson *et al*. (2010).
The presence of OTUs in the samples is indicated by dark shading. The origins of the sequences from an OTU in a sample are: D, DGGE; C, clone library; and S, isolated strain.

16S rRNA gene sequences from OTU 16ST57 were available and only one sequence (EU032366) belonged to the ITS28. The other two ITS sequences could not be aligned with this ITS type and were isolated from Lake Reid (Larsemann Hills, East Antarctica).

The study of the ITS clearly increases the phylogenetic resolution provided by the 16S rRNA genes. The sequences of TM2ULC129 and TM3FOS129 showed 97.3% 16S rRNA gene similarity to *Leptolyngbya* sp. ANT.LH52 (AY493584), but they were included in a new OTU (16ST80). Indeed, the ITS sequences from both strains were identical, but clearly not related to the *Leptolyngbya* sp. ANT.LH52 sequence. This emphasizes that, although they were at the limit of the OTU definition, they were distinct and, therefore, the ITS supported the creation of the new OTU 16ST80. The ITS analysis also confirmed that the same *Phormidium autumnale* population was present both in the terrestrial and in the aquatic samples. Indeed, two sequences from the Forlidas Pond shoreline water sample (TM2FOCH1 and TM2ULC130) and one sequence from the terrestrial mat (TM3FOCB5) shared identical 16S rRNA gene and ITS sequences (Fig. 3 and alignment in Fig. S2).

The distribution pattern of the ITS could be linked to the different physicochemical parameters or the distinct geographical origin of the samples. For instance, ITS02, which was divided into four subgroups, contained sequences from TM1 and TM2 (Forlidas Pond) and Lakes Ace (DQ181748, AY493632, AY493633) and Rauer 8 (DQ181759, DQ181761, DQ181762) (Vestfold Hills and Rauer Islands, respectively). These lakes are quite different, Lakes Rauer 8 and Ace being hyposaline (6.26 and 25.9 mS cm^{-1}, respectively) while the TM1 and TM2 sequences were isolated from hypersaline and oligosaline samples (142.37 and 0.227 mS cm^{-1}, respectively) (Table S1). The ITS27 is divided into two subgroups. The first contains sequences from Forlidas Pond and its catchment while the second is composed by sequences from Lake Fryxell (AF547634, AF547628), a brackish meromitic lake sampled in its freshwater moat (Taton *et al*., 2003). The physicochemical parameters differ considerably between these lakes and appear to coincide with the ITS subgroups. A positive correlation with local factors can be observed for ITS28, which contains two sequences (99.6% pairwise identity) originating from distant lakes sharing similar environmental parameters. The sequence EU032366 was isolated from Lake Miers (Miers Valley, Southern Victoria Land) while TM4LUCG9 comes from Lundström Lake. Both samples were taken at the shoreline and their physicochemical parameters were similar (Bell, 1967).

The ITS sequences recorded in Antarctica appear to be more similar among themselves than to non-Antarctic ITS. The type ITS23 contained two sequences from samples TM2 (TM2FOCH1) and TM3 (TM3FOCA9), which were related to five sequences from two Antarctic lakes (Ace Lake and Lake Fryxell, in the Vestfold Hills and McMurdo Dry Valleys, respectively) and to a strain (*Geitlerinema carotinosum* AICB 37; AY423710) isolated in Banloc (Romania). The Antarctic ITS sequences from ITS23 were 98.7% similar between themselves, but only 93% similar to the non-Antarctic ITS sequences. Similarly, only two Antarctic ITS from Lake Fryxell could be aligned within ITS27. They correspond to the OTU 16ST44, for which non-Antarctic sequences are available (16S and ITS), although these ITS were too dissimilar to be aligned into ITS27 (e.g. AM778715, EF18274). However, the low number of ITS sequences in the databases impedes a deeper analysis and further studies are required.

Impoverished diversity

The autotrophs recorded in the Dufek Massif appear limited to cyanobacteria and a few green algae and lichens. In addition, this area has been reported to harbor the most reduced metazoan terrestrial and freshwater ecosystems known from Antarctica (Hodgson *et al*., 2010). The only previous description of the ecology in this area was given by Neuburg *et al*. (1959). During this first visit to the region in the International Geophysical Year (1957/1958), some vegetation was collected from Forlidas Pond for microscopic observation and identified as '*Phormidium incrustatum* and possibly *Phormidium retzii*' (G. Llano & G. Prescott, pers. commun.). The fact that we did not find these species probably results from the different approach rather than a change in the biodiversity (Hodgson *et al*., 2010). The description of *P. retzii* could correspond to the morphotype illustrated in

Table 5. ITS types and subgroups present in the clone libraries and isolated strains

ITS type	ITS02		ITS23		ITS26	ITS27	ITS28	ITS29		ITS30
ITS sequences*	Subgroups	TM1ULC73†	Subgroups	TM3FOCA2	TM3FOS129†	TM2FOCH1	TM4LUCG9	Subgroups	TM4LUCC30	TM4ULC131
		TM1FOCA5		TM2FOCA9	TM2ULC129†	TM2FOCD1	EU032366			
		TM2FOCF1		DQ181751		TM3FOCB5				
		TM2FOCH9		AF547633		TM2ULC130†				
				AF547632						
				AF547630						
				AF547626					TM4LUCF10	EU032363
		DQ181759								EU032367
		DQ181761								
		AY493632		AY423710		AF547634			TM4LUCF12	DQ181755
		AY493633				AF547628				
		DQ181762								
		DQ181748								
Corresponding OTU‡	16ST63		16ST49		16ST80	16ST44	16ST57	16ST16 and 16ST92		16ST11

*The ITS sequences from Forlidas Pond, Lundström Lake and GenBank sequences that could be meaningfully aligned.
†Strains.
‡The OTU corresponding to the ITS sequences obtained in this study.
Sequences in the same types shared at least 75% pairwise identity. The OTUs based on 16S rRNA gene and corresponding to the ITS types are indicated.

Fig. 2b, but we identify this morphotype as *P. crassior* because of its ecology and the absence of truncated end cells, a very characteristic feature in the literature. Similarly, *P. incrustatum* shares several characteristics with the morphotype illustrated in Fig. 2b, but it has a narrower cell width.

Our data show a quite impoverished cyanobacterial diversity in Forlidas Pond (5 OTUs) and Lundström Lake (7 OTUs) compared with lakes in the coastal regions of Antarctica. Indeed, using the same methodology, we calculated an average of 8.5 OTUs in lakes of the McMurdo Dry Valleys and other sites in East Antarctica, ranging from 4 OTUs for Lake Rauer 8 (Rauer Islands, East Antarctica) to 15 OTUs in Lake Fryxell (McMurdo Dry Valleys) (Taton *et al.*, 2003, 2006a). Sample TM1 comes from a hypersaline brine, with a salinity four times that of seawater. Hypersaline conditions, in addition to the other extreme physical and chemical conditions present at these latitudes, are likely to account for the particularly low biodiversity (3 OTUs) in this sample.

Forlidas Pond and Lundström Lake shared only one OTU (16ST44). The high salinity of Forlidas Pond suggests a long history of evaporation in this lake. In contrast, Lundström Lake has a low salinity, with only a slight increase at the base of the water column (Table S1). Because of their different evaporation history and geomorphology, we can infer that these lakes have also been subject to different ecological processes, and this could be one reason for the differences in diversity. Moreover, geomorphological features that separate the two lakes, such as the Support Force Glacier, Argentina Range and Recovery Glacier, and the 420 km distance between them, could also play a role.

Spatial distribution

Four OTUs found in Forlidas Pond were also present in terrestrial habitats in its catchment (Fig. S3), showing that these taxa could inhabit both aquatic and terrestrial biotopes (Gordon *et al.*, 2000). Although some terrestrial mats near melting snow were damp, most terrestrial mats were completely dry at the time of sampling. It seems likely that the terrestrial mats were once growing on the bottom of the lake before its size began to shrink, and that they now survive due to water sourced from seasonally melting snow patches (Hodgson *et al.*, 2010). The ITS distribution shows a similar spatial distribution as observed by analysis of the 16S rRNA gene sequences (Fig. S4). Lundström Lake's ITS sequences are not shared with any sample from the Forlidas Valley while one ITS type is shared between the TM1 and the TM2 samples (ITS02) and three ITS types are shared between the TM2 and the TM3 samples (ITS23, ITS26, ITS27). In addition, when our ITS sequences were compared with the ITS sequences from GenBank, we observed that the intra-Antarctic variability was smaller than the differences between the Antarctic ITS and those of other continents.

Numerous terrestrial mats were present around Forlidas Pond and in the adjacent Davis Valley. We only found *Leptolyngbya*, *Geitlerinema* and *Phormidium* sp. in the sample taken from one of these terrestrial mats (TM3). Because of the large size of the mat, we would also expect to find ensheathed filamentous cyanobacteria producing large amounts of exopolysaccharides (i.e. *Coleodesmium* or *Stigonema*). However, the main cyanobacteria responsible for the production of these macroscopic structures remain uncertain, as *Leptolyngbya* are too small and rare in the samples, *Geitlerinema* does not possess sheaths and *Phormidium* produce only thin sheaths. Nevertheless, it is known that meshes of filaments of *P. autumnale* can provide physical support and protection against desiccation for other algae, mainly associated cyanobacterial filaments and diatoms (Wynn-Williams, 1996). Moreover, the importance of its soil-binding role for the physical structure of the community has been shown before (Mataloni *et al.*, 2000).

Endemism

The results of this study support the hypothesis that, in the more inhospitable regions of continental Antarctica, cyanobacterial communities are characterized by a low biodiversity, taxa tolerant of extreme cold and dry conditions, and marginal endemism (Hodgson *et al.*, 2010). Specifically, samples from Forlidas Pond contained only cosmopolitan OTUs while Lundström Lake included one potential Antarctic endemic OTU (16ST02). However, the 16S rRNA gene marker is highly conserved and might underestimate the phenomenon of endemism, as shown by Cho & Tiedje (2000). Cosmopolitan OTUs must possess features enabling the processes of dispersal, colonization and establishment between different continents and, thus, possess resistance capacities that could explain their presence in the two locations studied here. The higher colonization rates (Hodgson *et al.*, 2010 and references therein) attributed to cosmopolitan OTUs could also explain their abundance. However, Antarctic endemic OTUs will also have well-developed resistance adaptations, being recorded across distant locations within the Antarctic continent.

The harsh environmental conditions, together with geographical isolation, could explain the marginal endemism found in Lundström Lake (Vyverman *et al.*, 2007; Souza *et al.*, 2008; Hodgson *et al.*, 2010). The unique OTU from this study, which was exclusively recorded in Antarctica (OTU 16ST02), has been recorded in two very distant locations (*c.* 2600 km apart): in the Larsemann Hills (DQ181726) (East Antarctica) (Taton *et al.*, 2006a) and on the McMurdo Ice Shelf (AY541565) (Jungblut *et al.*, 2005). It is possible that OTU 16ST02 present in Lundström Lake originated in one of these Antarctic locations (or *vice versa*). However, we cannot definitively state that this OTU is not present in other (including temperate) regions, but is as yet unrecorded (Martiny *et al.*, 2006). Therefore, its description as endemic should be considered with caution. Other cyanobacterial sequences previously considered as endemic in 2006 are now known to be cosmopolitan, as new sequences have been added to the databases (e.g. OTUs 16ST07, 16ST11, 16ST14, 16ST16, 16ST53 in this study). We therefore use the term 'potentially endemic'. The ITS types defined in this study, except ITS23, are all potentially endemic to Antarctica, in contrast to the corresponding OTUs (based on 16S rRNA gene), which were all cosmopolitan. This is in agreement with the conclusion of Cho & Tiedje (2000), who showed higher endemicity levels for ITS than for 16S rRNA gene sequences. However, the ITS database remains fragmentary. As these databases expand, future research in inland continental Antarctica will shed more light on the geographical distribution and evolutionary isolation of cyanobacteria in these extreme habitats.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Photographs of the sampling locations, Forlidas Pond (a) and Lundström Lake (b).
Fig. S2. Alignment of the ITS sequences from the Forlidas Pond and Lundström Lake environmental samples and their closest relatives found by BLAST analysis.
Fig. S3. Spatial distribution of cyanobacterial OTUs in Forlidas Valley.
Fig. S4. Spatial distribution of ITS groups observed with the clone libraries of samples from the Forlidas Valley and Lundström Lake.
Table S1. Water chemistry of Forlidas Pond and Lundström Lake.
Table S2. Molecular diversity summary and richness indices.
Table S3. Definition of morphospecies described in this study.

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Culturable bacterial diversity at the Princess Elisabeth Station (Utsteinen, Sør Rondane Mountains, East Antarctica) harbours many new taxa

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ABSTRACT

We studied the culturable heterotrophic bacterial diversity present at the site of the new Princess Elisabeth Station at Utsteinen (Dronning Maud Land, East Antarctica) before construction. About 800 isolates were picked from two terrestrial microbial mat samples after incubation on several growth media at different temperatures. They were grouped using rep-PCR fingerprinting and partial 16S rRNA gene sequencing. Phylogenetic analysis of the complete 16S rRNA gene sequences of 93 representatives showed that the isolates belonged to five major phyla: *Actinobacteria*, *Bacteroidetes*, *Proteobacteria*, *Firmicutes* and *Deinococcus-Thermus*. Isolates related to the genus *Arthrobacter* were the most prevalent whereas the genera *Hymenobacter*, *Deinococcus*, *Cryobacterium* and *Sphingomonas* were also recovered in high numbers in both samples. A total of 35 different genera were found, the majority of which has previously been reported from Antarctica. For the genera *Aeromicrobium*, *Aurantimonas*, *Rothia, Subtercola, Tessaracoccus* and *Xylophilus,* this is the first report in Antarctica. In addition, numerous potential new species and new genera were recovered; many of them currently restricted to Antarctica, particularly in the phyla *Bacteroidetes* and *Deinococcus-Thermus*.

Introduction

Antarctica is characterised by its geographical and climatic isolation and most of the continent has experienced relatively little direct anthropogenic influence. Antarctic ecosystems are characterised by short food chains dominated by microbial organisms [30] which have evolved over thousands of years, confronted with extreme conditions such as low temperature, desiccation and UV-radiation. As a consequence, they experience a high selection pressure and potentially belong to local, as yet undescribed new taxa [31] that could be of interest for biotechnology. An increasing number of studies have investigated the bacterial diversity in Antarctic ecosystems and reported on the presence of a large bacterial diversity [10,30,47,52]. Nevertheless, few studies used a cultivation approach although the availability of cultures is important both for detailed taxonomical and biotechnological investigation. As human activity and climate change increasingly affect the continent, it is urgent to investigate the bacterial diversity in these Antarctic ecosystems before they are irreversibly damaged.

Microbial studies on continental Antarctica have focussed mainly on aquatic ecosystems, ranging from freshwater to hypersaline lakes [19,60], sea-ice [13] and marine areas [29]. Microbial studies of terrestrial environments have mostly been restricted to regions such as Victoria Land [5] and Wilkes Land [17]. By contrast, in the region of Dronning Maud Land only the Schirmacher Oasis [69] and the Syowa region [65] have been more extensively investigated. The Sør Rondane Mountains are located in Dronning Maud Land, East Antarctica about 200 km away from the coast. They consist of a large number of small nunataks (mountain summits protruding through surrounding ice sheets) and large ice-free mountains covering an area 200 km wide (latitudinal) and 100 km long (meridional) [49]. A biological study by Ohyama et al. [53] showed the presence of terrestrial Arthropods in the Sør Rondane Mountains which were different from the fauna observed in the eastern region of Dronning Maud Land, e.g. Syowa Station and Enderby Land. To our knowledge, no microbiological studies on this area have been reported.

In the Antarctic summer of 2007–2008, the "zero-emission" Princess Elisabeth Station was built near Utsteinen nunatak, a few kilometres north of the Sør Rondane Mountains that protect the new base from the heavy katabatic winds typical of Antarctica. Environmental samples were collected during an exploratory expedition to the site in January 2007, prior to construction. They represent the undisturbed, virgin state and are being studied to catalogue the prokaryotic and eukaryotic biodiversity present to enable future assessment of the environmental impact. In this

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Fig. 1. Pictures from the locations where the samples BB 50 (left) and BB115 (right) were taken.

study, we report on the culturable heterotrophic bacterial diversity of two microbial mat samples from Utsteinen nunatak and assess the geographic distribution of 16S rRNA phylotypes recovered.

Materials and methods

Source of samples

Samples were aseptically taken in January 2007 at Utsteinen (71°57′S, 23°20′E) (Dronning Maud Land, Antarctica), at the site of the planned "Princess Elisabeth Station". They were kept frozen until processing in Belgium. Sample BB50 (Fig. 1), originating from a gravel and green microbial/algal mat from the eastern side of the Utsteinen nunatak and sample BB115 (Fig. 1), from a black mat on gravel and rock debris from the bank of a frozen lake on the western side of the Utsteinen nunatak, were used for this study.

Enumeration and isolation of heterotrophic bacteria

One gram of sample was aseptically weighed and homogenized in 9 ml sterile physiological water (PW) (0.86% NaCl) using a vortex. Ten-fold dilution series were plated on four different media: Marine agar 2216 (MA) (BD Difco™), R2A (BD Difco™), 10 times diluted R2A (R2A/10), and Peptone-Yeast-Glucose-Vitamin (PYGV) medium (DSMZ medium 621). Incubation conditions used were 20, 15 and 4 °C under both aerobic and anaerobic (Anaerobe system, BD GasPak™ EZ, ≥15% CO_2) atmosphere. After an incubation period of 2–3 weeks under aerobic conditions or up to 8 weeks under anaerobic conditions, the colony numbers stabilized. The calculation of the total number of CFU/g for each condition was made for the plates showing between 20 and 400 colonies when the number of CFU's had reached a plateau. At the end of the incubation period, three colonies (or less in case of insufficient growth) of each morphological type were isolated and purified. Pure cultures were cryopreserved at −80 °C using broth medium plus 15% glycerol or the MicroBank™ system (Pro-Lab Diagnostics, Ontario, Canada).

To assess whether dilution series in physiological water (PW) might pose osmotic problems for halophilic bacteria, we included a dilution series in seawater (SW) (Instant Ocean®) plated on MA for sample BB50. The isolates from both conditions appeared to be very similar (data not shown) and therefore the seawater dilutions were not made for the second sample.

Genotypic fingerprinting

To eliminate duplicate isolates, a whole-genome fingerprinting technique, repetitive element palindromic (rep)-PCR was used, which permits the reduction of the large number of isolates to a smaller group of clusters and unique strains. DNA preparation was carried out as described by Baele et al. [8]. Rep-PCR fingerprinting using the GTG_5 primer (5′-GTG GTG GTG GTG GTG-3′) was performed according to Gevers et al. [35]. Resulting fingerprints were processed using BioNumerics (v 5.1.) software (Applied-Maths). Rep-PCR profiles were compared by calculating Pearson's correlation coefficients (r). A cluster analysis was performed on the resulting matrix using the Unweighted Pair Group Method using Arithmetic averages (UPGMA). A threshold of 80% r was used [35] in combination with visual inspection of band patterns to delineate clusters.

16S rRNA gene sequencing and phylotype identification

The 16S rRNA genes for the representatives of the different reptypes were amplified and sequenced as previously described [81]. PCR products were purified using a Nucleofast 96 PCR clean up membrane system (Machery-Nagel, Germany) and Tecan Genesis Workstation 200. The sequencing primers used were as listed by Coenye et al. [20]. Initially only approximately the first 400 bases were determined. The fragments obtained were cleaned with the BigDye® xTerminator™ Purification Kit according to the protocol of the supplier (Applied Biosystems). Sequence analysis was performed using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, USA). Pairwise similarity values were calculated to delineate phylotypes at 99.0% [70,71] 16S rRNA gene sequence similarity. For each phylotype, the 16S rRNA gene sequence of one representative was completed. Sequence assembly was performed using the Assembler module in BioNumerics (v 5.1.) (Applied-Maths). Using the FASTA algorithm, the closest related sequences were obtained from the EMBL database. Phylogenetic analysis using the sequences of type strains from all the species listed in the FASTA results completed with related taxa was performed using BioNumerics. A multiple sequence alignment was made and after visual inspection, distances were calculated using the Kimura-2 correction. A neighbour joining dendrogram [64] was constructed and bootstrap analysis was performed using 500 bootstrap replicates of the data.

Phylotypes were identified using a threshold of 99.0% 16S rRNA gene sequence similarity. This approach may underestimate the actual number of species because of the limited resolving power of the 16S rRNA gene sequence and there are examples of distinct species with identical or nearly identical 16S rRNA gene sequences [61,73]. Nevertheless, from a comparison of 16S rRNA gene homology with DNA-DNA reassociation values for members of the class *Actinobacteria*, Stach et al. [70] found that a 16S rRNA gene similarity level of 99.0% covered 70% of all DNA-DNA hybridization values of more than 70% (which is the golden standard for species defini-

Table 1
Number of phylotypes recovered with a currently cosmopolitan, cold, bipolar or Antarctic distribution.

Phylum/class	Cosmopolitan	Cold	Bipolar	Antarctic
Actinobacteria	20	1	0	9
Alphaproteobacteria	8	0	0	2
Betaproteobacteria	5	0	0	1
Gammaproteobacteria	0	0	0	1
Bacteroidetes	4	0	5	18
Firmicutes	7	0	0	0
Deinococcus-Thermus	1	0	0	11
Total	45	1	5	42

Phylotypes showing ≥99.0% 16S rRNA gene sequence similarity with environmental sequences or strains originating from Antarctica together with phylotypes without significant (≥99.0%) sequence similarity with environmental sequences.

tion [72]). We therefore opted to use the level of 99.0% sequence similarity to define phylotypes and regarded the phylotypes as pragmatic proxies for bacterial species. Consequently, phylotypes showing ≥99.0% 16S rRNA gene sequence similarity with a particular type strain were considered as belonging to this species. For the cases where phylotypes belong to complex clusters of species sharing more than 99.0% 16S rRNA gene similarity, these phylotypes were classified as potentially new species in the genus concerned. Phylotypes grouping within a particular genus, but with <99.0% 16S rRNA gene sequence similarity with named species were also classified as potential new species within this genus (Table 1). Phylotypes for which the 16S rRNA gene sequence similarities were equally low with all related genera, could not be assigned to a particular genus and were classified as potential new genera.

The complete 16S rRNA gene sequences determined in this study have been deposited in the EMBL database (FR682667–FR682760).

Geographic distribution of the taxa recovered

To assess the environmental range of our phylotypes, the 16S rRNA gene sequences were compared to both the prokaryote (PRO) and the environmental (ENV) subsections of the EMBL database. The ENV section contains sequences of clones and unnamed isolates from various environments. Additionally, sequences were compared to the nucleotide collection, the high throughput genome sequences and the environmental samples from GenBank using Blast. Based on the origin of high scoring (≥99.0% 16S rRNA gene sequence similarity) entries, we labelled our phylotypes as Antarctic (when no high scoring sequences from non-Antarctic origin were found), bipolar (only high scoring sequences from polar environments), cold (only high scoring sequences from cold environments) or cosmopolitan (at least one high scoring sequence from non-Antarctic/cold/polar environment).

Sample coverage

Rarefaction curves were calculated with an online rarefaction calculator (http://biome.sdsu.edu/fastgroup/cal_tools.htm). The Shannon biodiversity index was calculated as described by Magurran et al. [48].

Results

Yield on different media

The media R2A, R2A/10 and PYGV showed a rather good yield at 15 and 20 °C and under aerobic atmosphere (Fig. 2). Lower numbers of colonies were found at 4 °C, under anaerobic conditions and on the marine media for all conditions used. Some of the plates

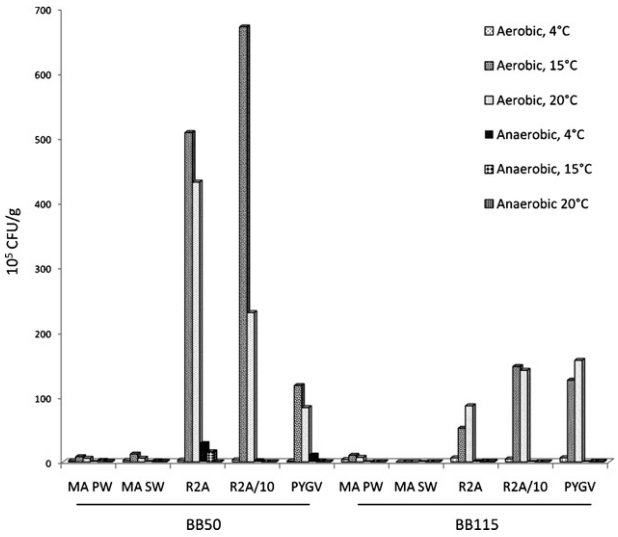

Fig. 2. Yield for the different incubation conditions and growth media for sample BB50 and BB115.

incubated under anaerobic atmosphere showed no growth at all. In general, the number of CFU for the different media and temperatures observed for sample BB115 was much lower than for sample BB50.

Interestingly, the majority of the colonies were pigmented, ranging from pale yellow to dark red. This has been observed before for bacteria isolated from Antarctica and cold environments [11,51]. Pigments provide protection against ultraviolet radiation [26] and Fong et al. [32] suggested that an increase of carotenoid production in cold conditions may contribute to membrane stability.

Isolation and grouping by rep-PCR fingerprinting

In total, 465 (BB50) and 331 (BB115) isolates were purified and included in rep-PCR. To confirm the significance of the cut-off level for rep-clusters, some representatives from large clusters with around 80% correlation were sequenced, and found to have identical sequences (data not shown). In total, 62 (BB50) and 48 (BB115) clusters were delineated and 95 (BB50) and 46 (BB115) isolates formed separate branches (Table S1). Only two of the clusters contained isolates from both samples.

Identification based on 16S rRNA gene sequences

The 16S rRNA gene for a representative of each of the 251 different rep-types was partially sequenced. Rep-types were thus grouped in 93 phylotypes and for each of these the 16S rRNA gene sequence of a representative was completed and compared with the EMBL database (Table S1). The genera *Arthrobacter*, *Rhodococcus*, *Microbacterium*, *Nocardioides*, *Cryobacterium, Sphingomonas, Brevundimonas, Variovorax, Massilia, Hymenobacter, Chryseobacterium* and *Deinococcus* were recovered from both samples. Mostly, this involved different species, although seven species (represented by phylotypes R-36475, R-36515, R-36533, R-36535, R-36544, R-36558 and R-38538) were found in both samples (Table S1). In addition, other genera were obtained only from one of the samples. For some phylotypes (R-36369, R-36375, R-36492 and R-36501), phylogenetic analysis did not allow us to reliably assign them to an existing genus because their 16S rRNA gene sequence similarities were equally low with all related genera. These were classified as potential novel genera (Table S1).

From sample BB115 a fungus was recovered. Sequencing of the 18S rRNA gene (EMBL number FR717359) resulted in identi-

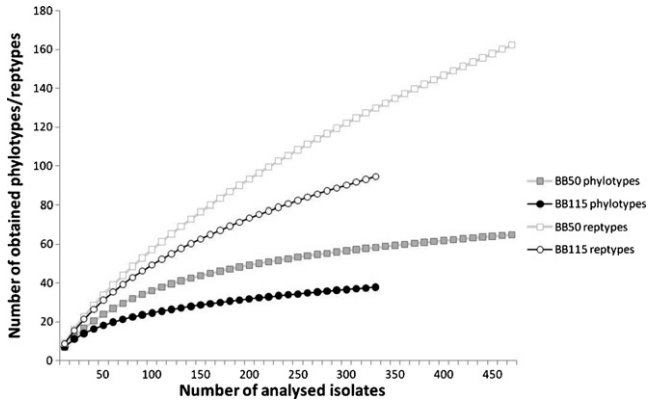

Fig. 3. Rarefaction curves constructed with the number of rep-clusters and phylotypes for both samples.

fication as *Thelebolus microspores* (99.9% 18S rRNA gene sequence similarity). This fungus belongs to the *Thelebolaceae* in the class of *Leotiomycetes* and was originally isolated from Antarctica [27]. The strain was deposited in the BCCM/MUCL (Agro)Industrial Fungi and Yeasts Collection as MUCL 52945.

Results for anaerobic incubation conditions

Only 80 isolates of BB50 and 23 isolates of BB115 were taken from the anaerobic plates. The anaerobic isolates belonged to phylotypes containing only anaerobic isolates (in Table S1 marked with AN) or both anaerobic and aerobic isolates (in Table S1 marked with A + AN). When tested for growth under aerobic atmosphere, all of them did grow, indicating they are in fact facultatively anaerobic. This is in line with the observation that most of them were identified as species or genera described as microaerobic or facultatively anaerobic. The low yield under anaerobic conditions and the recovery of mainly facultatively anaerobic bacteria was probably largely due to the not strictly anaerobic procedure followed during sampling, transport, storage and plating which would have inhibited any true anaerobes present.

Coverage of heterotrophic diversity and dominant taxa

Sample coverage was assessed using rarefaction curves (Fig. 3). The curves representing the phylotypes nearly reached a plateau whereas the curves for the rep-types continued to rise, indicating that not all diversity was recovered yet. Comparison of the numbers of isolates recovered for the different phylotypes revealed that for both samples, isolates related to the genus *Arthrobacter* were the most prevalent, whereas the genera *Hymenobacter*, *Deinococcus*, *Cryobacterium* and *Sphingomonas* were also isolated in rather high numbers from both samples. In general, phylotypes of the phylum *Actinobacteria* represented the majority of the isolates at 54.6% (BB50) and 65.7% (BB115) (Table S1). Isolates affiliated with the phylum *Bacteroidetes* were present at 16.9% (BB50) and 11.8% (BB115), whereas the genus *Deinococcus* represented 10.0% (BB50) and 7.5% (BB115) of the total number of isolates.

Geographical distribution of the taxa recovered

Comparison of our sequences with public databases was used to assess the geographic distribution for the different phylotypes (Table 1, Table S1). All phylotypes related to the phylum *Firmicutes* were found to be cosmopolitan (Table 1, Table S1). Furthermore, in both the *Alphaproteobacteria* and the *Betaproteobacteria*, most phylotypes were found to be cosmopolitan, whereas a minority of phylotypes, together with the single *Gammaproteobacteria* phylotype, showed no significant similarity (≥99.0%) with any sequence in public databases and could thus be regarded as new and currently known only from Antarctica. Half of the *Actinobacteria* phylotypes were cosmopolitan, whereas the other half comprised phylotypes which showed no significant similarity (≥99.0%) with any sequence in public databases except for phylotype R-36550 which was related to an isolate (accession number AM419018) from Antarctica and an isolate (accession number GU784867) from a glacier cryoconite in Austria. In the phylum *Bacteroidetes*, a large majority of phylotypes are currently known only from Antarctica while five and four phylotypes had a bipolar or cosmopolitan distribution, respectively. Phylotype R-36526 which was identified as *Chryseobacterium jeonii* (previously *Sejongia jeonii*), a species from terrestrial samples from King George Island [87], was found to be currently restricted to Antarctica. The five bipolar *Bacteroidetes* phylotypes were related to other sequences from polar habitats: phylotype R-36616 was highly related to the sequence from a clone recovered from Arctic snow and melt water from Svalbard, Norway (accession number FJ946523); R-36490, R-36364, R-42653 and R-43420 showed high similarity with clones found in Arctic sea ice (accession numbers AY198110, AF468332). In the phylum *Deinococcus-Thermus*, none of the phylotypes showed high similarity with other sequences except for three phylotypes which are related to species originally isolated from Antarctica and one phylotype with a cosmopolitan distribution.

Discussion

Cultivation conditions

To obtain a broad range of heterotrophic bacteria, several media were used, based on a review of literature. All growth media and temperatures used, gave access to some taxa that were only found under these conditions and were not isolated from the others. For instance the six isolates identified as *Arthrobacter phenanthrenivorans* were only recovered from the R2A/10 medium at 4 °C and the isolates related to *Lysobacter* sp. were only found on R2A at 20 °C. In general, the incubation conditions yielding the highest number of colonies (Fig. 2) were the oligotrophic media at 15 and 20 °C. Although our isolates originated from a much colder environment [56] and came from samples that were preserved frozen, indicating that they are resistant to cold temperatures, they seemed to grow better at moderate temperatures and are more likely psychrotolerant than psychrophilic. This phenomenon has been observed before in Antarctic marine bacteria that showed optimal growth levels at temperatures higher than those of their natural environment [28,34].

The marine medium was probably less successful because the samples were taken inland (about 180 km away from the ocean) and salinity requires specific adaptations for the organisms to survive [36]. The generally smaller diversity observed on the R2A/10 medium at all temperatures and on all media at 4 °C, may be explained by the fact that most isolates were taken after 2 or 3 weeks incubation whereas these conditions might have been more successful after a longer incubation period [25,66].

Diversity recovered

The rarefaction curves (Fig. 3) for both phylotypes and rep-types were higher for sample BB50 than for sample BB115. Additionally, the Shannon biodiversity index was slightly higher for sample BB50 (3.17) than for sample BB115 (2.89) indicating that sample BB50 was more diverse than sample BB115. The identifications of the phylotypes showed that there were important differences

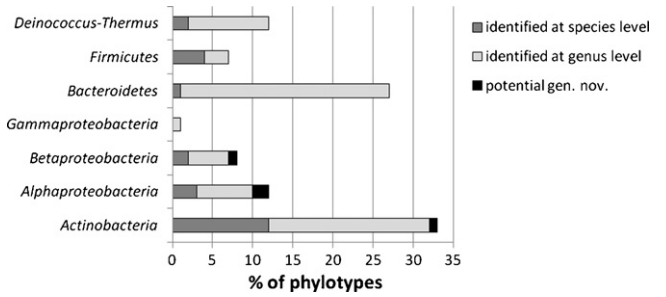

Fig. 4. Distribution of the phylotypes (%) over the different phyla and classes. The bar of each phylogenetic group is subdivided according to the different identification levels of the phylotypes. The group "identified at species level" contains those phylotypes that belong to existing species considering the threshold of 99.0% 16S rRNA gene sequence similarity. The group "identified at genus level" contains phylotypes that, based on phylogeny, belong to a particular genus and may represent a new species within that particular genus or an existing species within this genus showing less than 99.0% 16S rRNA gene sequence similarity with the type strain of this species. The group "potential gen. nov." contains those phylotypes that could not be assigned to a particular genus based on the phylogeny of the 16S rRNA gene.

in biodiversity of the two samples. A quarter of the isolates from each sample belonged to species or genera that were not found in the other sample and only two rep-clusters and seven phylotypes were in common. About 18.6% (BB50) and 16.9% (BB115) of the isolates belonged to phylotypes identified as named species. The majority of isolates (79.7% in BB50 and 83.1% in BB115) were identified as potentially novel species in existing genera. Most of these genera were represented in both samples, although generally by different species. Sample BB50 also contained four potentially new genera. In view of the limited resolving power of the 16S rRNA gene [33], for the phylotypes classified as potentially new taxa, a polyphasic approach will be necessary to clarify their taxonomic status. Additionally, it is important to note that the phylotypes grouping high numbers of isolates also comprise multiple rep-types (e.g. phylotype R-36707 contains 160 isolates, divided over 15 different rep-types). This indicated that these large phylotypes contain considerable genetic variation despite their high (>99.0%) 16S rRNA gene sequence similarity. This is in agreement with previous reports [2,55] and illustrates that rep-PCR fingerprinting has a higher taxonomic resolution than 16S rRNA gene sequencing [45]. Whether these phylotypes represent more than one species needs to be assessed by further characterization.

Our observation of *Actinobacteria*, *Bacteroidetes* and *Deinococcus-Thermus* as most dominant phyla (Fig. 4) recovered by cultivation from terrestrial microbial mats corroborates previous reports that found these groups dominant in Antarctic soils [5,86]. Twenty-one phylotypes could be identified as known species. Only the species *Arthrobacter flavus* [63], *A. antarcticus* [59], *Sphingomonas faeni* [16], *Chryseobacterium jeonii* (formerly *Sejongia jeonii*) [87], *Deinococcus marmoris* and *D. saxicola* [41] have previously been reported from Antarctica. For all the other species this is the first report in Antarctica, although their genera (except for *Aeromicrobium*, *Rothia* and *Subtercola*) do contain other Antarctic species. Interestingly, *Staphylococcus warneri* has been isolated from human skin [46] which could indicate that this phylotype might be a contaminant, for instance from the sampling or the processing during cultivation. However, other representatives of this genus have been reported from Antarctica [3] and from the stratosphere above Antarctica [57], indicating *S. warneri* may indeed be present in this pristine environment.

Other phylotypes were identified as potentially novel species in existing genera. The majority of these genera have previously been found in Antarctica [4–6,16,18,44,67,69,78,84]. However, for the genera *Tessaracoccus*, *Aurantimonas* and *Xylophilus* this is the first report from Antarctica. Especially in the phylum *Bacteroidetes* several new species within other genera were described from Antarctica in recent years [12,14,15,50,68,77,79,80,87,88].

In some genera, multiple potentially new species were found in this study, particularly in *Hymenobacter* (21), *Deinococcus* (10) and *Arthrobacter* (7) (Table S1). These genera already harbour a number of Antarctic species [40–42,59,63] and therefore seem particularly well represented in Antarctica. Furthermore, the presence in these genera of species from very diverse habitats including soil, water, clinical or veterinary samples, air and also extreme habitats such as Antarctic, alpine or Himalayan samples, deserts, hot springs and irradiated materials (list of Prokaryotic names with Standing in Nomenclature http://www.bacterio.net), points towards the extreme adaptability of these groups.

Besides the observation of a large number of potential new taxa and the first reports from Antarctica for several species and genera, another important outcome of this study is the availability of cultures of all these strains. These cultures will allow the detailed investigation of potential new taxa in order to describe them and explore their biotechnological potential. Furthermore, this study provides additional cultures for species that are, at present, only represented by one strain in culture collections (e.g. *Chryseobacterium jeonii* and *Arthrobacter antarcticus*) and for genera that contain at the moment only one species (e.g. *Saxeibacter*). The study of additional cultures will give more insight in the diversity present in these species and genera.

In addition to a large variation of heterotrophic bacteria, also a fungus was recovered. The isolation of the fungus *Thelebolus microspores* may be related to the presence of petrel nests on top of nunatak Utsteinen since this fungus is a psychrophile described as growing on guano of birds. A natural association with skuas, petrels and other birds was confirmed as they showed frequent intestinal colonisation by *Thelebolus microspores* [27].

Geographical distribution of the taxa recovered

Our sequences were compared with the EMBL and NCBI databases to assess the geographic distribution of the different phylotypes (Table 1, Table S1). A large majority of the phylotypes of the phyla *Bacteroidetes* and *Deinococcus-Thermus* and a minority of the *Actinobacteria* and *Alphaproteobacteria* were found to be currently known only from Antarctica (Table 1). The majority of the phylotypes affiliated with the *Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria* and all *Firmicutes* phylotypes had high 16S rRNA gene sequence similarities with environmental sequences from various origins indicating that these groups, including potential new species and genera, are more generally distributed cosmopolitans. It is important to note that the labels – Antarctic, bipolar or cold distribution (Table 1) – reflect current knowledge of bacterial diversity and ecology which is known to be limited [24]. The observations that bacteria in aerosols in the Antarctic atmosphere mainly belong to the phyla *Proteobacteria* and *Actinobacteria* [37,57,58] and that the intestinal flora of Antarctic birds is particularly composed of *Firmicutes*, *Proteobacteria* and *Actinobacteria* [9] indicate that assisted dispersal may influence the geographical distribution of these phylogenetic groups. However, information about the bacterial diversity in these niches is still very limited and more studies are needed to improve our understanding of the assisted distribution (wind-blown or via animals) of bacteria in Antarctica.

Of the potential new species and genera that were recovered, about 41% did not show significant similarity (≥99.0%) with any other sequence (Table 1 and Table S1). They are, for now, restricted to Antarctica. High levels of Antarctic endemicity were observed in some continental nunataks and mountain ranges for organisms such as lichens, tardigrades, diptera, mites, springtails, nematodes and cyanobacteria [7,21,22,39,54,62,74–76]. These reports support the glacial refugia hypothesis which suggests that some of these

ice-free mountains may have served as refuges for survival of biota during glacial periods [23]. Although the presence of refugia is difficult to prove and for many areas of the continent no field estimates for previous ice sheet thickness are available [23], there is evidence for the existence of refugia in the Bunger Hills [38] and the Larsemann Hills [43], both in East-Antarctica. Furthermore, according to Wand et al. [83] nunataks in Dronning Maud Land may have been exposed for 39,400 years and thus may have served as a refuge for survival of micro-organisms during the Last Glacial Maximum (~18,000 years ago)[1,23]. For bacteria, endemism is at present hard to prove because cultivated (and therefore named) diversity is estimated to represent only 1–3% of extant bacterial diversity [24] and because many habitats world-wide, including extreme ones [82] remain to be sampled for bacterial diversity. The observation in this study of a rather high percentage of potential new bacterial taxa (Table S1) currently restricted to Antarctica (Table 1) is in line with the glacial refuge hypothesis, although it does not provide solid support because some of these taxa may later prove to be more widely distributed.

In previous studies [34,82], the extent of sequence divergence with non-Antarctic sequences has been regarded as evidence for the separate evolution of Antarctic phylotypes. The average phylogenetic divergence of our phylotypes with Antarctic distribution and the nearest non-Antarctic named species was found to be 3.14% which decreased to 2.56% when unnamed environmental sequences were included. This value is lower than that reported in previous studies that date back 15 years [34,82], illustrating that the extent of the 16S rRNA gene databases which has increased significantly since 1996 [85], has a large influence on this percentage and emphasizing the importance of extending databases with microbial diversity studies in Antarctica and beyond.

Conclusion

This study provides the first data on the heterotrophic bacterial biodiversity in the region of the Princess Elisabeth Station in Utsteinen. Although only 2 samples were investigated, 796 isolates were obtained which showed a large diversity distributed over 35 different genera in 5 phyla. The most dominant phyla were *Actinobacteria*, *Bacteroidetes* and *Deinococcus-Thermus*. In addition to several genera that were previously reported from Antarctica, this is the first report in Antarctica for the genera *Aeromicrobium*, *Aurantimonas*, *Rothia, Subtercola, Tessaracoccus* and *Xylophilus*. Overall, 45% of the phylotypes are currently known only from Antarctica whereas 48% are cosmopolitan. A considerable number of potential new species and genera were recovered, distributed over 24 genera belonging to different phylogenetic groups. About 41% of the phylotypes representing new taxa are currently restricted to Antarctica. The rich microbial diversity found at the site indicates that this is an important region for further investigation.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.syapm.2011.02.002.

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RESEARCH LETTER

The *gyrB* gene is a useful phylogenetic marker for exploring the diversity of *Flavobacterium* strains isolated from terrestrial and aquatic habitats in Antarctica

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Abstract

Within the phylum *Bacteroidetes*, the *gyrB* gene, encoding for the B subunit of the DNA gyrase, has been used as a phylogenetic marker for several genera closely related to *Flavobacterium*. The phylogenies of the complete 16S rRNA gene and the *gyrB* gene were compared for 33 Antarctic *Flavobacterium* isolates and 23 type strains from closely related *Flavobacterium* species. *gyrB* gene sequences provided a higher discriminatory power to distinguish between different *Flavobacterium* groups than 16S rRNA gene sequences. The *gyrB* gene is therefore a promising molecular marker for elucidating the phylogenetic relationships among *Flavobacterium* species and should be evaluated for all the other type strains of described *Flavobacterium* species. Combining the phylogeny of both genes, the new Antarctic *Flavobacterium* strains constitute 15 *Flavobacterium* groups, including at least 13 potentially new species together with one group of isolates probably belonging to the species *Flavobacterium micromati* and one group close to *Flavobacterium gelidilacus*.

Introduction

Heterotrophic bacterial communities in Antarctica are highly diverse in aquatic (Bowman *et al*., 2000; Van Trappen *et al*., 2002) as well as in terrestrial (Aislabie *et al*., 2006; Babalola *et al*., 2009) habitats. A genus that has been isolated often from these environments is *Flavobacterium* (Brambilla *et al*., 2001; Humphry *et al*., 2001; Van Trappen *et al*., 2002), and several novel *Flavobacterium* species were described from Antarctic habitats (*Flavobacterium gelidilacus*, *Flavobacterium gillisiae*, *Flavobacterium hibernum*, *Flavobacterium micromati*, *Flavobacterium psychrolimnae*, *Flavobacterium xanthum*) or other cold environments (*Flavobacterium xinjangense* and *Flavobacterium omnivorum*). Other *Flavobacterium* species have been mainly isolated from freshwater fish (*Flavobacterium branchiophilum*, *Flavobacterium columnare*, *Flavobacterium psychrophilum*), temperate freshwater (*Flavobacterium aquatile*, *Flavobacterium flevense*, *Flavobacterium saccharophilum*) and from soil (*Flavobacterium johnsoniae*, *Flavobacterium pectinovorum*). Most *Flavobacterium* species are psychrotolerant and as they are able to hydrolyse several carbohydrates and biomacromolecules such as gelatine, casein and starch, they might be of biotechnological importance (Bernardet & Bowman, 2006).

The family *Flavobacteriaceae* (phylum *Bacteroidetes*) as well as the genus *Flavobacterium* have been revised and added to repeatedly over the years (Vandamme *et al*., 1994; Bernardet *et al*., 1996, 2002). *Flavobacterium* was created in 1923 for all bacteria that formed yellow- or orange-pigmented colonies and weakly produced acid from carbohydrates (Bergey *et al*., 1923). This broadly defined and taxonomically heterogeneous group was further refined using phenotypic characteristics (Holmes *et al*., 1984) and the determination of guanine plus cytosine (G+C) content (Reichenbach, 1989). The introduction of the 16S rRNA gene oligonucleotide catalogue (Paster *et al*., 1985), DNA–rRNA hybridization data (Bauwens & De Ley, 1981; Segers *et al*., 1993; Vandamme *et al*., 1994) and sequence data (Woese *et al*., 1990; Gherna & Woese, 1992) changed the family and the genus further and provided the framework for the present classification. Currently, strains are assigned to the genus *Flavobacterium* (including 71 species to date) based on fatty acid analysis, the G+C content and a number of

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morphological and phenotypical characteristics following the proposal of Bernardet *et al.* (1996) in combination with 16S rRNA gene sequence analysis (Bernardet *et al.*, 2002; Bernardet & Bowman, 2006).

Although DNA–DNA hybridizations (DDH) are the gold standard for species identification (Stackebrandt *et al.*, 2002), these experiments are technically challenging, laborious and time consuming. Sequence analysis of 16S rRNA genes is used for prokaryotic classification (Rossello-Mora & Amann, 2001) to provide a tentative identification. It can often limit the number of DDH experiments required. Nevertheless, the 16S rRNA gene has a limited resolving power at the species level (Fox *et al.*, 1992; Probst *et al.*, 1998). Within the genus *Flavobacterium*, values of 97.2–98.7% 16S rRNA gene sequence similarity are found between distinct *Flavobacterium* species (Bernardet & Bowman, 2006). As protein-encoding genes evolve faster, they are considered more appropriate for the phylogenetic analysis of closely related species. Within the genus *Flavobacterium*, protein-encoding genes have not yet been used for detailed phylogenetic study. The *gyrB* gene was found to be a successful marker for phylogenetic analysis in several groups in other phyla, for example *Acinetobacter* (*Proteobacteria*) (Yamamoto & Harayama, 1996) and *Micromonospora* (*Actinobacteria*) (Kasai *et al.*, 2000), but also in the phylum *Bacteroidetes* in the genus *Marinilabilia* and related taxa (Suzuki *et al.*, 1999). In these studies, phylogenetic analysis based on the *gyrB* gene sequences was shown to be consistent with DDH and phenotypic comparison (Yamamoto & Harayama, 1996). Suzuki *et al.* (2001) applied *gyrB* gene sequencing to study the phylogenetic relationships of marine isolates within the phylum *Bacteroidetes* and included two *Flavobacterium* species. In addition, more *gyrB* sequences from *Flavobacterium* species are becoming available in the frame of genome projects (Duchaud *et al.*, 2007).

In a previous study of aquatic and terrestrial microbial mats in Antarctica, several *Flavobacterium* strains were isolated that showed a low similarity to described *Flavobacterium* species, based on the partial or the full 16S rRNA gene sequences (Peeters *et al.*, submitted). In the present study, we determined the *gyrB* gene sequence of 33 of these new Antarctic isolates and of the type strains of related *Flavobacterium* species to study the diversity of our isolates in more detail and to elucidate the usefulness of *gyrB* as a phylogenetic marker for phylogeny in the genus *Flavobacterium*. We also compared with the phylogeny based on the near-complete 16S rRNA gene sequences.

Materials and methods

Strains used

The *Flavobacterium* strains studied here (Table 1) were obtained as part of a large study into the diversity of heterotrophic bacteria in microbial mats from Antarctica (Peeters *et al.*, submitted). The samples used in that study originated from a terrestrial sample, taken in the close neighbourhood of the Princess Elisabeth Station in Utsteinen, Dronning Maud Land (Peeters *et al.*, 2011a), and microbial mat samples from lakes in the Transantarctic Mountains (Peeters *et al.*, 2011b), the Schirmacher Oasis and on Pourquoi-Pas Island (Antarctic Peninsula) (for details, see Table 1). In these previous studies, isolates were first grouped by rep-PCR fingerprinting and representatives of all rep-types were tentatively identified by full or partial 16S rRNA gene sequencing (Peeters *et al.*, 2011a; Peeters *et al.*, 2011b; Peeters *et al.*, submitted). Several of these strains were identified as *Flavobacterium* and 33 of these were used in this study (Table 1). To elucidate their phylogenetic relationships, type strains of closely related *Flavobacterium* species were also included (Table 2).

16S rRNA gene sequence analysis

The complete 16S rRNA gene sequences of four Antarctic *Flavobacterium* isolates were available from previous studies (Peeters *et al.*, 2011a, 2011b). The 16S rRNA genes of the remaining 29 Antarctic *Flavobacterium* isolates were only partially sequenced (400 bp) (Peeters *et al.*, submitted). These sequences were completed in this study (accession numbers listed in Table 1) using the same method as that described before (Vancanneyt *et al.*, 2004). A multiple sequence alignment of all complete 16S rRNA gene sequences was performed using the BIONUMERICS (version 5.1.) software package (Applied-Maths) and a region of 912 bp, containing good sequence data for all strains, was delimited for further analysis. After visual inspection, distances were calculated using the Kimura-2 correction. A neighbour-joining dendrogram (Saitou & Nei, 1987) was constructed and bootstrapping analysis was performed using 500 bootstrap replicates. A maximum likelihood dendrogram was calculated using the program PHYML (Guindon & Gascuel, 2003). The reliability of the tree was checked using the approximate likelihood ratio test (aLRT) method (Anisimova & Gascuel, 2006).

gyrB gene sequence analysis

For *F. johnsoniae*, *F. aquatile* and *Myroides odoratus* the *gyrB* sequences were available in the EMBL database (Table 2). For the other strains used, the *gyrB* sequences were determined in this study. DNA preparation was carried out as described by Baele *et al.* (2003). Primers were designed in KODON 3.5 using all available *gyrB* sequences from *Flavobacterium* and species from closely related genera (*Bacteroides*, *Cytophaga*, *Flexibacter*, *Terrimonas*, *Porphyrobacter*, *Parabacteroides*, *Salinibacter* and *Prevotella*) in the EMBL database (September 2009). A *gyrB* segment about 1200 bp

Table 1. Strain numbers, accession numbers and isolation source of the Antarctic *Flavobacterium* isolates used

		Accession no.		
Species	Strain no.	16S rRNA gene	*gyrB* gene	Isolation source
Flavobacterium sp. 1	R-40838	FR682718*	FR772324	Terrestrial microbial mat, Utsteinen nunatak, Antarctica
	R-40949	FR772055	FR772296	Terrestrial microbial mat, Utsteinen nunatak, Antarctica
Flavobacterium sp. 2	R-36233	FR682719*	FR772292	Terrestrial microbial mat, Utsteinen nunatak, Antarctica
	R-36668	FR772052	FR772293	Terrestrial microbial mat, Utsteinen nunatak, Antarctica
	R-36669	FR772053	FR772294	Terrestrial microbial mat, Utsteinen nunatak, Antarctica
	R-36523	FR772054	FR772295	Terrestrial microbial mat, Utsteinen nunatak, Antarctica
Flavobacterium sp. 3	R-41499	FR772077	FR772318	Aquatic microbial mat, Schirmacher Oasis, Antarctica
Flavobacterium sp. 4	R-38377	FR772072	FR772313	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-37599	FR772073	FR772314	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-38423	FR772067	FR772308	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-40835	FR772071	FR772312	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-36964	FR691441*	FR772322	Aquatic microbial mat, Forlidas Pond, Antarctica
Flavobacterium sp. 5	R-38388	FR772056	FR772297	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
Flavobacterium sp. 6	R-38274	FR772058	FR772299	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-38352	FR772069	FR772310	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
Flavobacterium sp. 7	R-38477	FR772059	FR772300	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
Flavobacterium sp. 8	R-40837	FR772060	FR772301	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-38313	FR772065	FR772306	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-38503	FR772061	FR772302	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-41504	FR772062	FR772303	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
Flavobacterium sp. 9	R-38294	FR772063	FR772304	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-38296	FR772064	FR772305	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
Flavobacterium sp. 10	R-38392	FR772074	FR772315	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
Flavobacterium sp. 11	R-37608	FR772076	FR772317	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
Flavobacterium sp. 12	R-38474	FR772057	FR772298	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-38373	FR772070	FR772311	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
Flavobacterium sp. 13	R-40832	FR772078	FR772319	Aquatic microbial mat, Forlidas Pond, Antarctica
	R-36976	FR772080	FR772323	Aquatic microbial mat, Forlidas Pond, Antarctica
	R-36963	FR691440*	FR772321	Aquatic microbial mat, Forlidas Pond, Antarctica
	R-36961	FR772079	FR772320	Aquatic microbial mat, Forlidas Pond, Antarctica
Flavobacterium sp. 14	R-38349	FR772068	FR772309	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
Flavobacterium sp. 15	R-38420	FR772066	FR772307	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica
	R-37612	FR772075	FR772316	Aquatic microbial mat, Pourquoi-Pas Island, Antarctica

The 16S rRNA gene sequences marked with an asterisk were determined in previous studies (Peeters *et al*., 2011a, 2011b).

long was obtained with the primers *gyrB*-241F (5′-GA YACCGGWCGTGGTATTCC-3′) and *gyrB*-1588R (5′-TC DAYATCGGCATCACACAT-3′), which were used both for amplification and for sequencing reactions. For amplification, the reaction mix (50 μL) consisted of 5 μL GeneAmp® 10 × PCR buffer (Applied Biosystems), 5 μL dNTP's (2 mM), 0.5 μL of the forward and reverse primer (50 μM), 1 μL Taq polymerase (1 U μL^{-1}), 33 μL MilliQ water and 5 μL template DNA. After an initial denaturation step (95 °C for 5 min), three cycles of preamplification (95 °C for 1 min, 55 °C for 2 min 15 s and 72 °C for 1 min 15 s) and 25 cycles of amplification (95 °C for 35 s, 55 °C for 1 min 15 s and 72 °C for 1 min 15 s) were performed, finishing with 72 °C for 7 min. PCR products were purified using a Nucleofast 96 PCR cleanup membrane system (Machery-Nagel, Germany) and a Tecan Workstation 200. The sequencing PCR was performed as described before (Vancanneyt *et al*., 2004). Sequence assembly and phylogenetic analysis was performed with the BIONUMERICS (version 5.1) software package (Applied-Maths) using a region of 1006 bp, containing good sequence data for all strains. The multiple alignment was verified by comparison with an alignment of the corresponding amino acids. After visual inspection of the sequence alignments, distances were calculated using the Kimura-2 correction. A neighbour-joining dendrogram (Saitou & Nei, 1987) was constructed and bootstrapping analysis was performed using 500 bootstrap replicates. A maximum likelihood dendrogram was calculated using the program PHYML (Guindon & Gascuel, 2003). The reliability of the tree was checked using the aLRT method (Anisimova & Gascuel, 2006). Accession numbers of the *gyrB* gene sequence of the *Flavobacterium* strains and the type strains of the *Flavobacterium* species are listed in Tables 1 and 2, respectively.

Table 2. *Flavobacterium* species included in this study

Species	Strain no.	Accession no.		Isolation source	References
		16S rRNA gene	*gyrB* gene		
Flavobacterium antarcticum	LMG 25319^T	FM163401	**FR774016**	Terrestrial sample from the Antarctic	Yi *et al*. (2005)
Flavobacterium aquatile	LMG 4427^T	AM230485	AB034225	Deep well, Kent, England	Bernardet *et al*. (1996)
Flavobacterium degerlachei	LMG 21915^T	AJ557886	**FR774017**	Microbial mats in Antarctic lakes	Van Trappen *et al*. (2004)
Flavobacterium flevense	LMG 8328^T	D12662	**FR774018**	Freshwater lake, the Netherlands	Bernardet *et al*. (1996)
Flavobacterium frigidarium	LMG 21010^T	AF162266	**FR774019**	Marine sediment, Antarctica	Humphry *et al*. (2001)
Flavobacterium frigoris	LMG 21922^T	AJ557887	**FR850657**	Microbial mats in Antarctic lakes	Van Trappen *et al*. (2004)
Flavobacterium fryxellicola	LMG 22022^T	AJ811961	**FR774020**	Microbial mats in Antarctic lakes	Van Trappen *et al*. (2005)
Flavobacterium gelidilacus	LMG 21477^T	AJ440996	**FR774021**	Microbial mats in Antarctic lakes	Van Trappen *et al*. (2003)
Flavobacterium gillisiae	LMG 21422^T	U85889	**FR774014**	Antarctic coastal sea ice	McCammon & Bowman (2000)
Flavobacterium glaciei	LMG 25320^T	DQ515962	**FR774022**	China No.1 glacier	Zhang *et al*. (2006)
Flavobacterium hibernum	LMG 21424^T	L39067	**FR774023**	Freshwater Antarctic lake	McCammon *et al*. (1998)
Flavobacterium johnsoniae	LMG 1340^T	AM230489	AB034222	Soil or mud, Rothamsted or Cambridge, England	Bernardet *et al*. (1996)
Flavobacterium limicola	LMG 21930^T	AB075230	**FR774015**	Freshwater sediments	Tamaki *et al*. (2003)
Flavobacterium micromati	LMG 21919^T	AJ557888	**FR774024**	Microbial mats in Antarctic lakes	Van Trappen *et al*. (2004)
Flavobacterium omnivorum	LMG 21986^T	AF433174	**FR774025**	China No. 1 glacier	Zhu *et al*. (2003)
Flavobacterium psychrolimnae	LMG 22018^T	AJ585428	**FR774026**	Microbial mats in Antarctic lakes	Van Trappen *et al*. (2005)
Flavobacterium psychrophilum	LMG 13179^T	AB078060	**FR774027**	Kidney of salmon	Bernardet *et al*. (1996)
Flavobacterium reichenbachii	LMG 25512^T	AM177616	**FR774028**	Hard water rivulet, Germany	Ali *et al*. (2009)
Flavobacterium succinicans	LMG 10402^T	AM230492	**FR774029**	Eroded fin of salmon, Washington	Bernardet *et al*. (1996)
Flavobacterium swingsii	LMG 25510^T	AM934651	**FR774030**	Hard water rivulet, Germany	Ali *et al*. (2009)
Flavobacterium tegetincola	LMG 21423^T	U85887	**FR774031**	Antarctic cyanobacterial mat	McCammon & Bowman (2000)
Flavobacterium xanthum	LMG 8372^T	AF030380	**FR774032**	Pool mud, Syowa, Antarctica	McCammon & Bowman (2000)
Flavobacterium xinjiangense	LMG 21985^T	AF433173	**FR774033**	China No. 1 glacier	Zhu *et al*. (2003)
Myroides odoratus	NBRC 14945^T	M58777	AB034239	Urine and serum specimen	Vancanneyt *et al*. (1996)

Accession numbers for newly determined sequences are shown in bold.

Results and discussion

This study was carried out to resolve the relationships of 33 Antarctic *Flavobacterium* strains that were previously characterized by partial 16S rRNA gene sequencing and found to represent several potentially novel groups. We completed the 16S rRNA gene sequences for all the strains and performed a phylogenetic analysis including also the type strains of 23 related or Antarctic *Flavobacterium* species. Neighbour-joining and maximum likelihood trees (Fig. 1 and Supporting Information, Fig. S1) showed a similar topology with the *Flavobacterium* isolates forming 15 groups, labelled *Flavobacterium* sp. 1–15. *Flavobacterium* sp. 13 and *Flavobacterium* sp. 5 were located close to, respectively, *F. micromati* and *F. gelidilacus*, with 99.8% and 99.0% sequence similarity to the respective type strain. It is well known that because of its high conservation, the 16S rRNA gene sequence has limited resolving power at the species level (Rossello-Mora & Amann, 2001). Indeed, there are examples of distinct species with identical or nearly identical 16S rRNA gene sequences (Fox *et al*., 1992; Probst *et al*., 1998), microheterogeneity of the 16S rRNA genes within one species (Bennasar *et al*., 1996) or single organisms with two or more 16S rRNA genes with a relatively high sequence divergence (Nübel *et al*., 1996). In the genus *Flavobacterium*, several new species have been described with a rather high 16S rRNA gene sequence similarity, for example the type strains of *Flavobacterium weaverense* and *Flavobacterium segetis* share 98.9% 16S rRNA gene sequence similarity, and yet, they have a DDH value of only 34% (Yi & Chun, 2006). Because protein-encoding genes are generally less conserved (Ochman & Wilson, 1987), they may be more appropriate for phylogenetic analysis of closely related species. Several protein-encoding genes such as *glnA*, *recA* and *hsp60* have been used for typing and taxonomical purposes within genera in the *Bacteroidetes* (Gutacker *et al*., 2002; Sakamoto *et al*., 2010). In this study, the *gyrB* gene, encoding for the B subunit of the DNA gyrase, was selected because it was previously used successfully to distinguish between closely related taxa affiliated with the genus *Flavobacterium* (Suzuki *et al*., 1999, 2001). Izumi *et al*. (2003) reported on the use of *gyrB* primers in a PCR-restriction fragment length

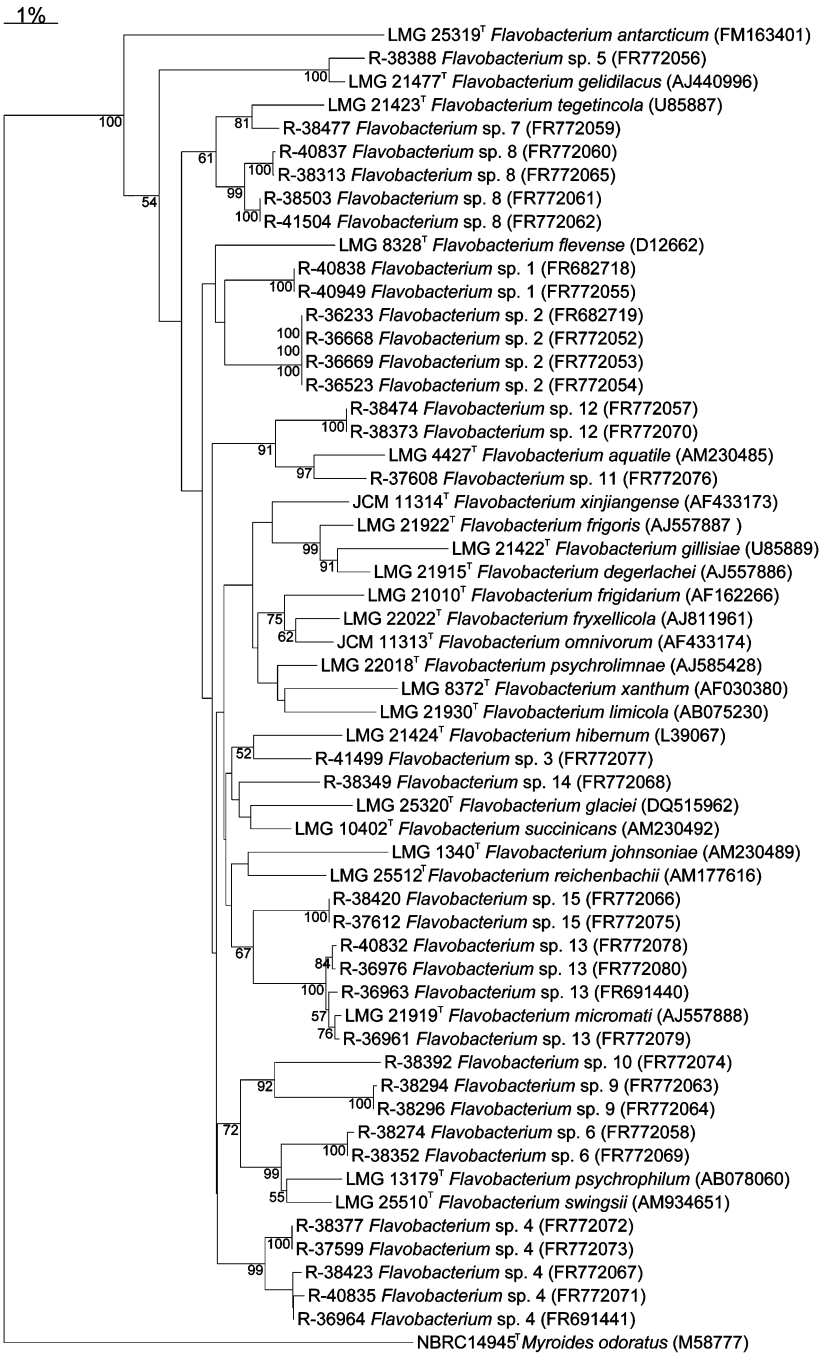

Fig. 1. Phylogenetic tree based on neighbour-joining analysis of the 16S rRNA gene sequence similarities of the *Flavobacterium* strains and closely related species. New *Flavobacterium* isolates are marked as *Flavobacterium* sp., followed by a number. The numbers at branch nodes are bootstrap values shown as percentages of 500 bootstrap replicates (only values > 50% are shown). Scale bar represents 1% estimated substitutions.

polymorphism analysis for the genotyping of *F. psychrophilum*, and Suzuki *et al.* (1999) designed *gyrB* primers to study the phylogenetic relationship for the genus *Marinilabilia* (*Bacteroidetes*) and related taxa. We tested all primers reported in these studies *in silico* on the *gyrB* sequences available from related genera and from the complete genome of *F. johnsoniae* DSM 2064 and found considerable mismatches with all groups included in the comparison. Therefore, more general primers were designed based on the available sequence information.

As expected for a more variable housekeeping gene, the distance between the *Flavobacterium* groups and the type strains is significantly higher in the *gyrB* gene dendrogram (Figs 2 and S2) in comparison with the 16S rRNA gene dendrogram (Figs 1, S1 and Table 3). The threshold for species definition has been suggested to be 98.7–99.0% 16S

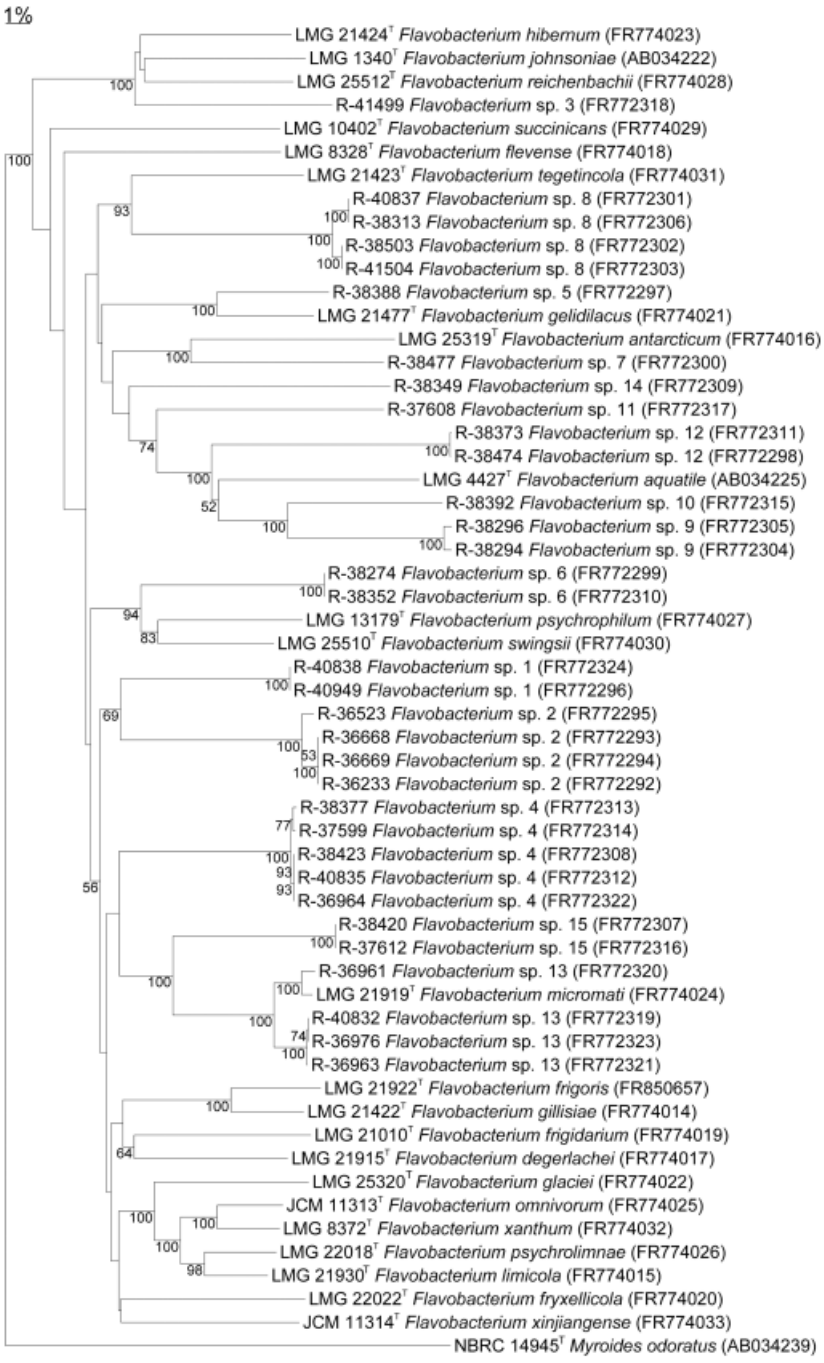

Fig. 2. Phylogenetic tree based on neighbour-joining analysis of the *gyrB* gene sequence similarities of the *Flavobacterium* strains and closely related species. New *Flavobacterium* isolates are marked as *Flavobacterium* sp., followed by a number. The numbers at branch nodes are bootstrap values shown as percentages of 500 bootstrap replicates (only values > 50% are shown). Scale bar represents 1% estimated substitutions.

rRNA gene sequence similarity by Stackebrandt & Ebers (2006), whereas for the *gyrB* phylogeny, this is less well documented. Suzuki *et al.* (2001) reported that the proposed limit for species identity, the 70% DNA reassociation value, corresponds to 88.8% *gyrB* sequence similarity in the subset of the *Bacteroidetes* they studied, whereas several other studies revealed a wide range of interspecies similarity values [60.0–89.0% *gyrB* gene sequence similarity within the genus *Helicobacter* (*Epsilonproteobacteria*) (Hannula & Hanninen, 2007), 75.4–95.0% within the genus *Bacillus* (*Firmicutes*) (Wang *et al.*, 2007), 85.0–97.5% within the genus *Aeromonas* (*Gammaproteobacteria*) (Yanez *et al.*, 2003), 77.5–97.6% within the genus *Gordonia* (*Actinobacteria*) (Kang *et al.*, 2009), 89.5–98.2% within the genus *Kribbella* (*Actinobacteria*) (Kirby *et al.*, 2010) and 70.1–98.7% within the genus *Streptococcus* (*Firmicutes*) (Itoh *et al.*, 2006)]. Among the

Table 3. Within-group similarity, closest related species and corresponding sequence similarity for the different Antarctic *Flavobacterium* groups based on the 16S rRNA and the *gyrB* gene phylogeny

16S rRNA gene				*gyrB* gene			
Antarctic species	Within group similarity (%)	Nearest neighbour	Similarity (%)	Antarctic species	Within group similarity (%)	Nearest neighbour	Similarity (%)
Flavobacterium sp. 1	100	*Flavobacterium psychrolimnae* LMG 22018^T	97.3	*Flavobacterium* sp. 1	100	*Flavobacterium limicola* LMG 21930^T	86.1
Flavobacterium sp. 2	100	*Flavobacterium succinicans* LMG 10402^T	96.4	*Flavobacterium* sp. 2	98.9–98.8	*Flavobacterium psychrolimnae* LMG 22018^T	86.6–86.4
Flavobacterium sp. 3		*Flavobacterium succinicans* LMG 10402^T	97.5	*Flavobacterium* sp. 3		*Flavobacterium hibernum* LMG 21424^T	87.2
Flavobacterium sp. 4	99.5–99.2	*Flavobacterium succinicans* LMG 10402^T	97.9–97.8	*Flavobacterium* sp. 4	99.8–99.7	*Flavobacterium degerlachei* LMG 21915^T	86.9–86.7
Flavobacterium sp. 5		*Flavobacterium gelidilacus* LMG 21477^T	99.0	*Flavobacterium* sp. 5		*Flavobacterium gelidilacus* LMG 21477^T	91.9
Flavobacterium sp. 6	99.9	*Flavobacterium swingsii* LMG 25510^T	97.9–97.8	*Flavobacterium* sp. 6	100	*Flavobacterium swingsii* LMG 25510^T	88.6
Flavobacterium sp. 7		*Flavobacterium tegetincola* LMG 21423^T	98.2	*Flavobacterium* sp. 7		*Flavobacterium antarcticum* LMG 25319^T	85.5
Flavobacterium sp. 8	99.1–100	*Flavobacterium tegetincola* LMG 21423^T	97.5–96.9	*Flavobacterium* sp. 8	100–99.0	*Flavobacterium tegetincola* LMG 21423^T	85.8–85.7
Flavobacterium sp. 9	99.9	*Flavobacterium swingsii* LMG 25510^T	95.6–95.5	*Flavobacterium* sp. 9	99.4	*Flavobacterium aquatile* LMG 4008^T	84.6
Flavobacterium sp. 10		*Flavobacterium swingsii* LMG 25510^T	96.1	*Flavobacterium* sp. 10		*Flavobacterium aquatile* LMG 4008^T	84.2
Flavobacterium sp. 11		*Flavobacterium aquatile* LMG 4427^T	97.8	*Flavobacterium* sp. 11		*Flavobacterium swingsii* LMG 25510^T	82.9
Flavobacterium sp. 12	100	*Flavobacterium aquatile* LMG 4427^T	97.1	*Flavobacterium* sp. 12	99.9	*Flavobacterium aquatile* LMG 4008^T	84.1–83.7
Flavobacterium sp. 13	99.6–99.4	*Flavobacterium micromati* LMG 21919^T	99.8–99.4	*Flavobacterium* sp. 13	99.9–97.2	*Flavobacterium micromati* LMG 21919^T	99.0–96.9
Flavobacterium sp. 14		*Flavobacterium succinicans* LMG 10402^T	97.7	*Flavobacterium* sp. 14		*Flavobacterium swingsii* LMG 25510^T	84.1
Flavobacterium sp. 15	100	*Flavobacterium succinicans* LMG 10402^T	97.2	*Flavobacterium* sp. 15	100	*Flavobacterium micromati* LMG 21919^T	88.5

Antarctic *Flavobacterium* groups for which no within-group similarity is listed consist of one strain.

type strains of the *Flavobacterium* species investigated in this study, the interspecies *gyrB* sequence similarity values varied from 79.1% between *F. aquatile* and *Flavobacterium reichenbachii* to 94.9% between *F. xanthum* and *F. omnivorum*.

The phylogenetic trees based on the *gyrB* sequences (Figs 2 and S2) show that the groups found in the 16S rRNA gene dendrogram (Figs 1 and S1) were confirmed. The Antarctic *Flavobacterium* groups generally showed lower *gyrB* gene sequence similarity to neighbouring groups and species, which confirmed their status as potentially new species. *Flavobacterium* sp. 13 and sp. 5, which, in the 16S rRNA gene phylogeny, were closely related to *F. micromati* and *F. gelidilacus*, respectively, also group with these species in the *gyrB* phylogeny. Both groupings are well supported; however, the *gyrB* similarity of *Flavobacterium* sp. 13 to *F. micromati* LMG 21919 (97.0%) is higher than that of *Flavobacterium* sp. 5 to *F. gelidilacus* LMG 21477 (91.9%). *Flavobacterium* sp. 13 probably belongs to *F. micromati* that was originally isolated from microbial mats in Antarctic lakes (Van Trappen *et al.*, 2004) as were the isolates of *Flavobacterium* sp. 13 (Table 1). *Flavobacterium* sp. 5 probably represents a new species in view of the rather low *gyrB* gene sequence similarity to *F. gelidilacus* in comparison with the higher similarity values obtained between some type strains. Nevertheless, the precise relation to *F. gelidilacus*, another species from Antarctic microbial mats (Van Trappen *et al.*, 2003), remains to be investigated further.

The similarities within the delineated *Flavobacterium* groups are generally very high for the 16S rRNA gene sequences (Table 3). The *gyrB* sequences were mostly also very similar within groups and ranged from 97.2% to 100% (Table 3). In *Flavobacterium* sp. 2, sp. 8 and sp. 13 (Figs 2 and S2) subclusters were observed with 97.2–99.0% sequence similarity. In other genera, comparable high intraspecies *gyrB* gene sequence similarities were observed, for example 98.5–100% *gyrB* gene sequence similarity within the genus *Streptomyces* (*Actinobacteria*) (Hatano *et al.*, 2003), 97.4–100% within the genus *Aeromonas* (*Gammaproteobacteria*) (Yanez *et al.*, 2003), 95.0–100% within the genus *Bacillus* (*Firmicutes*) (Wang *et al.*, 2007) and 94.6–100% within the genus *Helicobacter* (*Epsilonproteobacteria*) (Hannula & Hanninen, 2007).

It should be noted that all *Flavobacterium* groups studied here comprised several rep-types (Peeters *et al.*, submitted) and the strains were chosen to represent this diversity. The topologies of the neighbour-joining and the maximum likelihood dendrogram were slightly different for the 16S rRNA gene compared with the *gyrB* gene (Figs 1, 2, S1 and S2), as has also been observed for other groups (Yamamoto & Harayama, 1996). However, overall, the phylogenies of the 16S rRNA (Figs 1 and S1) and *gyrB* (Figs 2 and S2) gene were similar and confirmed the division of the Antarctic strains into 15 groups, one probably belonging to *F. micromati* and one close to *F. gelidilacus*. The other 13*Flavobacterium* groups formed separate groups in both the 16S rRNA gene and the *gyrB* gene phylogeny and probably represent new species. However, additional characterization is necessary to confirm this and to describe them as new species.

In conclusion, this study showed that within the genus *Flavobacterium*, the *gyrB* gene has a higher discriminatory power than the 16S rRNA gene. In comparison with the 16S rRNA gene sequence, the sequence similarities for the *gyrB* gene between the delineated groups are significantly lower whereas within the different groups they are still very high. Although there are differences in topology in the dendrograms based on either gene, the same groups of Antarctic *Flavobacterium* strains were recovered. Thus, the *gyrB* gene is a promising molecular marker to elucidate the phylogenetic relationships among *Flavobacterium* species and should be evaluated for all the other *Flavobacterium* species described. The phylogeny of both the 16S rRNA gene and the *gyrB* gene showed that the Antarctic *Flavobacterium* isolates studied here represent at least 13 potentially new species. These will be studied in more detail using various methods to confirm this and describe these groups appropriately.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Phylogenetic tree calculated using the maximum likelihood method based on the 16S rRNA gene sequences of the *Flavobacterium* strains and closely related species.
Fig. S2. Phylogenetic tree calculated using the maximum likelihood method based on the *gyrB* gene sequences of the *Flavobacterium* strains and closely related species.

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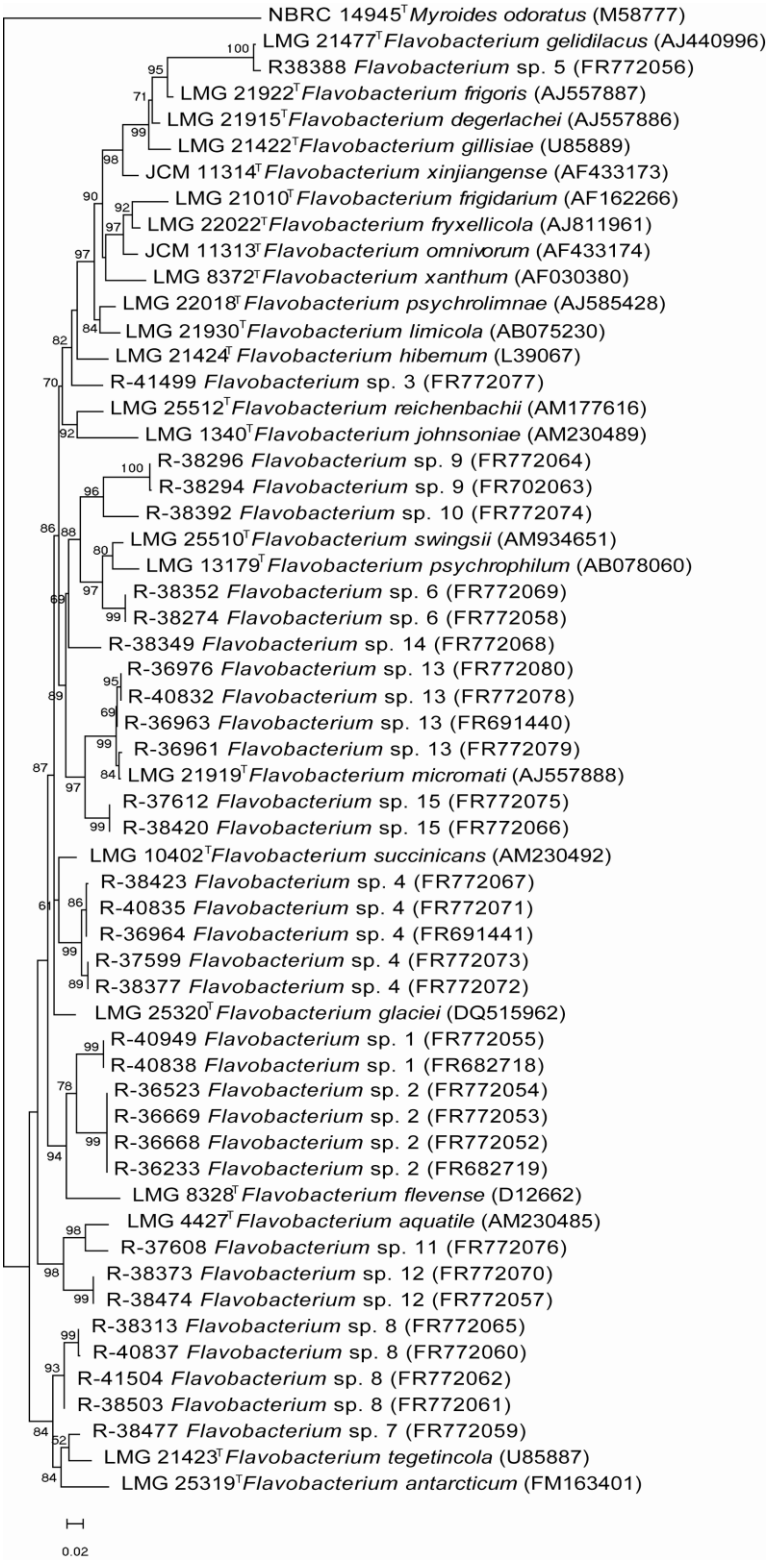

Fig. S1 Phylogenetic tree calculated using the maximum likelihood method based on the 16S rRNA gene sequences of the *Flavobacterium* strains and closely related species. Antarctic *Flavobacterium* isolates are indicated as *Flavobacterium* sp. followed by a number. The numbers at branch nodes are the aLRT branch support numbers (only values > 80% are shown). Bar represents 0.02% estimated substitutions.

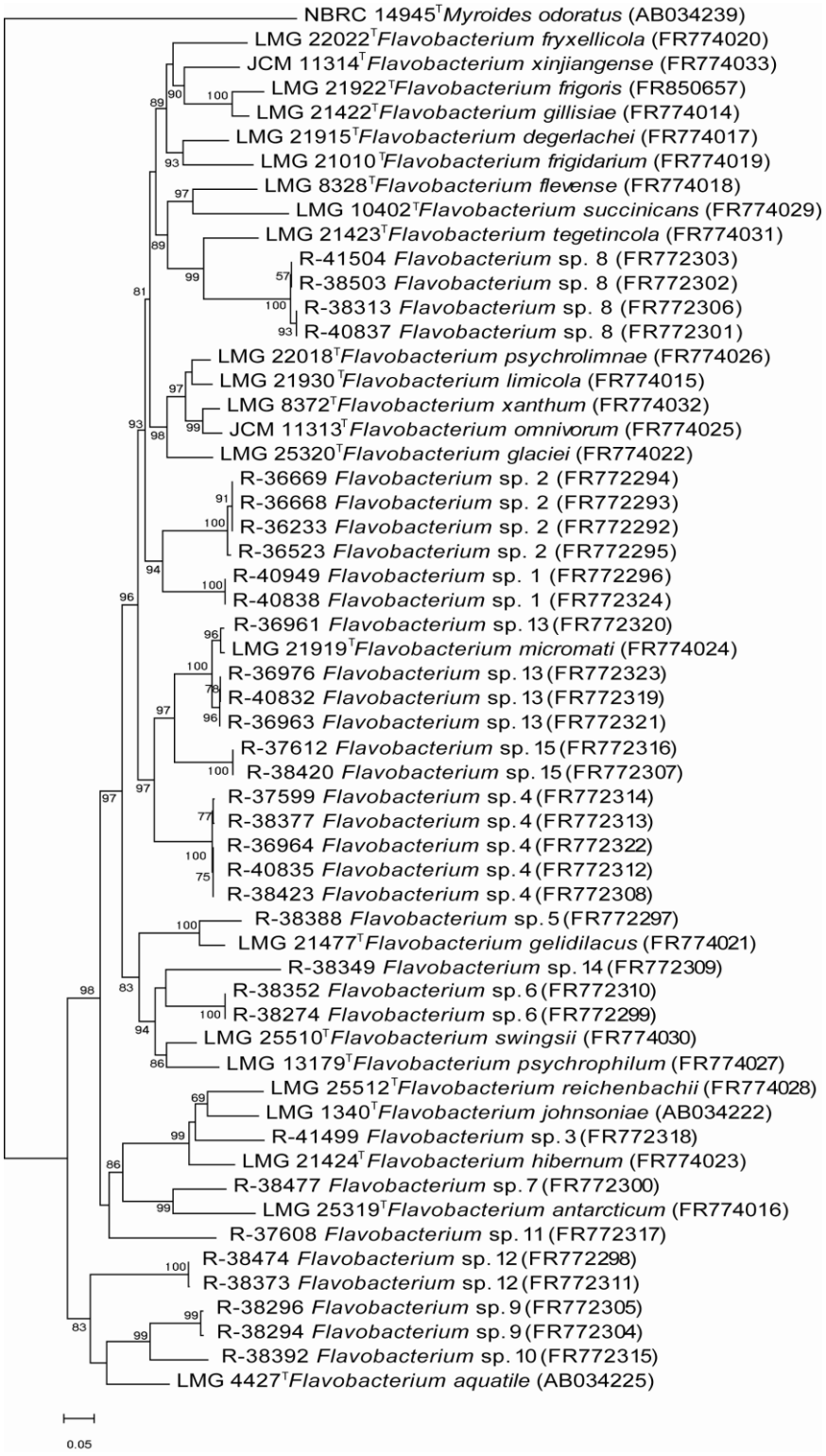

Fig. S2 Phylogenetic tree calculated using the maximum likelihood method based on the *gyrB* gene sequences of the *Flavobacterium* strains and closely related species. Antarctic *Flavobacterium* isolates are indicated as *Flavobacterium* sp. followed by a number. The numbers at branch nodes are the aLRT branch support numbers (only values > 80% are shown). Bar represents 0.05% estimated substitutions.

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ORIGINAL PAPER

Heterotrophic bacterial diversity in aquatic microbial mat communities from Antarctica

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Abstract Heterotrophic bacteria isolated from five aquatic microbial mat samples from different locations in continental Antarctica and the Antarctic Peninsula were compared to assess their biodiversity. A total of 2,225 isolates obtained on different media and at different temperatures were included. After an initial grouping by whole-genome fingerprinting, partial 16S rRNA gene sequence analysis was used for further identification. These results were compared with previously published data obtained with the same methodology from terrestrial and aquatic microbial mat samples from two additional Antarctic regions. The phylotypes recovered in all these samples belonged to five major phyla, *Actinobacteria*, *Bacteroidetes*, *Proteobacteria*, *Firmicutes* and *Deinococcus-Thermus,* and included several potentially new taxa. Ordination analyses were performed in order to explore the variance in the diversity of the samples at genus level. Habitat type (terrestrial vs. aquatic) and specific conductivity in the lacustrine systems significantly explained the variation in bacterial community structure. Comparison of the phylotypes with sequences from public databases showed that a considerable proportion (36.9%) is currently known only from Antarctica. This suggests that in Antarctica, both cosmopolitan taxa and taxa with limited dispersal and a history of long-term isolated evolution occur.

Keywords Microbial diversity · Cultivation · 16S rRNA gene sequencing · ASPA · PCA

Introduction

Microbial mats and surface crusts that may develop in wet Antarctic habitats (Vincent 2000; Laybourn-Parry and Pearce 2007) are dense communities of vertically stratified microorganisms and are believed to be responsible for much of the primary production under the extreme polar conditions. The mats and crusts typically consist of mucilage, in which cyanobacteria and other algal cells are embedded, together with other heterotrophic and chemoautotrophic microorganisms, sand grains and other inorganic materials (Fernández-Valiente et al. 2007). Particularly, the lacustrine ecosystems, which range from relatively deep freshwater and hypersaline lakes, to small ponds and seepage areas (Verleyen et al. 2011) act as true biodiversity and primary production hotspots in a matrix of polar desert and ice.

In recent years, Antarctic microbial mats have attracted a lot of scientific interest, with the photoautotrophic taxa such as cyanobacteria (Taton et al. 2006), green algae (De Wever et al. 2009) and diatoms (Sabbe et al. 2003) probably being the best-studied groups. Water depth (and

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hence light climate), liquid water availability and conductivity or related parameters are the most important variables in structuring these communities (Hodgson et al. 2004; Verleyen et al. 2010). Surprisingly, only a small number of studies have focussed on the heterotrophic bacterial diversity in these microbial mats (Brambilla et al. 2001; Van Trappen et al. 2002). Other land-based habitats in Antarctica that have been studied for their heterotrophic bacterial diversity include soils in dry valleys (Aislabie et al. 2006b) and maritime Antarctica (Chong et al. 2010), the plankton in freshwater lakes (Pearce. 2005) and anoxic waters in meromictic lakes (Franzmann et al. 1991). The few studies focussing on the heterotrophic bacterial diversity in aquatic microbial mats comprised samples from lakes in the McMurdo Dry Valleys, the Vestfold Hills and the Larsemann Hills and included culture-dependent as well as independent approaches. They reported a large diversity with an important number of previously unknown taxa (Brambilla et al. 2001; Van Trappen et al. 2002). As a result, several new species have been described in the phyla *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Firmicutes* (Reddy et al. 2002a, b, 2003a, b; Van Trappen et al. 2003, 2004a, b, c, d; Shivaji et al. 2005). The relationship between the bacterial diversity of microbial mats and environmental parameters has not yet been studied although Brambilla et al. (2001) suggested some general features expected of the organisms obtained based on their phylogenetic position.

The aims of this study were (1) to contribute to a better understanding of the diversity of heterotrophic bacteria in microbial mat communities from a range of terrestrial and aquatic habitats in coastal and inland ice-free regions in Continental and Maritime Antarctica and (2) to explore the relationship between the bacterial communities and a set of environmental parameters. We applied a cultivation-based approach using several media and growth conditions to access heterotrophic bacteria. A large number of isolates was obtained and identified through genotypic characterization using rep-PCR fingerprinting and phylogenetic analysis of the 16S rRNA gene sequences. Comparison of the sequences with those available in public databases allowed identification of the bacteria and an assessment of their geographical distribution.

Experimental procedures

Source of samples

Five samples (PQ1, LA3, SK5, WO10 and SO6) from lacustrine habitats in different locations in Continental Antarctica and the Antarctic Peninsula (Fig. 1) were

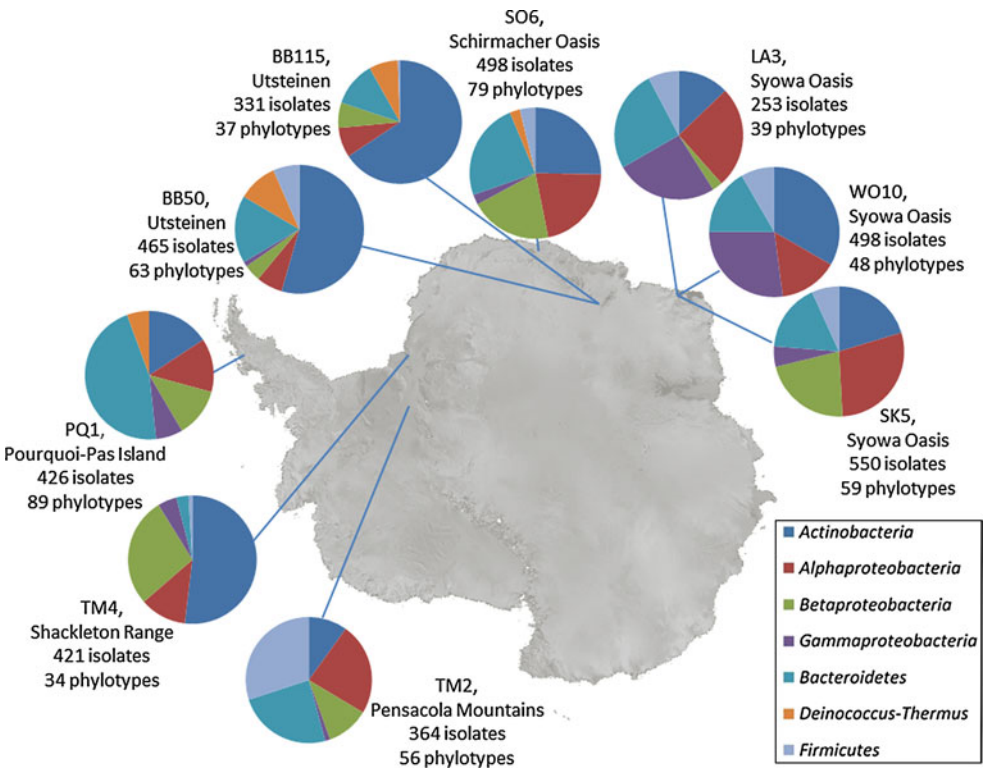

Fig. 1 Division of the phylotypes over the different phylogenetic groups. The number of obtained isolates and phylotypes are mentioned for the different samples. Information for samples BB50, BB115, TM2 and TM4 was based on Peeters et al. 2011a, b

analysed (Table 1). All samples were kept frozen continuously after collection (in January 2003 [PQ1] and January 2007 [LA3, SK5, WO10 and SO6]) until processing in the laboratory. Specific conductivity and pH were measured in the field using a YSI 600 m. Details regarding the analysis of the concentration of the major ions and nutrients have been described by Hodgson et al. (2010) and Verleyen et al. (2011).

Data for the new samples were also compared with information on four further samples previously studied using the same methods, including two terrestrial mat samples from Utsteinen (Sør Rondane Mountains, East Antarctica) (Peeters et al. 2011a) and two microbial mat samples from lakes in the Pensacola Mountains and the Shackleton Range (Peeters et al. 2011b).

Enumeration and isolation of heterotrophic bacteria

One gram of sample was aseptically weighed and homogenized in 9 ml sterile cold (4°C) physiological saline (0.86% NaCl) using a vortex. Tenfold dilution series (kept at 4°C) were plated on four different media (Marine agar 2216 (MA) (BD Difco™), R2A (BD Difco™), ten times diluted R2A (R2A/10), and PYGV (peptone-yeast-glucose-vitamin) medium (DSMZ medium 621)) and incubated at 20, 15 and 4°C. R2A (Difco) contains pyruvate, starch and dextrose as C sources and yeast extract, peptone and casaminoacids as N and C sources, and PYGV (DSMZ medium 621) contains peptone, yeast extract and glucose as C and/or N sources and additional vitamins and minerals. Both are considered oligotrophic media because the amounts of these components are at least two to ten times lower than in more general media such as nutrient broth. In addition to regular physiological saline (PS) dilution series, sea water (SW) dilutions were used for the LA3 and WO10 samples, which originated from lakes close to the ocean and had elevated conductivity values.

All plates were incubated for several weeks during which the number of colony forming units (CFUs) was counted. When the number of CFUs had stabilized, the total number of CFU/g for each combination of culture conditions was calculated for the plates showing between 20 and 400 colonies. At the end of the incubation period, three colonies (or less in case of insufficient growth) of each morphological type (colony parameters used include colour, margin, elevation, shape, diameter, surface appearance) were isolated and purified. Pure cultures were cryopreserved at −80°C using broth medium plus 15% glycerol or the MicroBank™ system (Pro-Lab Diagnostics, Ontario, Canada).

Genotypic fingerprinting

To reduce the large number of isolates, duplicates were eliminated using a whole-genome fingerprinting technique, repetitive element palindromic (rep)-PCR, resulting in a smaller number of clusters and unique isolates. DNA preparation was carried out as described by Baele et al. (2003). Rep-PCR fingerprinting using the GTG_5 primer (5′-GTG GTG GTG GTG GTG-3′) was performed according to Gevers et al. (2001). Resulting fingerprints were processed using the BioNumerics (v 5.1.) software (Applied-Maths). Rep-PCR profiles were compared by calculating pairwise Pearson's correlation coefficients (r). A cluster analysis was performed on the resulting matrix using the Unweighted Pair Group Method using Arithmetic averages (UPGMA). An 80% Pearson's correlation coefficient threshold was used (Gevers et al. 2001) in combination with visual inspection of bands to delineate rep-clusters. Rep-types included both rep-clusters and isolates grouping separately.

16S rRNA gene sequencing

The 16S rRNA genes of the representatives of all the different rep-types were amplified and partially sequenced as

Table 1 Overview of samples with their location, coordinates and description

Sample number	Place	Region	Latitude	Longitude	Sample description
PQ1	Narrows lake	Pourquoi-Pas Island, Antarctic Peninsula	67°42′S	67°27′W	Littoral cyanobacterial mat with green algae and diatoms
LA3	Langhovde lake 3	Syowa Oasis	69°13′S	39°48′E	Littoral brown crusts of cyanobacteria or diatoms from a small salt lake, sampling depth 0.2 m
SK5	Naka Tempyo	Syowa Oasis	69°28′S	39°40′E	Littoral epipsammic and interstitial microbial mat, brown or orange pigmented on top with a green surface layer, sampling depth 0.1 m
WO10	West Ongul Island, lake 10	Syowa Oasis	69°01′S	39°32′E	Littoral orange mat below a black decomposed mat. Shallow pool with evidence of higher lake level, sampling depth 0.15 m
SO6	Schirmacher Oasis, lake	Schirmacher Oasis	70°45′S	11°40′E	Littoral microbial mat sample from freshwater lake, sampling depth 0.1 m

previously described (Vancanneyt et al. 2004). PCR products were purified using a Nucleofast 96 PCR clean up membrane system (Machery-Nagel, Germany) and Tecan Workstation 200. The BKL1 primer was used for sequencing (Coenye et al. 1999). The fragments obtained (approximately 400 bp of the first and most variable part of the gene) were cleaned with the BigDye® xTerminator™ Purification Kit according to the protocol of the supplier (Applied Biosystems). Sequence analysis was performed using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, USA). Phylogenetic analysis was performed using the BioNumerics (v 5.1.) software package (Applied-Maths). The sequences were compared, and pairwise similarity values were calculated to delineate phylotypes at 99.0% 16S rRNA gene sequence similarity (Stach et al. 2003; Acinas et al. 2004). The classifier of the Ribosomal Database Project, containing the sequences of all described species, was used to obtain a genus identification for the phylotypes (Wang et al. 2007). Identifications with confidence estimates lower than 80% (Wang et al. 2007) were verified by phylogenetic analysis with all neighbouring taxa. A multiple alignment of the sequences was made, and after visual inspection, distances were calculated using the Kimura-2 correction. A neighbour joining dendrogram (Saitou and Nei 1987) was constructed, and bootstrap analysis was undertaken using 500 bootstrap replicates. When the analysis showed that a phylotype was not part of an existing genus and was either equally related to multiple genera or had 16S rRNA gene sequence similarities with neighbouring genera below the threshold value of 96.4% (Yarza et al. 2010), the phylotype was classified as a potentially new genus.

The 16S rRNA gene sequences determined in this study have been deposited in the EMBL database under accession numbers FR772052–FR772080 and FR772100–FR772289.

Sample coverage

Rarefaction curves were used to estimate how well our method covers the fraction of bacteria viable in the growth conditions used. They were calculated with an online rarefaction calculator (http://biome.sdsu.edu/fastgroup/cal_tools.htm). The Shannon biodiversity index was calculated as described by Magurran (1988).

Multivariate analysis

Direct and indirect ordinations were performed using CANOCO 4.5 for Windows (ter Braak and Smilauer 2002). A principal component analysis (PCA) was applied of the number of rep-types assigned to the different genera for each sample. Redundancy analysis (RDA) was applied to assess whether differences in bacterial community structure are underlain by differences in habitat type. Therefore, we created three dummy variables (Table S2). The forward selection procedure and unrestricted Monte Carlo permutations tests (499 permutations, $P = 0.05$) were used to select the minimal number of variables explaining the variation in the distribution of the different rep-types over the genera for the different samples. The importance of limnological variability was assessed for the lacustrine samples only, because no chemical data were available for the terrestrial samples.

Geographical distribution of the phylotypes

The 16S rRNA gene sequence of each phylotype was compared with sequences available in public databases (EMBL and NCBI) including cultured strains as well as environmental sequences (both from metagenomics and high-throughput sequencing). Based on the origin of sequences showing $\geq$99.0% sequence similarity, the phylotypes were classified as Antarctic (when no high scoring sequences, or only high scoring sequences originating from other Antarctic environments, were found), bipolar (only high scoring sequences from polar environments), cold (only high scoring sequences from cold environments) or cosmopolitan (at least one high scoring sequence from non-Antarctic/cold/polar environment) (Table 4). Phylotypes that showed no significant similarity with any other sequences were classified as Antarctic.

Results

Isolation, rep-PCR fingerprinting and 16S rRNA gene sequencing

Dilution series of the different samples (Table 1) were plated on four different media and incubated at three relatively cold temperatures compared to those used for more temperate bacteria. After 3-week incubation for plates at 20 and 15°C and 8 weeks for 4°C, the number of colony forming units (CFUs) was counted for the different conditions. When comparing the number of CFU/g for the five samples, there were clear differences (Table 2). Sample WO10 had the highest CFU/g of all samples. The highest value for samples PQ1 and SK5 was low in comparison with the other samples although a large diversity in colony morphologies was observed and consequently many isolates were taken (Fig. 1). For samples PQ1, SK5 and SO6, the highest number of CFU/g was found at 15 or 20°C, while for samples LA3 and WO10, 4°C gave best growth. The samples originating from saline and brackish lakes and ponds (LA3 and WO10) yielded the highest number of CFU/g on marine medium, whereas the other samples yielded the highest number of CFU/g on an oligotrophic medium.

Table 2 Plate counts (10^5 CFU/g) for the different growth conditions per sample

Medium	Temperature (°C)	PQ1	LA3	SK5	WO10	SO6
MA PS	4	0.00026	**21.6**	0.0008	**368.4211**	0.282759
	15	0.000341	17.78333	0.0021	177.7632	0.398276
	20	0.000345	16.13333	0.003	244.7368	0.614828
MA SW	4	nd	9.1	nd	52.28571	nd
	15	nd	11	nd	55.71429	nd
	20	nd	14.1	nd	48	nd
R2A	4	0.003245	0.000167	0.187	41.31579	8.241379
	15	0.0128	0.0003	0.86	57.63158	**79.2069**
	20	**0.02195**	0.000133	1.89	114.2105	19.91379
R2A/10	4	0.0022	0	0.16	9.013158	7.862069
	15	0.0148	0.00007	0.507	63.42105	26.44828
	20	0.0309	17.66667	0.9	30	24.34483
PYGV	4	0.00127	0.00007	0.2085	15.52632	7.034483
	15	0.0132	0.0007	1.38	34.73684	25.7069
	20	0.022	0.0001	**2.1**	37.89474	26.82759

The maximum plate count for each sample is shown in *bold* and *underlined*; *nd* not determined

Between 253 and 550 isolates (Fig. 1) were purified from the five new samples. This gave a total of 2,225 isolates that were grouped in 810 rep-types. To compare the diversity obtained under each culture condition, the relative diversity yield was calculated as the number of rep-types recovered from a sample for each medium and temperature combination, divided by the total number of rep-types obtained for that sample. The highest values are summarized in Table 3. For all samples, the highest values for the colony counts (Table 2) and the highest diversity (Table 3) were found on either oligotrophic media (R2A, R2A/10 and PYGV) or marine media (MA PS and MA SW). The highest CFU/g and diversities for each sample were in the same temperature categories (high-temperature category: 15–20°C; low-temperature category: 4°C) for samples PQ1, SK5 and SO6; however, for samples LA3 and WO10, the highest CFU/g was at 4°C, while the highest diversity was recovered at 20°C.

Representatives of the different rep-types were subjected to 16S rRNA gene sequence analysis. Based on these sequences, phylotypes were delineated at 99% sequence similarity. The number of phylotypes recovered per sample ranged from 39 (LA3) to 89 (PQ1) (Fig. 1). Interestingly, only an intermediate number of isolates was taken in this latter sample in comparison with the other samples, suggesting that it harbours a relatively large diversity. This was confirmed by the higher Shannon diversity index based on the number of isolates per rep-type: 5.17 for PQ1, compared to 4.24, 4.62, 4.54 and 4.82 for samples LA3, SK5, WO10 and SO6, respectively. Rarefaction curves (Fig. S1) were calculated to assess the coverage of the culturable diversity under these culture conditions. The curves for most samples approached a plateau. However, for sample PQ1, the rarefaction curve continued to rise despite a high number of isolates being recovered from this sample.

Distribution of the phylotypes over different phyla, classes, genera and samples

The different phylotypes were identified using the classifier tool of the Ribosomal Database Project and phylogenetic analysis of the 16S rRNA gene sequences. The diversity found in the different samples was considered at different taxonomic levels. At phylum level, for most samples, the phylotypes were affiliated with four major phylogenetic groups, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Firmicutes*. In addition, isolates of the *Deinococcus-Thermus* phylum were recovered from samples PQ1 and SO6 (Fig. 1). At genus level, variation between the five samples was larger: 70 genera were recovered as well as 18 potentially novel genera (Table S1). Only *Salinibacterium* and *Flavobacterium* were found in all five samples.

Previously, we studied two terrestrial samples, BB50 and BB115, from the Utsteinen region (Peeters et al.

Table 3 Highest relative values for the number of rep-types and corresponding conditions

Samples	PQ1	LA3	SK5	WO10	SO6
Highest relative diversity yield	0.167	0.271	0.274	0.258	0.294
Medium	R2A	MA PS	PYGV	MA PS	PYGV
Temperature (°C)	15	20	15	20	20

2011a), and two aquatic microbial mat samples, TM2 and TM4, from the Pensacola Mountains and the Shackleton Range, respectively (Peeters et al. 2011b), using the same isolation conditions and the same characterization methods. Later, we compare our new findings with those from these four samples. To facilitate comparison and to provide an overview, bacterial genus diversity data from these two studies are also included in Table S1. No genera were recovered from all nine samples. The genera *Arthrobacter*, *Brevundimonas* and *Hymenobacter* were found in eight samples, whereas *Cryobacterium*, *Rhodococcus*, *Sphingomonas*, *Flavobacterium* and *Bacillus* were found in seven of the nine samples. Some 38% (31/82) of the genera were recovered from only one sample (e.g. *Frigoribacterium*, *Saxeibacter*, *Aurantimonas*, *Caulobacter*, *Lysobacter*, *Maribacter*, *Brevibacillus*).

The genus *Arthrobacter* (Table S1) was best represented among the isolates (733 isolates, representing 20 different phylotypes), although the largest number of different phylotypes (50) was found in the genus *Hymenobacter*, which also had a rather high number of isolates (230). Other well-represented genera based on either the number of isolates or the number of phylotypes included *Brevundimonas*, *Flavobacterium*, *Polaromonas*, *Psychrobacter*, *Massilia, Sphingopyxis*, *Sphingomonas* and *Deinococcus*.

At the phylotype level, none of the phylotypes was found in all nine locations (Table S1). Only one phylotype (R-36741), identified as *Brevundimonas,* was found in eight samples. Phylotype R-36538, identified as *Arthrobacter,* was isolated from six samples. Furthermore, phylotypes belonging to the genera *Brevundimonas*, *Rhodococcus*, *Salinibacterium*, *Sphingomonas* and *Massilia* were found in five samples, and phylotypes belonging to the genera *Arthrobacter*, *Cryobacterium*, *Rothia*, *Polaromonas*, *Bacillus*, *Paenibacillus* and a potentially new genus in the class *Betaproteobacteria* were found in four samples. Additionally, 15 (4.2%) of the 356 phylotypes were recovered from three samples, 68 (19.1%) were found in two samples, and 260 (73.0%) were restricted to a single sample. Table 4 shows the distribution of shared phylotypes over the different samples. Sample SK5 shared the highest percentage of phylotypes with other samples, especially with samples PQ1, LA3 and SO6. Also, samples TM2 and WO10 and TM4 and SO6 shared an important percentage (≥10%) of phylotypes.

In all nine samples, only 3.4% (47) of the rep-types contained isolates from more than one sample. The majority of these mixed rep-types contained isolates from two different samples, and only two comprised isolates from three different samples. All samples contained isolates that were part of these mixed rep-types, whereas the highest number was shared between samples SK5 and SO6. A large portion of the mixed rep-types was affiliated with *Actinobacteria,* while the remainder was related to all other classes and phyla obtained except for the *Deinococcus-Thermus* phylum. The mixed rep-types belonged to diverse genera, with several from the genera *Arthrobacter*, *Brevundimonas*, *Hymenobacter*, *Pedobacter* and *Rothia*.

Bacterial community structure in relation to environmental conditions

Also here, we included information from our previous studies (Peeters et al. 2011a, b) to enhance the comparison. The principal component analysis at genus level (Fig. 2) confirmed the differences observed between the nine samples. The two terrestrial samples from Utsteinen (BB50 and BB115) are located relatively close to each other in the top half of the scatter plot. The two samples from the saline lakes (LA3 and WO10) and the brackish lake (TM2) are situated on the negative side of the first ordination axis. A redundancy analysis revealed that the dummy variable denoting the difference in habitat type and grouping terrestrial and freshwater habitats significantly explained 27.3% of the differences in community composition between terrestrial and aquatic samples. This indicates that the samples from saline lakes are different to those from freshwater systems and terrestrial environments. In the subset of the samples from aquatic habitats for which limnological data are available, RDA confirmed that

Table 4 Number of phylotypes, defined at 99% sequence similarity (lower left triangle), and percentage of phylotypes (upper right triangle) shared between the samples

Sample	PQ1	LA3	SK5	WO10	SO6	BB50[a]	BB115[a]	TM2[b]	TM4[b]
PQ1	x	5%	11%	4%	9%	5%	2%	2%	4%
LA3	7	x	11%	7%	4%	1%	1%	3%	7%
SK5	16	11	x	7%	14%	7%	4%	5%	8%
WO10	5	6	7	x	8%	0%	2%	10%	5%
SO6	15	5	20	10	x	5%	6%	4%	10%
BB50	7	1	8	0	7	x	7%	3%	4%
BB115	3	1	4	2	7	7	x	4%	7%
TM2	3	3	6	10	6	4	4	x	9%
TM4	5	5	7	4	11	4	5	8	x

[a] Data from Peeters et al. 2011a

[b] Data from Peeters et al. 2011b

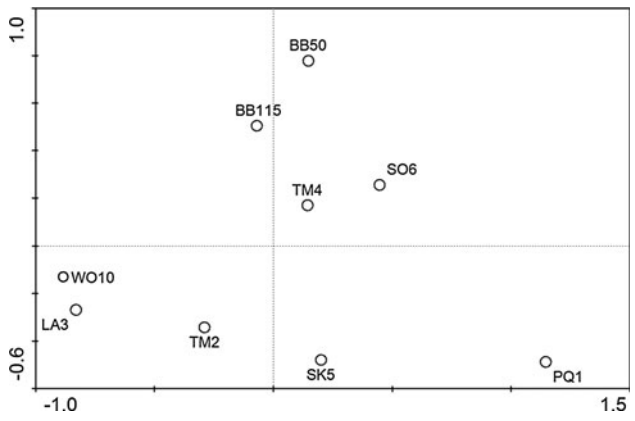

Fig. 2 Principal component analysis (PCA) of the samples showing the differences in bacterial diversity (at genus level) based on the number of rep-types. Information for samples BB50, BB115, TM2 and TM4 was based on Peeters et al. 2011a, b

conductivity significantly explained 34.4% of the variation in community structure at genus level.

Geographical distribution of the phylotypes

The sequences of the different phylotypes were compared with public databases to assess their geographical distribution. For the five new samples, a large number of the phylotypes (36.0–64.6%) showed a cosmopolitan distribution as was also found in the four previously studied samples (Table 5). All nine samples also contained a large number of phylotypes currently known only from Antarctica (20.6–58.4%), and many of these shared no significant similarity (≥99.0%) with any other sequence in public databases. In general, only small numbers of phylotypes have been classified as cold (≤10.4%) or bipolar (≤8.3%). It is clear that for most phyla/classes, the phylotypes were mainly cosmopolitan (Table 5). Notable exceptions were the phyla *Bacteroidetes* and *Deinococcus-Thermus,* of which the majority of phylotypes were currently known only from Antarctica, many of them without significant sequence similarity with any other sequence.

Discussion

We studied the cultured diversity of the heterotrophic bacteria recovered under standardized conditions from five aquatic microbial mat samples from different locations in Maritime and Continental Antarctica and compared the results with previously published data from terrestrial and aquatic microbial mats from two additional regions. Although only a limited number of isolates was studied from each sample, and the culturable diversity represents only a fraction of the total diversity present (Amann et al. 1995), some clear differences between the samples were apparent. The most diverse sample was PQ1, with the highest Shannon diversity index and the largest number of phylotypes recovered, despite only an intermediate number of isolates obtained in comparison with the other samples (Fig. 1). This relatively high diversity may be explained by the location of the sampling site on the Antarctic Peninsula where environmental conditions are less extreme than on the Antarctic continent.

The distribution of the different phyla, classes and genera varied considerably. In most samples, the phylotypes belonged to four major phylogenetic groups (*Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Firmicutes*) that have been reported frequently from various Antarctic habitats including aquatic microbial mats, soil from continental Antarctica and the sub-Antarctic islands and from sediments (Bowman et al. 2000a; Brambilla et al. 2001; Van Trappen et al. 2002; Bowman and McCuaig 2003; Aislabie et al. 2006b, 2008; Babalola et al. 2009; Cary et al. 2010; Chong et al. 2010; Selbmann et al. 2010). The phylum *Deinococcus-Thermus* was only recovered from four samples (BB50, BB115, PQ1 and SO6), including both terrestrial and aquatic samples. The genus *Deinococcus* has been found previously in Antarctic soils and especially in the McMurdo Dry Valleys (Aislabie et al. 2006a, 2008; Niederberger et al. 2008; Cary et al. 2010) although several other studies focussing on Antarctic soils (Shivaji et al. 2004; Gesheva 2009) as well as on marine environments (Bowman et al. 2000b, 2003) and microbial mats in Antarctic lakes (Brambilla et al. 2001; Van Trappen et al. 2002) did not report the presence of this taxon. Most of the frequently occurring genera (genera that were found in more than four samples or from which more than 100 isolates were recovered) have been reported previously from Antarctica (Irgens et al. 1996; Van Trappen et al. 2002; Busse et al. 2003; Shivaji et al. 2004; Ah Tow and Cowan 2005; Selbmann et al. 2010).

Besides genera found in multiple samples, also some phylotypes were found in more than one sample. The observation that sample PQ1, the only sample originating from the Antarctic Peninsula, shared comparable percentages of phylotypes with all samples (Table 4), irrespective of geographical distance is interesting. Moreover, these percentages are in the same range as those shared between the other samples. For some higher organisms such as Acari and Nematoda, a strong boundary has been observed between the species present in the Antarctic Peninsula and continental Antarctica, although for Tardigrada and Bryophyta, no continental/maritime divide has been found (Convey et al. 2008). Our results suggest that this boundary probably does not exist for bacterial taxa.

The above-mentioned differences between the samples are related to lake water conductivity and the type of

Table 5 Number of phylotypes recovered with cosmopolitan, cold, bipolar or Antarctic distribution for the different classes and phyla and the different samples

Distribution type	PQ1	LA3	SK5	WO10	SO6	BB50[a]	BB115[a]	TM2[b]	TM4[b]
Actinobacteria									
Cosmopolitan	8/14	4/5	7/12	10/16	13/20	12/20	10/13	4/5	12/13
Cold	4/14	1/5	2/12	4/16	2/20	0/20	1/13	0/5	0/13
Bipolar	0/14	0/5	0/12	0/16	0/20	0/20	0/13	1/5	0/13
Antarctic[c]	2/14 (1)	0/5 (0)	3/12 (3)	2/16 (2)	5/20 (5)	8/20 (7)	2/13 (2)	0/5 (0)	1/13 (1)
Alphaproteobacteria									
Cosmopolitan	10/12	8/10	15/17	6/7	15/17	5/7	5/5	8/13	6/7
Cold	0/12	0/10	0/17	0/7	0/17	0/7	0/5	1/13	0/7
Bipolar	0/12	0/10	0/17	0/7	0/17	0/7	0/5	0/13	0/7
Antarctic[c]	2/12 (1)	2/10 (2)	2/17 (2)	1/7 (0)	2/17 (2)	2/7 (2)	0/5 (0)	4/13 (3)	1/7 (1)
Betaproteobacteria									
Cosmopolitan	8/11	1/1	10/13	0/0	14/16	5/6	2/2	5/6	4/5
Cold	0/11	0/1	1/13	0/0	1/16	0/6	0/2	0/6	0/5
Bipolar	0/11	0/1	1/13	0/0	0/16	0/6	0/2	1/6	0/5
Antarctic[c]	3/11 (1)	0/1 (0)	1/13 (1)	0/0 (0)	1/16 (0)	1/6 (1)	0/2 (0)	0/6 (0)	1/5 (1)
Gammaproteobacteria									
Cosmopolitan	4/6	2/10	1/3	7/13	2/2	0/1	0/0	2/3	1/2
Cold	0/6	0/10	0/3	1/13	0/2	0/1	0/0	0/3	0/2
Bipolar	0/6	1/10	0/3	3/13	0/2	0/1	0/0	0/3	0/2
Antarctic[c]	2/6 (1)	7/10 (3)	2/3 (0)	2/13 (0)	0/2 (0)	1/1 (1)	0/0 (0)	1/3 (0)	1/2 (0)
Bacteroidetes									
Cosmopolitan	1/41	1/10	1/10	2/8	4/19	4/15	0/12	4/11	1/4
Cold	1/41	0/10	0/10	0/8	0/19	1/15	0/12	0/11	0/4
Bipolar	0/41	0/10	0/10	1/8	1/19	2/15	2/12	0/11	0/4
Antarctic[c]	39/41 (31)	9/10 (5)	9/10 (8)	5/8 (0)	14/19 (14)	8/15 (7)	10/12 (10)	7/11 (6)	3/4 (3)
Firmicutes									
Cosmopolitan	0/0	3/3	4/4	3/4	3/3	6/6	1/1	15/18	3/3
Cold	0/0	0/3	0/4	0/4	0/3	0/6	0/1	0/18	0/3
Bipolar	0/0	0/3	0/4	0/4	0/3	0/6	0/1	1/18	0/3
Antarctic[c]	0/0 (0)	0/3 (0)	0/4 (0)	1/4 (0)	0/3 (0)	0/6 (0)	0/1 (0)	2/18 (1)	0/3 (0)
Deinococcus-Thermus									
Cosmopolitan	1/5	0/0	0/0	0/0	0/2	1/8	0/4	0/0	0/0
Cold	0/5	0/0	0/0	0/0	0/2	0/8	0/4	0/0	0/0
Bipolar	0/5	0/0	0/0	0/0	0/2	0/8	0/4	0/0	0/0
Antarctic[c]	4/5 (2)	0/0 (0)	0/0 (0)	0/0 (0)	2/2 (2)	7/8 (5)	4/4 (3)	0/0 (0)	0/0 (0)
All isolates									
% cosmopolitan	36.0	48.7	64.4	58.3	64.6	52.4	48.6	67.9	79.4
% cold	5.6	2.6	5.1	10.4	3.8	1.6	2.7	1.8	0.0
% bipolar	0.0	2.6	1.7	8.3	1.3	3.2	5.4	5.4	0.0
% Antarctic[c]	58.4 (41.6)	46.2 (25.6)	28.8 (23.7)	22.9 (4.2)	30.4 (29.1)	42.9 (36.5)	43.2 (40.5)	25.0 (17.9)	20.6 (17.6)

Distribution types were assigned to phylotypes by evaluating the geographical origin of highly similar sequences (≥99.0%) present in public databases and originating from cultured strains as well as environmental samples and clone libraries

[a] Data from Peeters et al. 2011a

[b] Data from Peeters et al. 2011b

[c] In brackets, the number/percentage of phylotypes that shared no significant similarity with any other sequence in the public database

habitat (terrestrial vs. aquatic) as revealed by direct ordination analyses. The importance of conductivity was also evident from the fact that the medium used affected the colony yield and the diversity recovered for each sample. For example, the highest yield was obtained using the marine medium for the samples derived from saline and brackish lakes. A number of genera were only obtained from the saline lakes (e.g. *Loktanella*, *Halomonas*, *Gelidilacus* and *Algoriphagus*), whereas only small numbers of the less salt tolerant class *Betaproteobacteria* (Philippot et al. 2010) were isolated in these samples. Only the genera *Aeromicrobium* and *Micrococcus* were isolated both from terrestrial and saline samples. Interestingly, conductivity appears to be more important than the type of habitat, as revealed by the ordination analysis. Although our results may be influenced by the limited number of isolates and samples studied, this observation corroborates previous studies (Philippot et al. 2010; Tamames et al. 2010), reporting that the diversity obtained from freshwater samples is more comparable with that of terrestrial samples than with saline ones. The importance of conductivity and related variables rather than extremes of temperatures, pH, or other physical and chemical factors (Tamames et al. 2010) corroborates findings in other microbial organisms in Antarctic lakes, including diatoms and cyanobacteria (Verleyen et al. 2010).

In the nine samples, a significant number of phylotypes were found to represent potentially novel genera. From the terrestrial samples (BB50 and BB115), the saline samples (TM2, LA3 and WO10) and the freshwater samples (TM4, PQ1, SK5 and SO6), respectively, 4, 12 and 22 phylotypes represented potentially new genera. The majority of potentially new genera were found in the classes *Alphaproteobacteria* and *Betaproteobacteria* (35% each) and in samples SO6 (19%), SK5 (16%) and LA3 (16%). Further polyphasic studies are necessary to confirm their status and classification. The isolated taxa can be investigated for antimicrobial activities or other products of biotechnological significance (examples reviewed in Margesin and Feller 2010). Moreover, several phylotypes obtained here belonged to genera which at present contain only one species or even one strain (e.g. *Rhodoglobus*, *Saxeibacter*, *Enhydrobacter* and the recently described *Marisedimicola*). The additional cultures obtained in this work may give more insight into the diversity present in these genera.

A comparison of our sequences to those available in public databases (including sequences from cultured strains as well as environmental community samples and clone libraries) revealed that the majority of the taxa showed a cosmopolitan distribution (Table 5). Although the geographical distribution reflects current and therefore limited knowledge of bacterial diversity and ecology (Curtis and Sloan 2004), some interesting observations can be made. For the BB samples, an important number of phylotypes are currently restricted to Antarctica. This may be explained partly by the terrestrial, more exposed nature of these samples from the pristine environment of the new Princes Elisabeth Station in Utsteinen. These samples were also taken inland, whereas most previous microbial studies on terrestrial samples in Antarctica have focussed on regions closer to the coast and generally in close vicinity to research stations (Shivaji et al. 2004; Aislabie et al. 2006b; Chong et al. 2009). The other samples in our comparison originated from locations closer to the ocean and may have experienced inflow of non-Antarctic species, which may have contributed to the lower percentage of phylotypes with an Antarctic distribution. In addition, some strains may have been isolated previously in one of the few earlier studies in the regions of the Schirmacher and Syowa Oasis (Satoh et al. 1989; Shivaji et al. 2004). An important percentage of phylotypes currently restricted to Antarctica was also recovered from sample PQ1, although this sample was taken on the Antarctic Peninsula, closer to the ocean and to civilization.

Comparing the geographical distribution of the phylotypes in more detail, it is clear that the majority of those belonging to the *Actinobacteria*, *Proteobacteria* and *Firmicutes* have a more general distribution, whereas most *Bacteroidetes* and *Deinococcus-Thermus* phylotypes are currently restricted to the Antarctic continent. This high number of Antarctic phylotypes within the *Bacteroidetes,* with several potentially new taxa, is in agreement with the increasing number of new species described from Antarctica within this phylum (Shivaji et al. 1992; Bowman et al. 1997, 1998; Hirsch et al. 1998; McCammon et al. 1998; Bowman and Nichols 2002; Van Trappen et al. 2003, 2004b, c; Yi et al. 2005; Yi and Chun, 2006). Our observations therefore appear to indicate that both cosmopolitan and specific Antarctic phylotypes, possibly with a limited dispersal capacity, are present.

Conclusion

Although only a limited number of microbial mat samples were studied, these revealed a large diversity of culturable heterotrophic bacteria. There were important differences between the taxa obtained from each of the samples, and only limited overlap was observed between the diversity obtained. Phylotypes belonged to five major phylogenetic groups (*Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, *Firmicutes* and *Deinococcus-Thermus*) and several represented potentially new taxa. The bacterial diversity was found to relate to conductivity and habitat type. A comparison of our data with sequences in public databases showed that an important proportion of phylotypes (36.9%) are currently known only from the Antarctic continent,

although a large proportion of cosmopolitan taxa (56.3%) were also recovered. This suggests that, in Antarctica, cosmopolitan taxa as well as taxa with limited dispersal, which potentially evolved in isolation, occur.

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Culturable Diversity of Heterotrophic Bacteria in Forlidas Pond (Pensacola Mountains) and Lundström Lake (Shackleton Range), Antarctica

Karolien Peeters · Dominic A. Hodgson · Peter Convey · Anne Willems

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Abstract Cultivation techniques were used to study the heterotrophic bacterial diversity in two microbial mat samples originating from the littoral zone of two continental Antarctic lakes (Forlidas Pond and Lundström Lake) in the Dufek Massif (within the Pensacola Mountains group of the Transantarctic Mountains) and Shackleton Range, respectively. Nearly 800 isolates were picked after incubation on several growth media at different temperatures. They were grouped using a whole-genome fingerprinting technique, repetitive element palindromic PCR and partial 16S rRNA gene sequencing. Phylogenetic analysis of the complete 16S rRNA gene sequences of 82 representatives showed that the isolates belonged to four major phylogenetic groups: *Actinobacteria*, *Bacteroidetes*, *Proteobacteria* and *Firmicutes*. A relatively large difference between the samples was apparent. Forlidas Pond is a completely frozen water body underlain by hypersaline brine, with summer thaw forming a slightly saline littoral moat. This was reflected in the bacterial diversity with a dominance of isolates belonging to *Firmicutes*, whereas isolates from the freshwater Lundström Lake revealed a dominance of *Actinobacteria*. A total of 42 different genera were recovered, including first records from Antarctica for *Albidiferax*, *Bosea*, *Curvibacter*, *Luteimonas*, *Ornithinibacillus*, *Pseudoxanthomonas*, *Sphingopyxis* and *Spirosoma*. Additionally, a considerable number of potential new species and new genera were recovered distributed over different phylogenetic groups. For several species where previously only the type strain was available in cultivation, we report additional strains. Comparison with public databases showed that overall, 72% of the phylotypes are cosmopolitan whereas 23% are currently only known from Antarctica. However, for the *Bacteroidetes*, the majority of the phylotypes recovered are at present known only from Antarctica and many of these represent previously unknown species.

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Introduction

Microbial mats in Antarctic lakes harbour complex microbial communities adapted to extreme environmental conditions including low temperatures, UV irradiation, freeze–thaw cycles, dehydration, osmotic stress and low nutrient concentrations. Although a number of studies have focussed on the cyanobacterial diversity of Antarctic microbial mats [28, 29, 58, 59], only limited attention to date has been devoted to their heterotrophic bacterial diversity [12, 61], despite reports of a large diversity with several new taxa.

The Transantarctic Mountains (TM) geologically separate East and West Antarctica. As the longest range in Antarctica, they stretch 3,500 km across the continent, between the Ross Sea and the Weddell Sea [7, 57], and include a number of separately named mountain groups that are often again subdivided into smaller ranges. The summits and dry valleys of the TM and the nearby

Shackleton Range are some of the few places not covered by ice in Antarctica, due to extremely limited precipitation coupled with ablation [60]. In this study, a lake in the Transantarctic Mountains was studied, Forlidas Pond (Dufek massif, Pensacola Mountains) together with Lundström Lake (Shackleton Range), just beyond the northern limit of the Transantarctic mountains.

Forlidas Pond in the Forlidas Valley (unofficial name) is located in the Dufek Massif (Fig. 1), a range of peaks in the Pensacola Mountains that are situated between the Support Force Glacier and the Foundation Ice Stream [25]. As evaporation and sublimation have dominated over precipitation in this area, Forlidas Pond has evaporated down to a small remnant of a once much larger lake [60]. About 400 km away, Lundström Lake is situated in the Shackleton Range (Fig. 1) which is located at the north-western edge of the stable East Antarctic Craton [63]. The physical environments of Forlidas Pond and Lundström Lake are largely comparable. At the time of sampling, the lakes were both completely frozen to their base with a thin layer of slush underlying the ice at the lake bottom, which was highly saline in Forlidas Pond but only marginally saline in Lundström Lake [24, 25]. Visible biota was extremely limited at either site, and macroscopic vegetation appeared to be restricted to cyanobacterial mats and some small lichens. Cyanobacterial mats were found both as benthic mats in lakes and forming a clumped distribution on the ice-free ground [25]. Analyses of the cyanobacterial molecular diversity in and around Forlidas Pond and Lundström Lake showed that the richness was lower than in Antarctic coastal lakes (Fernández-Carazo, personal communication) and the cyanobacterial diversity was not limited to specific aquatic or terrestrial habitats. The invertebrate fauna within the area was equally impoverished. No arthropods were found, rotifers were rare and tardigrades were commonly found but belonged to only three different species [25].

The area is interesting for biological studies because of the lack of human visitation and impact, even by Antarctic standards. It was briefly visited during the International Geophysical Year (1957), by the US Geological Survey (1978–1979) and by a team from the British Antarctic Survey in 2003 [25]. Forlidas Pond is designated as an Antarctic Specially Protected Area (ASPA No. 119; http://cep.ats.aq/cep/apa/introduction/index.html). The present study is the first report on the culturable heterotrophic bacterial diversity in microbial mats originating from lakes in the Transantarctic Mountains and Shackleton Range. A large number of isolates was obtained and identified through genotypic characterization using repetitive element palindromic (rep)-PCR fingerprinting of the genome and phylogenetic analysis of 16S rRNA gene sequences. This

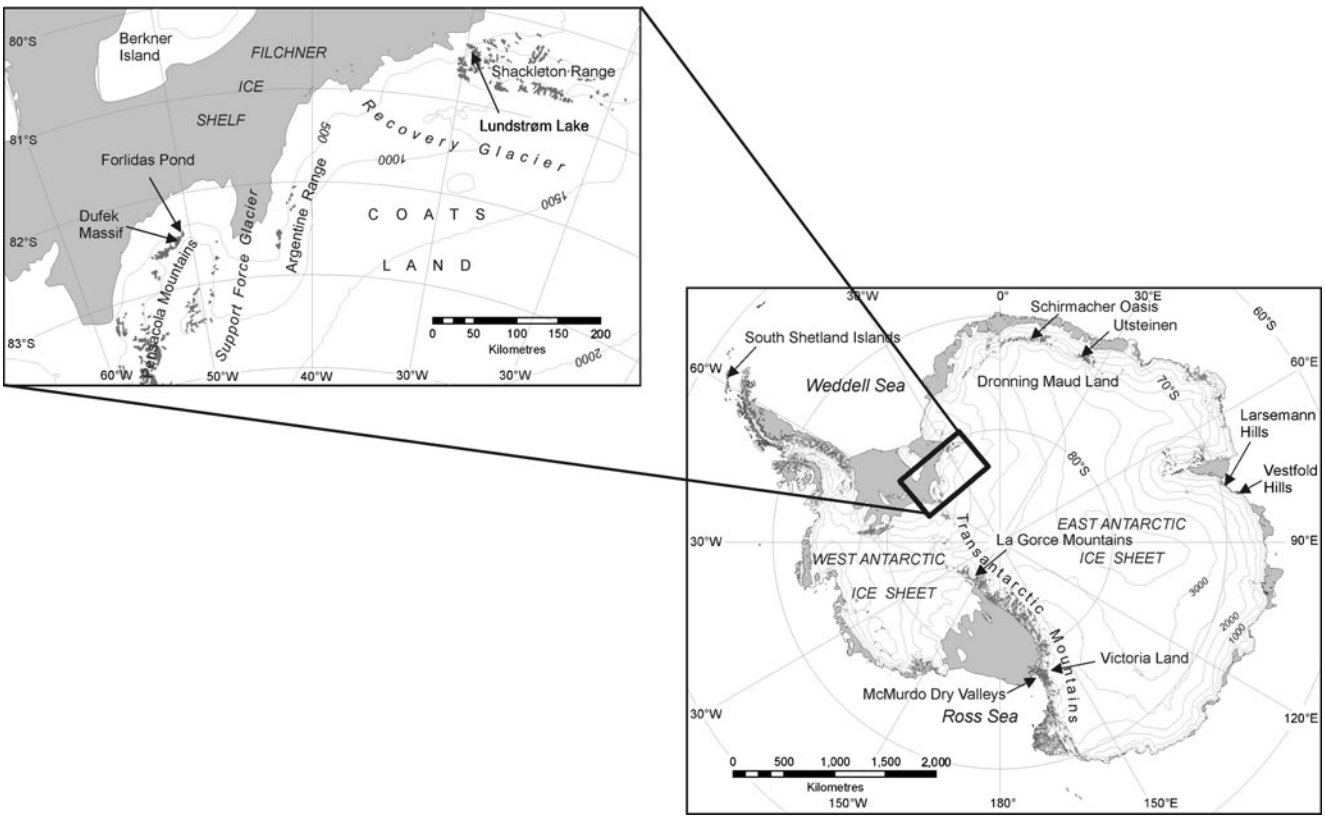

Figure 1 Locations of Forlidas Pond (Pensacola Mountains) and Lundström Lake (Shackleton Range)

study forms part of a more broad investigation of this region including also the exploration of limnology and biology [25], cyanobacterial (Fernández-Carazo, unpublished data) and eukaryotic diversity (Verleyen et al., unpublished data).

Methods

Source of Samples

Samples were collected aseptically during an expedition to the Transantarctic Mountains and Shackleton Range in December 2003. They were kept frozen in the field and during transport to Belgium via the British Antarctic Survey's Rothera Research Station. Sample TM2 was obtained from Forlidas Pond (51°16′W, 82°27′S) in the Pensacola Mountains (Fig. 1, Fig. S1) and described as a cyanobacterial mat that was actively growing in the littoral zone situated under 15 cm of clear ice and 15 cm of water. Air bubbles on the mat surface and trapped under ice were taken as evidence of recent photosynthetic activity [25]. Sample TM4 was taken from Lundström Lake (29°29′W, 80°27′S) in the Shackleton Range (Fig. 1, S1) and was described as a cyanobacterial mat from the littoral zone of the lake which at that time had an open freshwater moat [24].

Enumeration and Isolation of Heterotrophic Bacteria

One gramme of sample was aseptically weighed and homogenised in 9 ml sterile physiological water (0.86% NaCl) using a vortex. Tenfold dilution series were plated on four different media: marine agar 2216 (BD Difco™), R2A (BD Difco™), ten times diluted R2A (R2A/10), and pepton–yeast–glucose–vitamin (PYGV) medium (DSMZ medium 621). Incubation temperatures used were 20°C, 15°C, and 4°C. The plates were incubated for several weeks during which the number of colony-forming units (CFU) was counted. When the number of CFU's reached a plateau, the calculation of the total number of CFU/g for each condition was made for the plates showing between 20 and 400 colonies. At the end of the incubation period, three colonies of each morphological type (or less in case of rare types) were isolated and purified. Pure cultures were cryopreserved at −80°C using broth medium plus 15% glycerol or the MicroBank™ system (Pro-Lab Diagnostics, Ontario, Canada).

Genotypic Fingerprinting

To eliminate duplicate isolates, a whole-genome fingerprinting technique, rep-PCR was used, which permits reduction of the large number of isolates to a smaller number of clusters and unique isolates. DNA preparation was carried out as described by Baele et al. [6]. Rep-PCR fingerprinting using the GTG_5 primer (5′-GTG GTG GTG GTG GTG-3′) was performed according to Gevers et al. [20].

Resulting fingerprints were processed using BioNumerics (v 5.1.) software (Applied-Maths). Rep-PCR profiles were compared by calculating pairwise Pearson's correlation coefficients. A cluster analysis was performed on the resulting matrix using the unweighted pair group method using arithmetic averages. An 80% Pearson correlation coefficient threshold was used [20] in combination with visual inspection of bands, to delineate rep-clusters. Rep-type numbers were assigned to all rep-clusters as well as to isolates grouping separately.

16S rRNA Gene Sequencing and Analysis

The 16S rRNA gene of a representative of each rep-type was amplified and sequenced as previously described [62]. PCR products were purified using a Nucleofast 96 PCR clean up membrane system (Machery-Nagel, Germany) and Tecan Workstation 200. The sequencing primers used were as listed by Coenye et al. [14]. The fragments obtained were cleaned with the BigDye® xTerminator™ Purification Kit according to the protocol of the supplier (Applied Biosystems). Sequence analysis was performed using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, USA). Initially, approximately the first 400 bp of the 16S rRNA genes of representatives of all rep-types were determined. Pairwise similarity values were calculated to delineate phylotypes at 99.0% 16S rRNA gene sequence similarity. Although 97.0% 16S rRNA gene sequence similarity is generally accepted as the threshold to regard bacterial species as different [56], higher than that there is no minimum required similarity level that defines a bacterial species [27, 54] and current practice requires DNA-DNA hybridizations or multi-locus sequence analysis (MLSA) to establish species identity [19, 55]. More recently, Stackebrandt and Ebers [54] proposed that 98.7–99.0% could be regarded as a threshold range above which DNA–DNA hybridizations are required for species identification. Clearly in large-scale diversity (cultivation or metagenomic) studies, the requirement of hybridizations for species identification is not practical and MLSA data currently do not yet cover the breadth of all bacterial phyla. Furthermore, from a comparison of 16S rDNA homology with DNA–DNA reassociation values for members of the class *Actinobacteria*, Stach et al. [53] found that a 16S rDNA similarity level of 99.0% covered 70% of all DNA–DNA hybridization values of more than 70%. We therefore opted to use the level of 99.0% sequence similarity to define phylotypes and regarded the phylotypes as pragmatic

proxies for bacterial species, although the threshold of 99.0% in some cases may underestimate the actual number of species because of the limited resolving power of the 16S rRNA gene sequence.

For each phylotype, the 16S rRNA gene sequence of one representative was completed (±1,500 bp). Sequence assembly and phylogenetic analysis were performed using the BioNumerics (v 5.1.) software package (Applied-Maths). An approximate identification for the phylotypes was obtained by comparison with the European Molecular Biology Laboratory (EMBL) database using the FASTA algorithm. A phylogenetic analysis was performed using the sequences of type strains from all the species listed in the FASTA results completed with type strains of the related taxa, to obtain a more precise identification (Table 1). A multiple sequence alignment was made taking into account the homologous nucleotide positions after discarding unknown bases and gaps. After visual inspection, distances were calculated using the Kimura-2 correction. A neighbour-joining dendrogram [49] was constructed and bootstrapping analysis was undertaken by using 500 bootstrap replicates of the data. Phylotypes showing ≥99.0% 16S rRNA gene sequence similarity with a particular type strain were considered as belonging to this species. For the cases where phylotypes belong to complex clusters of species sharing more than 99.0% 16S rRNA gene similarity, these phylotypes were given only a generic identification. Phylotypes with <99.0% 16S rRNA gene sequence similarity with named species were also only identified at genus level (Table 1).

Geographic Distribution of the Phylotypes Recovered

To assess the geographic distribution of the phylotypes, the 16S rRNA gene sequences were compared to the prokaryote and the environmental divisions, containing sequences from various environmental samples, of the EMBL database. Additionally, sequences were compared to the nucleotide collection, the high throughput genome sequences and the environmental samples from GenBank using Blast. Based on the origin of the high scoring entries (≥99.0% sequence similarity was considered significant), we labelled our phylotypes as follows: Antarctic (no high scoring sequences from non-Antarctic origin), polar (only high scoring sequences from both polar regions), cold (only high scoring sequences from cold environments) or cosmopolitan (at least one high scoring sequence from non-Antarctic/cold/polar environment; Table 1, last column).

Sample Coverage

The Good's non-parametric coverage estimator was calculate according to the equation $C = 1 - (n1/N)$, where $n1$ is the number of rep-types/phylotypes for which only one isolate was recovered and N is the total number of isolates, as described by Good [22]. The Shannon biodiversity index was calculated as described by Magurran et al. [35].

Nucleotide Sequence Accession Numbers

The 16S rRNA gene sequences determined in this study have been deposited in the EMBL database (accession numbers are given in Table 1).

Results

Isolation and Grouping by Rep-PCR Fingerprinting

Generally, colonies became visible after 3–4 days at 15 and 20°C and 12 days at 4°C. Higher numbers of colonies were observed on the marine medium for all temperatures in comparison with the other media for sample TM2 (Supplementary data, Figs. S2 and S3). For sample TM4, the media R2A, R2A/10 and PYGV showed a good yield at 15°C and 20°C. At 4°C, lower numbers of colonies were found for both samples. In general, the number of colony-forming units observed for sample TM4 was much lower than for sample TM2 although a larger diversity of colony morphologies was noticed in TM4 which resulted in a higher number of isolates (see below). The majority (67%) of the colonies were pigmented, as previously reported for bacteria isolated from Antarctica and other cold environments [8, 37]. Based on differences in morphology and colour, isolates were selected. In total, 364 (TM2) and 421 (TM4) isolates were picked up and included in rep-PCR. Cluster analysis of rep-profiles resulted in 147 rep-types for sample TM2 (including 61 rep-clusters and 86 ungrouped isolates with a separate rep-profile) and 118 rep-types for sample TM4 (including 46 rep-clusters and 72 ungrouped isolates; Table 1). Only one of the rep-clusters contained isolates from both samples.

Identification Based on 16S rRNA Gene Sequencing

Analysis of the partial 16S rRNA gene of a representative of each of the 265 rep-types resulted in 82 phylotypes and for one representative of each of these the sequence was completed. Only eight of these were found to contain isolates from both sample TM2 and TM4, one of them contained a rep-type with isolates from both samples. Identification of the different phylotypes showed that the genera *Arthrobacter*, *Sphingomonas*, *Brevundimonas*, *Devosia*, *Polaromonas*, *Hymenobacter*, *Pedobacter*, *Bacillus* and *Pseudoxanthomonas* were recovered from both samples while other genera were obtained only from one of the samples (Table 1).

Table 1 Overview of the species recovered from samples TM2 and TM4 based on the phylogenetic affiliation of a completely sequenced representative for each phylotype

Identification[a]	Number of isolates	Representative strain	Accession number strain	Nearest phylogenetic neighbour			Sample number	Rep-type labels (number of isolates)	Distribution[b]
				Species	Accession number of type strain	Sequence similarity (%)			
Actinobacteria (32 (TM2) and 218 (TM4) isolates)									
Arthrobacter agilis	25	R-43938	FR691389	*Arthrobacter agilis*	X80748	99.8	TM4	206 (18), 350 (3), 973 (1), 2039 (1), 2040 (1), 2041 (1)	Cosmopolitan
Arthrobacter flavus	53	R-43110	FR691390	*A. flavus*	AB537168	100.0	TM2	135 (3), 165 (5), 176 (3), 943 (2), 2034 (1), 2038 (1)	Cosmopolitan
							TM4	201 (8), 216 (16), 225 (2), 455 (2), 857 (1), 894 (2), 940 (4), 2035 (1), 2036 (1), 2037 (1)	
Arthrobacter sp. 1	120	R-37013	FR691391	*Arthrobacter agilis*	X80748	99.4	TM4	196 (26), 199 (19), 200 (53), 239 (6), 240 (7), 312 (3), 2043 (1), 2044 (1), 2045 (1), 2046 (1), 2047 (1), 2048 (1)	Cosmopolitan
Arthrobacter sp. 2	10	R-39621	FR691392	*Arthrobacter oxydans*	X83408	98.9	TM4	223 (8), 2049 (1), 2050 (1)	Cosmopolitan
Arthrobacter sp. 3	1	R-38429	FR691393	*Arthrobacter tumbae*	AJ315069	98.4	TM4	2042 (1)	Antarctic[c]
Cryobacterium sp. 1	1	R-37019	FR691394	*Cryobacterium psychrotolerans*	DQ515963	97.5	TM4	2055 (1)	Cosmopolitan
Marisediminicola antarctica	6	R-36750	FR691395	*M. antarctica*	GQ496083	99.6	TM2	142 (2), 327 (2), 361 (2)	Cosmopolitan
Marisediminicola sp. 1	1	R-38315	FR691396	*M. antarctica*	GQ496083	98.9	TM4	2054 (1)	Cosmopolitan
gen. nov. *Actinobacteria* 1	1	R-36733	FR691397	*Yonghaparkia alkaliphila*	DQ256087	97.4	TM2	2058 (1)	Cosmopolitan
Knoellia aerolata	3	R-43433	FR691398	*K. aerolata*	EF553529	99.6	TM4	529 (2), 2064 (1)	Cosmopolitan
Kocuria palustris	2	R-39201	FR691399	*Kocuria palustris*	Y16263	100.0	TM4	897 (1), 2053 (1)	Cosmopolitan
Leifsonia (*Rhodoglobus*) *rubra*	6	R-36754	FR691400	*L. rubra*	AJ438585	99.8	TM2	169 (1), 2059 (1), 2060 (1), 2061 (1), 2062 (1), 2063 (1)	Polar
Rhodoglobus sp. 1	4	R-36762	FR691401	*L. rubra*	AJ438585	98.9	TM2	182 (3),2057 (1)	Cosmopolitan
Microbacterium lacus	1	R-43968	FR691402	*M. lacus*	AB286030	100.0	TM4	2056 (1)	Cosmopolitan
Micrococcus sp. 1	2	R-43944	FR691403	*Micrococcus yunnanensis*	FJ214355	99.6	TM4	2051 (1), 2052 (1)	Cosmopolitan
Rhodococcus sp. 1	10	R-37022	FR691404	*Rhodococcus baikonurensis*	AB071951	99.7	TM4	203 (9), 243 (1)	Cosmopolitan
Rhodococcus sp. 2	4	R-37551	FR691405	*Rhodococcus fascians*	X79186	99.5	TM4	218 (3), 2065 (1)	Cosmopolitan
Alphaproteobacteria (88 (TM2) and 54 (TM4) isolates)									
Bosea sp. 1	4	R-38307	FR691406	*Bosea massiliensis*	AF288309	99.0	TM2	191 (4)	Cosmopolitan
Brevundimonas sp. 1	48	R-36741	FR691407	*Brevundimonas subvibrioides*	AJ227784	99.8	TM2	152 (3), 153 (3), 154 (3), 190 (2), 315 (2), 2070 (1)	Cosmopolitan
							TM4	195 (32), 2068 (1), 2069 (1)	
Brevundimonas sp. 2	22	R-36759	FR691408	*Brevundimonas alba*	AJ227785	98.7	TM2	133 (2), 141 (4), 138 (7), 139 (4), 293 (2), 2074 (1), 2075 (1), 2076 (1)	Cosmopolitan
Brevundimonas sp. 3	12	R-37030	FR691409	*Brevundimonas subvibriodes*	AJ227784	98.7	TM2	633 (1)	Cosmopolitan
							TM4	202 (9), 2071 (1), 2073 (1)	
Brevundimonas sp. 4	2	R-37024	FR691410	*Brevundimonas vesicularis*	AJ007801	99.9	TM4	229 (2)	Cosmopolitan
Brevundimonas sp. 5	4	R-37014	FR691411	*Brevundimonas alba*	AJ227785	99.0	TM4	208 (2), 2066 (1), 2067 (1)	Cosmopolitan
Devosia sp. 1	1	R-36938	FR691412	*Devosia limi*	AJ786801	97.5	TM2	171 (1)	Cosmopolitan
Devosia sp. 2	27	R-36756	FR691413	*Devosia limi*	AJ786801	97.3	TM2	124 (3), 150 (6), 151 (4), 178 (4), 179 (4), 192 (1), 313 (2), 900 (2), 2081 (1)	Antarctic

Table 1 (continued)

Identification[a]	Number of isolates	Representative strain	Accession number strain	Nearest phylogenetic neighbour			Sample number	Rep-type labels (number of isolates)	Distribution[b]
				Species	Accession number of type strain	Sequence similarity (%)			
Devosia sp. 3	1	R-43424	FR691414	*Devosia limi*	AJ786801	97.1	TM2	358 (1)	Cold
Devosia sp. 4	1	R-43964	FR691415	*Devosia insulae*	EF012357	97.3	TM4	2080 (1)	Antarctic[c]
gen. nov. *Alphaproteobacteria* 1	1	R-39199	FR691416	*Microvirga guangxiensis*	EU727176	96.4	TM2	370 (1)	Antarctic[c]
gen. nov. *Alphaproteobacteria* 2	1	R-36935	FR691417	*Sphingosinicella microcystinivorans*	AB084247	94.2	TM2	2079 (1)	Antarctic[c]
Paracoccus marcusii	1	R-42686	FR691418	*P. marcusii*	Y12703	100.0	TM4	638 (1)	Cosmopolitan
Rhodobacter sp. 1	3	R-36943	FR691419	*Rhodobacter changlensis*	AM399030	97.0	TM2	187 (2), 2077 (1)	Antarctic[c]
Sphingomonas aerolata	2	R-36940	FR691420	*S. aerolata*	AJ429240	99.7	TM2 TM4	339 (1) 339 (1)	Cosmopolitan
Sphingopyxis flavimaris	8	R-36742	FR691421	*S. flavimaris*	AY554010	99.6	TM2	148 (8)	Cosmopolitan
gen. nov. *Alphaproteobacteria* 3	4	R-36760	FR691422	*Novosphingobium panipatense*	EF424402	95.0	TM2	319 (3), 2078 (1)	Cosmopolitan
Betaproteobacteria (38 (TM2) and 103 (TM4) isolates)									
Albidiferax sp. 1	1	R-37567	FR691423	*Albidiferax ferrireducens*	AF435948	98.5	TM4	2083 (1)	Cosmopolitan
Curvibacter sp. 1	1	R-36930	FR691424	*Curvibacter delicatus*	AF078756	98.2	TM2	173 (1)	Cosmopolitan
gen. nov. *Betaproteobacteria* 2, sp. 1	2	R-37018	FR691425	*Herminiimonas saxobidens*	AM493906	96.7	TM4	245 (1), 430 (1)	Cosmopolitan
gen. nov. *Betaproteobacteria* 2, sp. 2	1	R-38301	FR691426	*Herbaspirillum seropedicae*	Y10146	96.4	TM4	972 (1)	Cosmopolitan
gen. nov. Betaproteobacteria 3	1	R-43960	FR691427	*Variovorax soli*	DQ432053	97.7	TM4	2084 (1)	Antarctic[c]
gen. nov. *Betaproteobacteria* 1	1	R-36978	FR691428	*Janthinobacterium lividum*	Y08846	97.6	TM2	2099 (1)	Cosmopolitan
Hydrogenophaga sp. 1	2	R-38517	FR691429	*Hydrogenophaga taeniospiralis*	AF078768	98.2	TM2	432 (1), 2085 (1)	Polar
Polaromonas sp. 1	22	R-36732	FR691430	*Polaromonas vacuolata*	U14585	97.8	TM2	140 (5), 167 (5), 168 (8), 364 (2), 2086 (1), 2087 (1)	Cosmopolitan
Polaromonas sp. 2	4	R-38520	FR691431	*Polaromonas vacuolata*	U14585	98.1	TM2	162 (3), 2088 (1)	Cosmopolitan
Polaromonas sp. 3	106	R-37550	FR691432	*Polaromonas naphthalenovorans*	AY166684	98.6	TM2 TM4	180 (3), 181 (3), 2097 (1), 2098 (1) 197 (3), 198 (6), 209 (3), 210 (6), 211 (2), 212 (5), 213 (8), 219 (2), 220 (4), 232 (2), 244 (1), 270 (6), 323 (1), 324 (1), 369 (1), 376 (2), 450 (3), 559 (1), 891 (2), 892 (19), 893 (1), 898 (1), 899 (1), 903 (9), 2089 (1), 2090 (1), 2091 (1), 2092 (1), 2093 (1), 2094 (1), 2095 (1), 2096 (1)	Cosmopolitan
Gammaproteobacteria (5 (TM2) and 19 (TM4) isolates)									
Luteimonas sp. 1	1	R-37032	FR691433	*Luteimonas aquatica*	EF626688	96.8	TM4	2305 (1)	Cosmopolitan
Marinobacter psychrophilus	1	R-36953	FR691434	*M. psychrophilus*	DQ060402	99.4	TM2	2082 (1)	Cosmopolitan
Pseudoxanthomonas sp. 1	19	R-37036	FR691435	*Pseudoxanthomonas sacheonensis*	EF575564	98.1	TM2 TM4	2303 (1) 215 (6), 237 (3), 387 (1), 407 (1), 701 (1), 706 (1), 946 (1), 2300 (1), 2301 (1), 2302 (1), 2304 (1)	Antarctic
Psychrobacter glacincola	3	R-36959	FR691436	*P. glacincola*	AJ312213	99.9	TM2	175 (3)	Cosmopolitan

Bacteroidetes (89 (TM2) and 17 (TM4) isolates)									
Aequorivita sp. 1	1	R-36724	FR691437	*Aequorivita antarctica*	AY027802	98.1	TM2	2318 (1)	Antarctic[c]
Algoriphagus antarcticus	9	R-36749	FR691438	*A. antarcticus*	AJ577142	99.9	TM2	906 (4), 907 (4), 2308 (1)	Cosmopolitan
Algoriphagus sp. 1	4	R-36727	FR691439	*A. antarcticus*	AJ577142	96.5	TM2	129 (4)	Antarctic[c]
Flavobacterium micromati	32	R-36963	FR691440	*F. micromati*	AJ557888	99.7	TM2	131 (29), 2315 (1), 2316 (1), 2317 (1)	Cosmopolitan
Flavobacterium sp. 1	1	R-36964	FR691441	*Flavobacterium succinicans*	AM230492	97.8	TM2	2314 (1)	Cosmopolitan
Flavobacterium sp. 2	2	R-36968	FR691442	*Flavobacterium pectinovorum*	AM230490	97.7	TM2	2312 (1), 2313 (1)	Cosmopolitan
Gelidibacter algens	24	R-36722	FR691443	*G. algens*	U62914	99.4	TM2	158 (11), 159 (12), 2320 (1)	Antarctic
Gillisia sp. 1	6	R-36928	FR691444	*Gillisia limnaea*	AJ440991	96.1	TM2	177 (3), 344 (2), 2319 (1)	Antarctic[c]
Hymenobacter sp. 1	2	R-36960	FR691445	*Hymenobacter soli*	AB251884	96.1	TM2	172 (2)	Antarctic[c]
Hymenobacter sp. 2	2	R-37565	FR691446	*Hymenobacter roseosalivarius*	Y18833	98.4	TM4	217 (2)	Antarctic[c]
Hymenobacter sp. 3	3	R-37569	FR691447	*Hymenobacter soli*	AB251884	97.4	TM4	382 (2), 2306 (1)	Antarctic[c]
Pedobacter sp. 1	1	R-36962	FR691448	*Pedobacter daechungensis*	AB267722	94.9	TM2	2310 (1)	Antarctic[c]
Pedobacter sp. 2	11	R-38393	FR691449	*Pedobacter oryzae*	EU109726	95.5	TM4	204 (3), 222 (4), 294 (3), 2309 (1)	Antarctic[c]
Pontibacter sp. 1	7	R-36965	FR691450	*Pontibacter korlensis*	DQ888330	94.5	TM2	161 (4), 890 (2), 2307 (1)	Antarctic[c]
Spirosoma sp. 1	1	R-37560	FR691451	*Spirosoma rigui*	EF507900	92.9	TM4	2311 (1)	Cosmopolitan
Firmicutes (112 (TM2) and 10 (TM4) isolates)									
Aerococcus sp. 1	1	R-38529	FR691452	*Aerococcus urinaeequi*	D87677	99.9	TM2	2013 (1)	Cosmopolitan
Bacillus neizhouensis	1	R-43422	FR691453	*B. neizhouensis*	EU925618	100.0	TM2	2011 (1)	Cosmopolitan
Bacillus sp. 1	6	R-37580	FR691454	*Bacillus aerophilus*	AJ831844	97.8	TM2	303 (1), 304 (1), 2001 (1), 2002 (1)	Cosmopolitan
							TM4	889 (1), 2003 (1)	Cosmopolitan
Bacillus sp. 2	7	R-36721	FR691455	*Bacillus krulwichiae*	AB086897	93.9	TM2	155 (2), 183 (4), 2004 (1)	Cosmopolitan
Bacillus sp. 3	1	R-43946	FR691456	*Bacillus butanolivorans*	EF206294	94.3	TM4	514 (1)	Cosmopolitan
Carnobacterium funditum	9	R-36987	FR691457	*C. funditum*	S86170	99.3	TM2	184 (5), 224 (2), 2032 (1), 2033 (1)	Antarctic
Carnobacterium sp. 1	40	R-36982	FR691458	*Carnobacterium pleistocenium*	AF450136	99.6	TM2	166 (14), 174 (12), 309 (2), 505 (4), 2031 (1)	Cosmopolitan
							TM4	205 (7)	Cosmopolitan
Jeotgalibacillus marinus	2	R-42990	FR691459	*J. marinus*	AJ237708	99.0	TM2	500 (1), 2008 (1)	Cosmopolitan
Ornithinibacillus sp. 1	1	R-38538	FR691460	*Ornithinibacillus bavariensis*	Y13066	96.5	TM2	2012 (1)	Cosmopolitan
Paenibacillus sp. 1	1	R-36731	FR691461	*Paenibacillus wynnii*	AJ633647	98.2	TM2	2030 (1)	Polar
Paenibacillus sp. 2	4	R-36746	FR691462	*Paenibacillus macquariensis subsp. macquariensis*	AB073193	97.7	TM2	221 (2), 440 (1), 2029 (1)	Antarctic[c]
Paenisporosarcina sp. 1	1	R-36744	FR691463	*S. antarctica*	EF154512	98.9	TM2	2010 (1)	Cosmopolitan
Paenisporosarcina sp. 2	13	R-36758	FR691464	*S. antarctica*	EF154512	98.7	TM2	156 (12), 2009 (1)	Cosmopolitan
Planococcus antarcticus	28	R-36948	FR691465	*P. antarcticus*	AJ314745	99.3	TM2	136 (8),186 (3), 348 (3), 2015 (1), 2016 (1), 2017 (1), 2018 (1), 2019 (1), 2020 (1), 2021 (1), 2022 (1), 2023 (1), 2024 (1), 2025 (1), 2026 (1), 2027 (1), 2028 (1)	Cosmopolitan

Table 1 (continued)

Identification[a]	Number of isolates	Representative strain	Accession number strain	Nearest phylogenetic neighbour			Sample number	Rep-type labels (number of isolates)	Distribution[b]
				Species	Accession number of type strain	Sequence similarity (%)			
Planococcus sp. 1	1	R-36970	FR691466	*Planococcus donghaensis*	EF079063	98.6	TM2	2014 (1)	Cosmopolitan
Planococcus sp. 2	1	R-36952	FR691467	*Planococcus donghaensis*	EF079063	97.8	TM2	185 (1)	Cosmopolitan
Staphylococcus equorum subsp. equorum	2	R-36936	FR691468	*Staphylococcus equorum subsp. equorum*	AB009939	100.0	TM2	2006 (1), 2007 (1)	Cosmopolitan
Staphylococcus haemolyticus	2	R-38532	FR691469	*Staphylococcus haemolyticus*	D83367	99.9	TM2	508 (1), 2005 (1)	Cosmopolitan
Staphylococcus warneri	1	R-38534	FR691470	*S. warneri*	L37603	99.9	TM2	66 (1)	Cosmopolitan

The genetic diversity within each species is documented in the columns listed (rep-type labels and number of isolates in each rep-type). Geographical distribution is based on the origin of highly similar sequences in public databases

[a] Proposed identification is based on a species threshold of 99.0% 16S rRNA gene sequence similarity for species identification and considering phylogeny (see 16S rRNA Gene Sequencing and Analysis)

[b] Distribution represents the geographic distribution of our phylotypes. The 16S rRNA gene sequences were compared to the prokaryote and the environmental divisions of the EMBL database and to the nucleotide collection, the high throughput genome sequences and environmental samples of the GenBank database. Based on the origin of the high scoring (≥99.0%) entries, phylotypes were labelled as cosmopolitan, cold, polar or Antarctic

[c] This Antarctic phylotype showed no significant similarity with sequences in the public databases

Eight phylotypes could not be assigned to a particular genus as their 16S rRNA gene sequences were approximately equally related to several different genera. They potentially belong to one of these genera or alternatively may represent new genera. In this study, we therefore tentatively classified them as potential new genera (Table 1) although they should be studied in more detail using a polyphasic approach to determine their taxonomic status. Within the phylum *Actinobacteria*, a potential new genus was found. Phylotype R-36733 showed 97.4% 16S rRNA gene sequence similarity with *Yonghaparkia alkaliphila* and 96.7% with *Microcella putealis*, but the mutual sequence similarity between these two type strains was 98.2%. This observation and the topology of the neighbour-joining tree (data not shown) indicate that phylotype R-36733 cannot be reliably assigned to either genus and should be studied further using a polyphasic approach.

Three potential new genera were found in the class *Alphaproteobacteria*. Phylotype R-39199 formed part of a cluster with the genera *Microvirga* (96.4%), *Bosea* (93.8–94.4%) and *Balneimonas* (94.9%). Because the sequence similarities with the most closely related genera were equally low, this may be a new genus that should be studied in more detail to verify this. Phylotype R-36935 showed low 16S rRNA gene sequence similarity values with neighbouring taxa (93.9–94.2% with *Sphingosinicella* and 91.0–93.4% with *Sphingobium*) and therefore represents a potential new genus. Also phylotype R-36760 showed low 16S rRNA gene sequence similarity values with neighbouring taxa (92.5–94.9% with *Sphingopyxis* and 92.1–95.0% with *Novosphingobium*) and thus may represent a new genus.

Four phylotypes related to the class *Betaproteobacteria* could not be assigned to an existing genus. Phylotype R-36978 showed low 16S rRNA gene sequence similarity values with neighbouring taxa (97.3–97.6% with *Janthinobacterium*, 95.2–97.3% with *Massilia* and 96.2–96.5% with *Duganella*). The topology of the neighbour-joining tree (data not shown) indicates that phylotype R-36978 should be studied further to verify whether it represents a new genus. Phylotype R-37018 formed part of a cluster with the genera *Herminiimonas* (96.0–96.7%) and *Herbaspirillum* (96.0–96.6%) and may represent a new genus. Phylotype R-38301 showed 98.7% 16S rRNA gene sequence similarity with the former phylotype R-37018, suggesting they may belong to the same potential new genus. Phylotype R-43960 showed low 16S rRNA gene sequence similarity values with neighbouring taxa (97.2–97.7% with *Variovarax*, 97.2–97.5% with *Curvibacter* and 97.1–97.3% with *Ramlibacter*) and may represent a new genus. All these phylotypes potentially representing new genera should be studied further to establish their most appropriate classification.

Coverage of Heterotrophic Diversity and Dominant Taxa

Calculation of Good's estimator indicated that at phylotype level 94.4% (TM2) and 96.9% (TM4) of the diversity culturable in our conditions was retrieved and at the finer taxonomic level of rep-type only 77.7% (TM2) and 82.4% (TM4) was isolated. Table 1 also lists the number of isolates obtained for the different phylotypes. For TM2, most isolates belonged to the phylum *Firmicutes*, whereas the *Bacteroidetes* and *Alphaproteobacteria* were also well represented. *Betaproteobacteria* and *Actinobacteria* each represented about 10% of the isolates. The most prevalent genera among TM2 isolates, each representing more than 5% of the isolates, were *Carnobacterium*, *Brevundimonas*, *Flavobacterium*, *Polaromonas*, *Planococcus*, *Devosia* and *Gelidibacter*. For sample TM4, *Actinobacteria* represented more than half of the isolates, while *Betaproteobacteria* and *Alphaproteobacteria* were also well represented. A smaller number of isolates belonged to the *Bacteroidetes* and very few to the *Firmicutes*. The most prevalent genera among TM4 isolates were *Arthrobacter*, *Polaromonas* and *Brevundimonas*. Very few isolates from either sample belonged to the *Gammaproteobacteria*.

Table 2 Summary of the number of phylotypes recovered with cosmopolitan, cold, polar or Antarctic distribution

Phylum/class	Cosmopolitan	Cold	Polar	Antarctic[a]
TM2				
Actinobacteria	4	0	1	0
Alphaproteobacteria	8	1	0	4
Betaproteobacteria	5	0	1	0
Gammaproteobacteria	2	0	0	1
Bacteroidetes	4	0	0	7
Firmicutes	15	0	1	2
Total TM2	38	1	3	14
TM4				
Actinobacteria	12	0	0	1
Alphaproteobacteria	6	0	0	1
Betaproteobacteria	4	0	0	1
Gammaproteobacteria	1	0	0	1
Bacteroidetes	1	0	0	3
Firmicutes	3	0	0	0
Total TM4	27	0	0	7

[a] Both phylotypes showing ≥99.0% 16S rRNA gene sequence similarity with environmental sequences originating from Antarctica and phylotypes without any significant sequence similarity

Geographic Distribution of the Phylotypes Recovered

Comparing the sequences of the phylotypes with sequences in the public databases showed that all our *Actinobacteria* phylotypes had a cosmopolitan distribution (Tables 1 and 2) except for *Arthrobacter* phylotype R-38429 (TM4) which had no significant similarity with any other environmental sequence and *Leifsonia* (*Rhodoglobus*) phylotype R-36754 (TM2) which had a polar distribution as it has been found in Wright Valley, McMurdo Dry Valleys, Antarctica (EMBL accession #AJ438585), in Antarctic sea ice (EMBL accession #FJ889621) and in the Arctic (EMBL accession #AY771758).

The majority of our *Alphaproteobacteria* phylotypes also had a cosmopolitan distribution (Tables 1 and 2) except for four phylotypes without significant similarity with other environmental sequences. The sequence of *Devosia* phylotype R-43424 (TM2) was reported from cold environments such as a glacier in Austria (EMBL accession #GU441678) and *Devosia* phylotype (R-36756 (TM2)) is currently known only from Antarctica because it only matched a sequence reported from Lake Reid, Larsemann Hills, Antarctica (EMBL accession #AJ440974).

The majority of our *Betaproteobacteria* phylotypes had a cosmopolitan distribution (Tables 1 and 2) except for phylotype R-43960 (TM4), one of the potential new genera that had no significant similarity with other environmental sequences. Additionally, *Hydrogenophaga* phylotype R-38517 (TM2) showed a polar distribution as the sequence was similar only to sequences found in the Arctic (EMBL accession #AY771764) and in Ace Lake, Vestfold Hills, Antarctica (EMBL accession #AJ441011).

Within the *Gammaproteobacteria*, three phylotypes were found to be cosmopolitan (Tables 1 and 2). The sequence of *Pseudoxanthomonas* phylotype R-37036 (TM2, TM4) was found before in Lake Vida, McMurdo Dry valleys (EMBL accession #DQ521479) and Scott Base, Antarctica (EMBL accession #AY571839).

About half of the *Bacteroidetes* phylotypes showed no significant similarity with any sequences in the public databases (Tables 1 and 2) and may represent new taxa. Three phylotypes were assigned to existing species and one of these (*Gelidibacter algens* phylotype R-36722 (TM2)) is currently known only from Antarctica as it only matches sequences from microbial mats from Ace Lake, Vestfold Hills, Antarctica (EMBL accession #AJ441008).

The majority of the *Firmicutes* phylotypes showed a cosmopolitan distribution (Tables 1 and 2). *Paenibacillus* phylotype R-36731 (TM2) showed a polar distribution as it matched sequences found in the Canadian high Arctic (EMBL accession #DQ444985). *Paenibacillus* phylotype R-36746 showed no significant similarity with sequences in public databases. Phylotype R-36987 matched only with sequences of *Carnobacterium funditum*, a species originally described from Ace Lake, Antarctica [18].

Discussion

Species and Genus Identification

This study represents the first assessment of the heterotrophic bacterial diversity associated with cyanobacterial mats from two lakes in the Transantarctic Mountains and the Shackleton Range. Seven hundred and eighty-five isolates were grouped into 265 rep-types (107 rep-clusters and 158 separate isolates) and found to represent 82 phylotypes (Table 1). Overall, 28.6% (TM2) and 20.6% (TM4) of the phylotypes were assigned to existing species. For TM2, these were distributed over most of the phylogenetic groups recovered, with a majority in *Firmicutes*, whereas for sample TM4 they were all related to *Actinobacteria* and *Alphaproteobacteria*. Several of these phylotypes belong to species for which up to now only the type strain was available in culture collections (*Arthrobacter flavus*, *Bacillus neizhouensis*, *Knoellia aerolata*, *Leifsonia rubra*, *Marisediminicola antarctica*, *Microbacterium lacus* and *Sphingopyxis flavimaris*). The additional strains identified in our study may give more insight in the diversity present in these species.

Furthermore, 60.7% (TM2) and 67.7% (TM4) of the phylotypes could not be assigned to existing species based on 16S rRNA gene sequences and were therefore identified only at genus level. They may represent new species and some genera contained a remarkably high number of potential new species, e.g. *Brevundimonas*, *Devosia* (Table 1). These phylotypes were distributed over most of the phylogenetic groups, with a majority in *Firmicutes* for sample TM2 and in *Actinobacteria* for sample TM4. All will require further polyphasic characterization to verify this preliminary identification.

Finally, 8.9% (TM2) and 8.8% (TM4) of the phylotypes could not be identified at genus level and may represent potential new genera. These phylotypes are affiliated with the *Betaproteobacteria* for both samples and with the *Actinobacteria* and the *Alphaproteobacteria* for sample TM2. Also these phylotypes need to be studied in more detail to determine the most appropriate identification.

For some phylotypes, the identification was not straightforward. Phylotypes R-36754 and R-36762 showed, respectively, 99.8% and 98.9% 16S rRNA gene sequence similarity with *L. rubra* which was only distantly related (94.9%) with its type species *Leifsonia aquatica. L. rubra* formed part of a cluster with the genera *Rhodoglobus* [51] and *Salinibacterium* [23] (Supplementary data, Fig. S4) which have been created shortly after each other. Recently, An et al. [4] proposed a revision of the phylogenetic relationships among *L. rubra*, *Leifsonia aurea* and the genera *Rhodoglobus* and *Salinibacterium.* Unfortunately, the type strain of *L. rubra* was no longer available [5] and the species could not be renamed. For this reason, we tentatively classify our phylotype R-36754 as *Leifsonia* (*Rhodoglobus*) *rubra* and phylotype R-36762 as *Rhodoglobus* sp. although these groups need to be investigated with a polyphasic approach to clarify the relations between the different species and to determine an appropriate identification for the phylotypes.

Phylotypes R-36758 and R-36744 showed the highest 16S rRNA gene sequence similarity (98.7% and 98.9%, respectively) with *Sporosarcina antarctica* although in a neighbour-joining tree these strains, together with *S. antarctica*, were part of the *Paenisporosarcina* cluster (bootstrap value 100, Supplementary data, Fig. S5). *Paenisporosarcina* was recently created for the former *Sporosarcina macmurdoensis* and an additional new species [31]. In view of the 16S rRNA gene phylogeny, *S. antarctica* may also need to be reclassified as *Paenisporosarcina*. Consequently, the phylotypes R-36758 and R-36744 were identified as *Paenisporosarcina* sp. and as they have a mutual 16S rRNA gene sequence similarity of only 98.8%, they are classified as two different potential new species.

Using this cultivation approach, which is known to reveal only a small percentage of the real diversity [3], sample TM2 yielded a larger diversity of rep-types and phylotypes than sample TM4. Indeed, the bacterial diversity obtained from sample TM2 was distributed over 32 different genera, whereas only 21 genera were found in sample TM4. This was also reflected in the Shannon Biodiversity Index which showed a higher diversity for sample TM2 (Shannon index: 3.39) than for sample TM4 (2.34). Nevertheless, these differences in diversity may be influenced by the fact that only one sample for each lake has been studied which may not be representative for all diversity present in the lakes.

Comparison with Previous Antarctic Reports

Within the phylum *Actinobacteria*, nine genera were recovered in this study, all of which have previously been reported from Antarctica [45, 50, 61]. Several phylotypes could be identified at species level while others could only be assigned a generic identification. Only phylotypes R-43110 (*A. flavus*), R-36754 (*Leifsonia* (*Rhodoglobus*) *rubra*) and R-36750 (*M. antarctica*) represent species discovered in Antarctica [32, 43, 46] whereas all the other species represented have never before been reported from Antarctica.

The phylum *Proteobacteria* was represented by the classes *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*.

Within the class *Alphaproteobacteria*, three phylotypes could be identified at species level. Phylotype R-36940, which contains isolates from both lake samples, was identified as *Sphingomonas aerolata*. This species has not previously been reported from Antarctica, although the genus

Sphingomonas has been reported from the Schirmacher Oasis, East Antarctica [52]. Phylotypes R-42686 was identified as *Paracoccus marcusii.* This species has not previously been reported from Antarctica, although the genus *Paracoccus* has been found in Terra Nova Bay (Ross Sea) [36] and in Antarctic sea ice [10]. Phylotype R-36742 was identified as *S. flavimaris* and this is the first report in Antarctica for both this species and this genus.

Some other phylotypes were identified at genus level (Table 1). *Brevundimonas* has previously been reported from Antarctica [21]. The genus *Devosia* has also been found in Antarctica, in the region of the Princess Elisabeth station at Utsteinen. The genus *Rhodobacter* has been reported from Lake Fryxell [30]. The genus *Bosea* has never before been observed in Antarctica.

Within the class *Betaproteobacteria*, none of the phylotypes could be identified at species level. Four genera were represented of which *Polaromonas* and *Hydrogenophaga* have been observed in Antarctica [26, 39]. This is the first report in Antarctica for the genera *Albidiferax* and *Curvibacter.*

Within the class *Gammaproteobacteria*, two phylotypes could be identified at species level. *Psychrobacter glacincola* has been found previously in sea ice in Antarctica [8]. The species *Marinobacter psychrophilus* has been isolated from sea ice in the Arctic [67] but has not been reported from Antarctica, although other species within this genus have been isolated from marine sediment from the South Shetland Islands [38]. The genera *Pseudoxanthomonas*, present in both samples, and *Luteimonas* have not previously been reported from Antarctica.

In the phylum *Bacteroidetes*, three phylotypes could be identified at species level as *Algoriphagus antarcticus*, *Flavobacterium micromati* (both isolated from microbial mats from Antarctica [61]) and *G. algens* (observed in sea ice in Antarctica [9]). Some other phylotypes could be identified at genus level (Table 1). The genera *Hymenobacter*, recovered from both samples, and *Gillisia* have also been found in Victoria Land, Antarctica [1]. *Aequorivita* has been observed in seawater in Antarctica [11] and *Pedobacter* has been isolated at Utsteinen, Dronning Maud Land [41]. The genus *Spirosoma*, which has been found in Arctic permafrost soil [16] and *Pontibacter* are new records from Antarctica.

Within the phylum *Firmicutes*, seven phylotypes could be identified at species level. The species *Planococcus antarcticus* and *C. funditum* originate from Antarctica [18, 47]. This is also the first report from Antarctica for two of the three *Staphylococcus* species recovered, while *Staphylococcus warneri* has also been found at Utsteinen, Dronning Maud Land [41]. The species *Bacillus neizhouensis* has not been reported previously in Antarctica, although the genus *Bacillus* is present [17, 40, 50]. Finally, this is the first report in Antarctica for the species and genus *Jeotgalibacillus marinus*. Other previously known Antarctic genera recovered include *Aerococcus* [13], *Paenisporosarcina* and *Paenibacillus* [33, 44] while *Ornithinibacillus* is a new record.

In summary, about half of the species found in this study have never before been reported from Antarctica, although the majority of them belong to genera that have been observed there previously. Nevertheless, our data provide the first reports in Antarctica for the genera *Albidiferax*, *Bosea*, *Curvibacter*, *Luteimonas*, *Ornithinibacillus*, *Pseudoxanthomonas*, *Sphingopyxis* and *Spirosoma.*

Geographic Distribution of the Phylotypes Recovered

Comparison of our sequences with public databases was used to assess the geographic distribution for the different phylotypes. In general, the majority of the phylotypes related to *Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria* and *Firmicutes* were found to have a cosmopolitan distribution (Table 2). A small number of phylotypes within these groups had a cold, polar or Antarctic distribution. Only few phylotypes belonging to the *Gammaproteobacteria* were recovered and, of these, three showed a cosmopolitan distribution whereas one is currently only known from Antarctica. Remarkably, within the phylum *Bacteroidetes*, more than half of the phylotypes are at present only known from Antarctica. This observation is in line with previous reports [1, 65]. Temperate *Bacteroidetes* representatives apparently do not often possess the adaptations necessary for survival in Antarctic conditions as would seem to be the case in *Actinobacteria*, *Proteobacteria* and *Firmicutes* where more cosmopolitan species were recovered (Table 2). Comparing the two samples, some 68% of the phylotypes of sample TM2 appeared cosmopolitan and 32% have so far been reported only from Antarctica, polar or cold environments whereas, for sample TM4, more than three quarters of the phylotypes showed a cosmopolitan distribution and 21% of the sequences were only found in Antarctic environments. About 20% of the phylotypes for both samples showed no significant sequence similarity with sequences available in public databases and thus are at present only known from Antarctica.

It is important to note that these labels reflect current knowledge of bacterial diversity and ecology which is known to be limited [15]. The current undersampling of bacterial diversity in most habitats worldwide implies that taxa which are only known in Antarctica today may turn out to be cosmopolitan as the number of microbial diversity studies increases. Indeed, our searches revealed that some species that were originally described for Antarctic isolates, have since been reported from other places (Table 1, e.g. *P. antarcticus*, *F. micromati*, *M. antarctica*). Nevertheless,

overall 23% of the phylotypes recovered in this study are apparently currently only known in Antarctica.

Relation to Water Chemistry

Although available water chemistry data are limited (Table 3), they show that the conductivity values and the concentrations of several ions were elevated in sample TM2 in comparison with those of sample TM4. This may be a result of the evaporation and sublimation processes that have dominated over precipitation in the Dufek Massif [24] and caused Forlidas Pond to evaporate down to a small remnant of a once much larger water body. Roberts et al. [48] identified the environmental variables Cl, Na, Mg and Ca to be correlated with salinity. As the values for these ions are elevated for sample TM2 (Table 3) in comparison with those for sample TM4, this may indicate that sample TM2 is more saline than sample TM4. This is also reflected in the bacterial yield on the different media (Fig. S2) showing more colonies on the marine medium for sample TM2 in comparison with the other media and with sample TM4. In literature, salinity has been found to be the major environmental determinant of microbial community composition rather than extremes of temperature, pH, or other physical and chemical factors [34]. In this study, a large number of the phylotypes isolated from sample TM2 were related to the *Firmicutes*. This phylum was found to be prevalent in soil with extreme salinity [2]. In view of the limited number of isolates we studied, definite correlations cannot be drawn, however, we do note that the *Alphaproteobacteria*, which are often found in saline waters [42], were recovered in higher numbers from sample TM2 than from sample TM4, whereas the less salt tolerant *Betaproteobacteria* [42] were recovered only in low numbers from sample TM2 and in higher numbers from sample TM4. Finally, several genera that were only recovered from sample TM2 and not from sample TM4 have also been described as halotolerant, for instance *Gillisia* [2], *Sporosarcina* [64] and *Psychrobacter* [66] although most of the other species and genera found in this sample are not specifically associated with saline environments.

Conclusion

In this study, the heterotrophic bacterial diversity in microbial mat samples from the littoral zone from two different lakes in the Transantarctic Mountains and the Shackleton Range was investigated. A total of 785 isolates were characterised and showed a large diversity distributed over 42 different genera in four phyla. The most dominant phylogenetic groups recovered were *Firmicutes*, *Bacteroidetes* and *Alphaproteobacteria* for TM2 and *Actinobacteria*, *Betaproteobacteria* and *Alphaproteobacteria* for sample TM4. In addition to several genera previously recorded from Antarctica, this is the first report of the genera *Albidiferax*, *Bosea*, *Curvibacter*, *Luteimonas*, *Ornithinibacillus*, *Pseudoxanthomonas*, *Sphingopyxis* and *Spirosoma* in Antarctica. A large number of potential new species and genera were recovered. When compared with public databases, 72% of phylotypes recovered showed a cosmopolitan distribution and 23% are at present only known in Antarctica. In the phylum *Bacter-*

Table 3 Water chemistry data for Forlidas Pond and Lundström Lake (modified from [24])

Parameter	Forlidas Pond (TM2)	Lundström Lake (TM4)
Conductivity (mS/cm)	2.220	0.22702
Temperature (°C)	0.7	1.48
pH	8.15	9.04
Al (mg/L)	<0.002	0.005
Fe (mg/L)	0.004	<0.001
Mg (mg/L)	13.9	1.18
Ca (mg/L)	11.4	3.34
K (mg/L)	1.36	0.612
Na (mg/L)	45	3.47
Cl (mg/L)	88.6	60.1
SO4-S (mg/L)	17.5	27.9
TN (mg/L)	4.3	0.18
TOC (mg/L)	0.97	0.89
DOC (mg/L)	1.04	0.96
NO3-N (mg/L)	4.42	<0.100
NH4-N (mg/L)	0.043	0.026
PO4-P (mg/L)	<0.005	<0.005

oidetes, more than half of phylotypes recovered are currently known only in Antarctica. While TM2 was more diverse than TM4, the bacterial diversity found in both samples showed very limited overlap. These differences may be partly explained by variations in the water chemistry, with TM2 being more saline than TM4, and by their location about 400 km apart, separated by the Recovery and Support Force Glaciers. A cultivation-independent study of our samples is currently ongoing.

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Evidence for widespread endemism among Antarctic micro-organisms

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Abstract

Understanding the enormous diversity of microbes, their multiple roles in the functioning of ecosystems, and their response to large-scale environmental and climatic changes, are at the forefront of the international research agenda. In Antarctica, where terrestrial and lacustrine environments are predominantly microbial realms, an active and growing community of microbial ecologists is probing this diversity and its role in ecosystem processes. In a broader context, this work has the potential to make a significant contribution to the long-standing debate as to whether microbes are fundamentally different from macroorganisms in their biogeography. According to the ubiquity hypothesis, microbial community composition is not constrained by dispersal limitation and is solely the result of species sorting along environmental gradients. However, recent work on several groups of microalgae is challenging this view. Global analyses using morphology-based diatom inventories have demonstrated that, in addition to environmental harshness, geographical isolation underlies the strong latitudinal gradients in local and regional diversity in the Southern hemisphere. Increasing evidence points to a strong regionalization of diatom floras in the Antarctic and sub-Antarctic regions, mirroring the biogeographical regions that have been recognized for macroorganisms. Likewise, the application of molecular-phylogenetic techniques to cultured and uncultured diversity revealed a high number of Antarctic endemics among cyanobacteria and green algae. Calibration of these phylogenies suggests that several clades have an ancient evolutionary history within the Antarctic continent, possibly dating back to 330 Ma. These findings are in line with the current view on the origin of Antarctic terrestrial metazoa, including springtails, chironomids and mites, with most evidence suggesting a long history of geographic isolation on a multi-million year, even pre-Gondwana break-up timescale.

Keywords: Microbial biogeography; Isolation; Dispersal; Ubiquity hypothesis; Evolution

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1. Introduction

Until recently, ice sheet models implied that as a result of successive Neogene and Late-Pleistocene glacial maxima, ice-free regions in Antarctica would have been overridden by ice for extended periods of time. Consequently, virtually all terrestrial life on the Antarctic continent would have been driven to extinction at glacial maxima and, as a consequence the present biodiversity would probably be derived from recent colonizations of regions that have become ice-free since the start of the Holocene. However, recent work on terrestrial invertebrates, including springtails, chironomids and mites, has challenged this view (reviewed in Convey et al., 2008, 2009). Fossil evidence, time-calibrated molecular phylogenies and population genetic studies, provide strong evidence that a significant fraction of the contemporary Antarctic terrestrial metazoa must have been continuously isolated on a multi-million year, even pre-Gondwana break-up timescale. As a corollary, these findings imply that throughout time persistent active basal foodwebs must have been present, sustaining the productivity of these higher trophic levels. The questions then naturally arise as to what extent evolutionary history has been preserved in the modern microbial biota in Antarctica, and to what extent microbial biodiversity shows the same degree of geographical differentiation as Antarctic metazoa.

Providing answers to these questions also has a broader relevance to understanding the rates and nature of diversification and adaptation of microbes. In particular, they have the potential to contribute to the long-standing debate as to whether microbes have a biogeography similar to macroscopic organisms. Indeed, as early as the 19th century, Darwin and de Candolle had observed that small organisms generally have larger distributional ranges than larger species. This idea was further developed by the Dutch microbiologist Baas-Becking (1934) in his famous dictum: ''everything is everywhere but the environment selects'' and more recently, by Fenchel and Finlay (Fenchel, 1993; Finlay et al., 1996; Finlay, 2002) in the so-called Ubiquity hypothesis. Fundamental to this hypothesis is the idea that the enormous population sizes of microbes combined with an easy dispersal lead to low global diversity of micro-organisms and geographical distributions that are only constrained by the availability of suitable habitats. As a consequence, most microbial species should therefore be virtually cosmopolitan.

Here we summarize results from our recent work within the framework of the Belgian projects HOLANT and AMBIO (both contributing to SCAR EBA Work Package 1: Evolutionary history of Antarctic organisms). We show that, based upon different independent approaches, clear biogeographical patterns are emerging for some groups of phototrophic microbes, and hypothesize that these are to a large extent the result of geographical isolation. These examples also serve as a framework to discuss a number of general issues related to microbial biodiversity and biogeography in Antarctica.

2. Strong latitudinal gradients in taxonomic richness and turnover rates of diatoms

One prediction of the ubiquity hypothesis is that, as a result of global dispersal, latitudinal gradients in diversity should be weak or absent once ecological controls are factored out (Hillebrand and Azovsky, 2001; Finlay and Fenchel, 2004). Demonstrating large-scale trends in microbial diversity is not as straightforward as it might seem. Studies of large-scale trends in local and regional diversity of microbes have been hampered by uneven and/or incomplete sampling over large geographical scales (in Antarctica this also holds for metazoa, see Adams et al., 2006; Chown and Convey, 2007), by the lack of standardization in the delineation of taxonomical units on the basis of morphological criteria, and more recently, also in the molecular markers used to delimit OTUs (operational taxonomic units) and the techniques used to produce inventories of genetic diversity for single samples.

In a recent study, a large global data set consisting of diatom inventories of nearly 2000 lakes and pools was used to investigate latitudinal trends in diversity (Vyverman et al., 2007). This data set was assembled by merging several regional datasets used in paleolimnological studies. Although the overall sampling methodology and the analytical protocols for diatom enumeration were comparable in all studied regions, it was not possible to merge species lists because of nomenclatural problems and inconsistencies and, in particular, because of differences in species concepts and cryptic diversity (Vyverman et al. l.c.). These problems were alleviated by investigating diversity patterns at the genus level. As in many groups of organisms, genus richness is a power function of species richness, which indicates that any trends observed at the genus level should also reflect similar trends at the species level. Grouping the lakes into equal-sized grid cells of 10 000 km^2 and extensive resampling was applied to account for differences in sampling effort in different geographic regions, and to calculate mean local richness (the average number of

genera occurring in lakes in a grid cell) and mean regional richness (the total number of genera present in a grid cell).

The results of this analysis were strikingly different from the predictions based on the ubiquity hypothesis. Both local and regional diversity showed strongly asymmetric latitudinal gradients (Fig. 1). In the Southern Hemisphere, local and regional richness declined in a linear fashion with increasing latitude. In contrast, in the Northern Hemisphere, the gradient between local richness and latitude was hump-shaped between 55° and 70°N, while the gradient between regional richness and latitude was virtually flat. Limnological conditions that are known to strongly influence diatom community structure however were not significantly different between Northern and Southern Hemisphere lake districts. In contrast, the degree of geographical isolation and connectivity between lakes were good predictors of regional and local diatom diversity, respectively. Furthermore, these results are consistent with the inverse relationship between taxonomic turnover rates and connectivity observed in the same data set (Verleyen et al., 2009). This does not mean that ecological factors do not play a role in structuring diatom communities, but rather that ecological constraints play a minor role in comparison to dispersal limitation with increasing geographical extent.

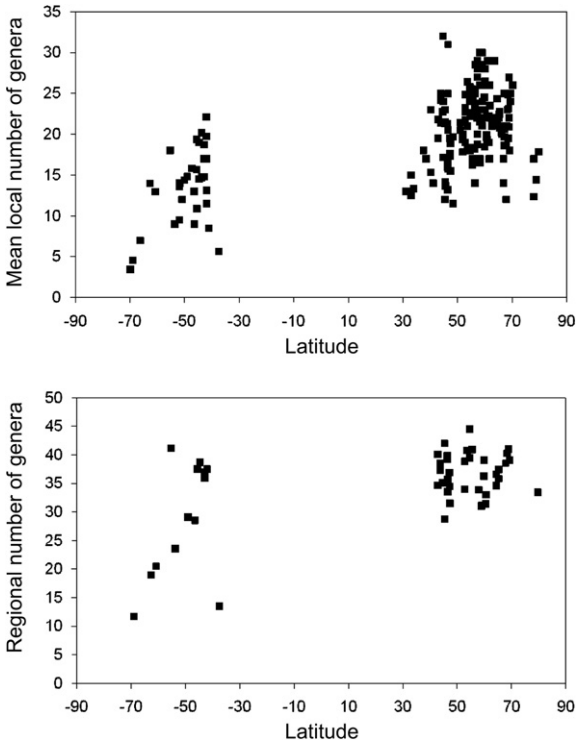

Fig. 1. The interhemispheric asymmetry in mean local (a) and mean regional (b) lacustrine diatom richness showing that highly connected sites such as those in lake rich regions in the Northern hemisphere hold a significantly higher number of genera than Antarctic lakes and the isolated islands in the Southern Ocean. Lakes were grouped into equal-sized grids of 100 by 100 km. For regional richness data, only grids containing more than 10 samples were included. Mean local richness between 70 and 40°S decreases linearly with latitude in the Southern Hemisphere ($y = 0.3394x + 30.502$, $R^2 = 0.3914$, $p < 0.001$) while in the Northern Hemisphere it shows a hump-shaped relationship with latitude and peaks between 55 and 70°N ($y = -0.0134x^2 + 1.5866x - 24.489$, $R^2 = 0.194$, all constants significant at $p < 0.05$). Mean Regional richness strongly declines with latitude in the Southern Hemisphere ($y = 0.8823x + 75.228$, $R^2 = 0.695$, $p < 0.001$), whereas it is virtually flat in the Northern Hemisphere and no significant regression line can be calculated. Modified from Vyverman et al. (2007).

3. Indications of endemism and bioregionalization of diatom floras

If our assertion is correct; that dispersal constraints – resulting from either pure geographical isolation or from geographical variation in propagule flux associated with migrating birds and animals or with atmospheric circulation patterns – influence global patterns in regional and local diatom diversity, it can be expected that Antarctic and Sub-Antarctic diatom floras would be characterized by a high degree of endemism.

Several fine-grained taxonomic revisions of regional diatom floras suggest that in some areas within Antarctica at least 40 per cent of the species are endemics (Schmidt et al., 1990; Sabbe et al., 2003; Van de Vijver et al., 2005; Spaulding et al., in press). Extending these studies, we have established a fully revised and intercalibrated database of diatom inventories of nearly 500 lakes covering most of the Antarctic and sub-Antarctic (Van de Vijver, unpubl., Verleyen et al. unpubl.). Preliminary analyses of this data set confirm that the diatom floras of Sub-Antarctic and Antarctic regions are significantly less diverse in their diatom floras than (Sub)Arctic floras, and that they show a high degree of endemism (Plate 1), with species varying in their geographical ranges. Furthermore, different biogeographical provinces can be identified that correspond to major boundaries in the distribution of invertebrate faunas and higher plants, indicating that the large-scale and long-term controls of the biogeography of macroorganisms and microbes must be similar.

While a robust time-calibrated phylogeny for the diversification within major clades of diatoms is not yet available, fossil evidence may shed light on the

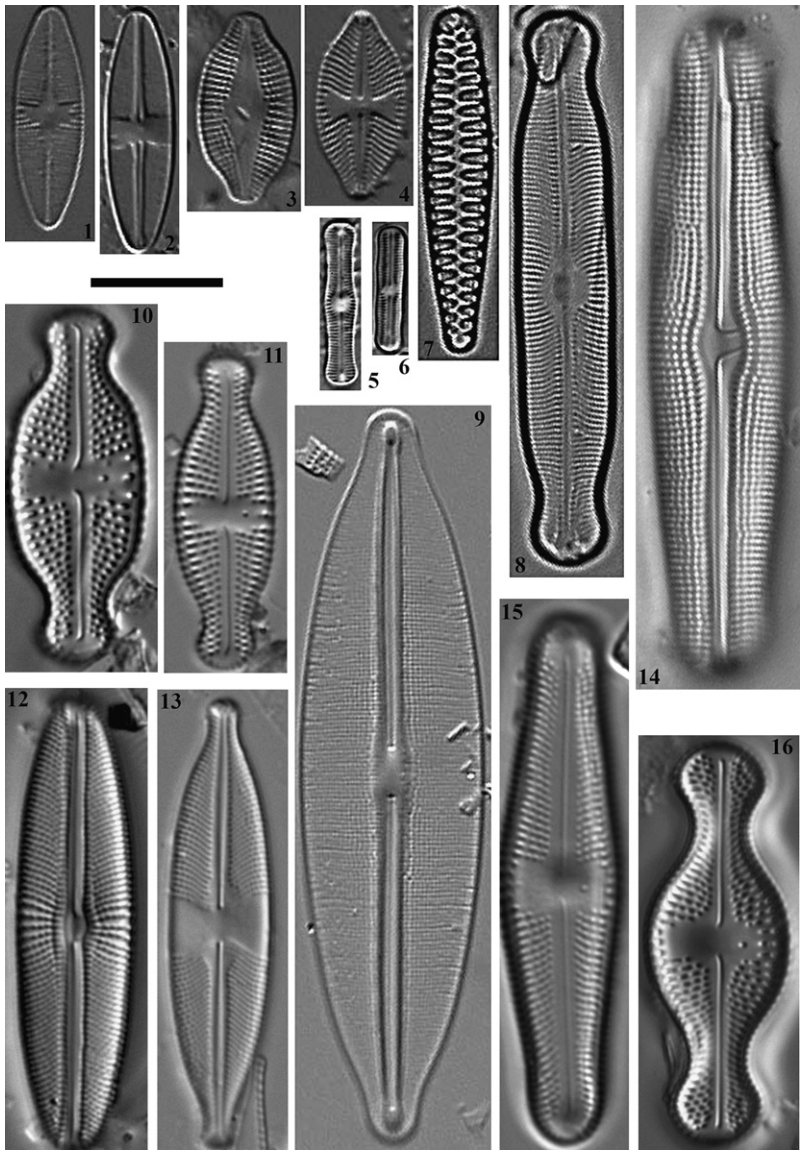

Plate 1. Endemic diatom taxa from the Sub-Antarctic Region (SA), the Maritime Antarctic Region (MA) and the Antarctic Continent (CA). SA: (1) and (2) *Psammothidium incognitum* (Krasske) Van de Vijver, (3) and (4) *Psammothidium manguinii* (Germain) Van de Vijver, (5) *Diadesmis ingeae* Van de Vijver, (6) *Diadesmis crozetikerguelensis* Le Cohu & Van de Vijver, (7) *Staurosira jolinae* Van de Vijver, (8) *Adlafia bryophiloides* (Manguin) Van de Vijver, (9) *Frustulia pulchra* Manguin. MA: (10) *Luticola muticopsis* (Van Heurck) Mann, (11) *Luticola austroatlantica* Van de Vijver & Kopalová, (12) *Muelleria regigeorgiensis* Van de Vijver & Spaulding, (13) *Stauroneis latistauros* Van de Vijver & Lange-Bertalot. CA: (14) *Muelleria peraustralis* (West & West) Spaulding & Stoermer, (15) *Luticola murrayi* (West & West – Mann, (16) *Luticola gaussii* (Heiden) Mann. Scale bar represents 10 μm.

changes that have occurred during the past millions of years and provide evidence of the persistence of genera and species. The few studies available to date suggest that there is great potential for detailed studies of geological deposits to understand the evolution of diatom floras in Antarctica. In the Marine Isotope Stage 5e (the last interglacial period, spanning about 130–117,000 years ago) sediments of proglacial Progress Lake in the Larsemann Hills (Hodgson et al., 2006) several diatom species were found that are today absent from the Antarctic continent but occur on several sub-Antarctic islands. This indicates that species' ranges are dynamic over longer time scales and may change in response to a combination of

changing climate dynamics, atmospheric circulation patterns and ability to disperse and colonise new environments; similar to the latitudinal shifts observed in terrestrial vegetation in the Northern Hemisphere during successive glacial cycles. Alternatively, these diatoms may have been present in Antarctica until they were finally driven to extinction during the last glaciation. In a recent study, Lewis et al. (2008) described a well-preserved sequence of fossil diatoms from a moraine-dammed basin in the western Olympus Range in the Mc Murdo Dry Valleys sector. The assemblages that were living in the lake around 14 million years ago were very different at the genus level from modern diatom communities in Antarctic lakes and bear a high resemblance to low-ionic strength lakes of the Arctic. Studies of these assemblages at the species level would provide evidence of their similarities to modern Arctic and/or Antarctic floras and inform us about their rates of change and divergence.

4. Evidence for long evolutionary histories of green algae

In diatoms, the elaborate structure of the siliceous cell walls offers a wealth of information that is used to delineate species. Repeatedly, studies combining morphological and molecular-genetic approaches with experimental breeding studies have demonstrated a good agreement between morphological, molecular and reproductive data (e.g. Behnke et al., 2004; Casteleyn et al., 2008, 2009; Vanormelingen et al., 2007). In green algae, however, delimitation of species and higher taxa based on morphology is contentious, especially in groups that are morphologically conservative and exhibit convergent evolution towards reduced morphology (e.g. Huss et al., 1999). For Antarctica, most data on green algae are largely restricted to morphology-based taxonomic inventories (Broady, 1996; Cavacini, 2001; Mataloni and Pose, 2001; Adams et al., 2006; Fermani et al., 2007; Zidarova, 2007) and suggest a high degree of cosmopolitanism. The currently available molecular data are fragmentary, and consist of a number of isolated taxonomic and ecophysiological studies on individual taxa (e.g. Moro et al., 2002; Pocock et al., 2004; Fell et al., 2006).

However, in a recent study (De Wever et al., 2009), molecular-phylogenetic techniques, including molecular clock analysis of nuclear encoded 18S rRNA gene sequences were used to characterize 61 Antarctic microchlorophyte isolates from 13 maritime and 30 continental Antarctic samples. Phylogenetic analysis showed that 14 distinct freshwater taxa were distributed among the classes Chlorophyceae and Trebouxiophyceae. Apart from one strain, all Antarctic sequences were different from non-Antarctic sequences currently available in GenBank. Although there is no generally accepted threshold of 18S rRNA sequence divergence for defining green algal taxa, most Antarctic sequences are divergent enough to be considered distinct species, genera or even higher-order taxa. These results thus indicate a wide phylogenetic diversity of apparently endemic Antarctic lineages at different taxonomic levels. They further contrast with earlier morphological studies and support the notion that Antarctica has developed a distinct regional flora rather than being dominated by cosmopolitan taxa. Consequently, given the high morphological similarity between unrelated microchlorophyte species that has resulted from convergent evolution, future studies should use molecular data, in addition to morphological data, to study green algal biodiversity and biogeography in Antarctica (Plate 2).

Estimated ages of the Antarctic green algal lineages based on a time-calibrated phylogeny ranged from 2.7–9.9 Ma for a number of chlamydomonad isolates (Chlorophyceae) to over 17–84 Ma for the majority of the sequences and 330–708 Ma for a trebouxiophycean isolate SC2-2. The widely differing branch lengths of the Antarctic lineages point to several independent but rare colonization events over a long time frame, and long-term survival in glacial refugia. As the phylogenetic tree was calibrated at only a single node (Chlorophyta–Streptophyta split between 700 and 1500 Ma) and relied on sequence data that were available on GenBank, the divergence times of the different Antarctic microchlorophyte strains should be regarded as rough estimates. The majority of the lineages (16 out of 26) have estimated ages between 17 and 84 Ma and probably diverged from their closest relatives around the time of the opening of Drake Passage (30–45 Ma) during the Eocene, which initiated the first transient glaciations on the continent. The lineages with longer branch lengths, including SC2-2 (330–708 Ma), have estimated ages that precede the break-up of Gondwana (65–100 Ma). Together, these findings suggest the existence of refugia being present during successive glacial cycles (Convey and Stevens, 2007; Convey et al., 2008, 2009) and conflict with the recolonization hypothesis, which proposes that fast colonization rates have resulted in the dominance of cosmopolitan species on Antarctica.

Our data also shed light on green algal dispersal within the Antarctic continent. The majority of

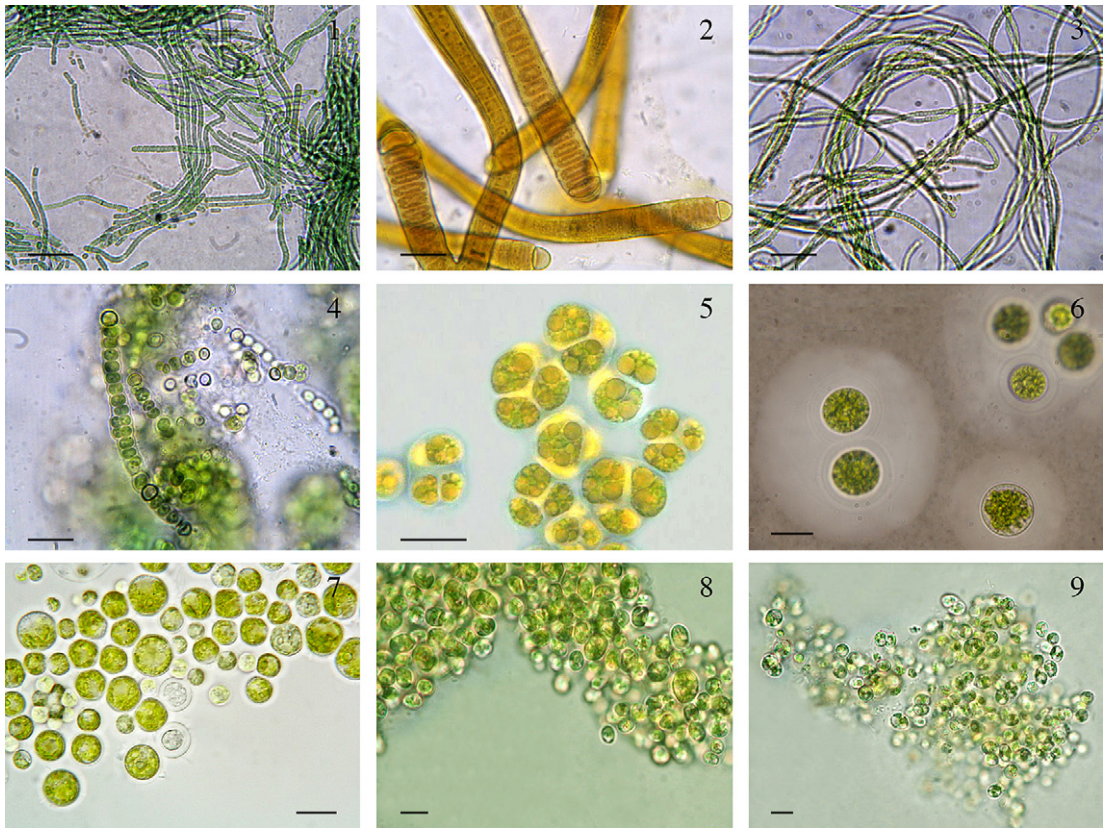

Plate 2. Antarctic cyanobacteria (1–4) and green algal (5–9) taxa. (1) *Plectolyngbya hodgsonii*, an Antarctic endemic representative. (2) *Calothrix* S16. (3) *Phormidium pristleyi* S26. (4) *Nostoc* S50. (5)–(9) Green algal strains, respectively VI8, VPL9-5, I6, WO8L-2 (*Scenedesmus* sp.) and EO5-4 (*Chlorella* sp.). Scale bars represent 10 μm.

Antarctic taxa were detected in only one Antarctic region, while none of the taxa was found in more than three of the five regions sampled. This implies that dispersal rates within Antarctica could be low and/or that immigrants are competitively excluded as a result of priority effects. Only two taxa, belonging to the genera *Chlorella* and *Scenedesmus*, were detected in three of the five regions, suggesting that these taxa may have more easily dispersed over the Antarctic continent. This is in agreement with Lawley et al. (2004), who found a similar lack of overlap between the eukaryotic biota of 'patterned ground soils' using a clone library approach from several widely separated Antarctic locations, with only a few taxonomic units showing apparently wider distributions. However, we have to bear in mind that these results were obtained from a small number of sampling sites that may differ in habitat properties. Extended taxon sampling and the use more variable molecular markers (such as rDNA internally transcribed spacer – ITS) will thus be required to elucidate phylogeographic patterns of green algal taxa within Antarctica.

5. Hidden diversity and endemism in cyanobacteria

Cyanobacteria are a particularly successful group in Polar Regions. They are abundant in lakes and ponds where they form benthic microbial mats, but also occur in the water column as well as in soils, cryoconite holes, and cryptoendolithic habitats (Vincent, 2000). Although several studies indicate the existence of genotypes found only in Antarctica (e.g. Priscu et al., 1998; Bowman et al., 2000; Smith et al., 2000; Vincent, 2000; Nadeau et al., 2001; Christner et al., 2003; de la Torre et al., 2003; Taton et al., 2003; Casamatta et al., 2005; Jungblut et al., 2005), comprehensive studies of the species diversity and geographical distribution are still in their infancy. As for green algae, molecular diversity of cyanobacteria is higher and therefore does not always coincide with the morphological diversity at ecological and biogeographical levels. Moreover, they are a very ancient group, responsible for the oxygenation of the Precambrian atmosphere, about 2.3 billion years ago. Recent and

ongoing studies, based on combined morphological studies and sequencing of 16S and ITS rDNA of strains as well as studies of uncultured diversity using clone libraries, lend further support to the idea that also for cyanobacteria much of the diversity remains to be discovered and that this diversity includes many potentially endemic taxa in Antarctica (Taton et al., 2006a,b) (Plate 2). However, these authors also found a number of OTUs containing non-polar sequences. Interestingly, these OTUs appear to be more widely distributed within Antarctica, which would support the idea that cosmopolitan OTUs are well adapted to transport and colonization and thus were quite successful in their dispersal and occupation of new habitats in different regions of Antarctica. Furthermore, OTU's based on molecular markers such as the 16S rRNA gene or ITS sequences might encompass significant microdiversity as suggested by slight variation in the sequence data. Such microheterogeneities might correspond to different ecotypes (Fuhrman and Campbell, 1998), and may further increase the genotypic diversity.

6. Diversity of bacteria

In parallel to the above-mentioned studies of other groups of micro-organisms, the isolation of heterotrophic bacteria from lacustrine and terrestrial habitats on a selection of different media and their subsequent preliminary identification based on partial 16S rRNA gene sequences is revealing a large diversity and variation between sites. Furthermore, fingerprint profiles based on rep-PCR indicate that very few fingerprint types are recovered in more than one site (3.5%). Depending on the site, approximately 5–50% of the 16S rRNA gene sequences recovered display less than 97% sequence similarity to named entries in the EMBL database (Peeters et al., unpublished), indicating that they represent unrecorded taxa which are potentially new to science. Previous studies of Antarctic bacterial diversity have also reported significant numbers of new groups (Brambilla et al., 2001; Van Trappen et al., 2002) and in recent years many new species and genera have been described from Antarctica (for example, Mevs et al., 2000; Van Trappen et al., 2004a,b, 2005; Hirsch et al., 2004; Yi et al., 2005; Lee et al., 2007). Most of these have not been reported from elsewhere and may be endemic to Antarctica. However, as bacterial diversity in general is estimated to be several orders of magnitude larger than the currently named species and genera (Hugenholtz et al., 1998; Schloss and Handelsman, 2004), it cannot be excluded that the taxa found in Antarctica may be found in other extreme cold habitats elsewhere on the planet. For example, *Shewanella hanedai* has been reported from the Arctic Ocean and from Ellis Fjord, Antarctica (Bowman et al., 1997); *Shewanella frigidimarina* was isolated from ice and cyanobacterial mats in Antarctica (Bowman et al., 1997) and also recovered from water in the Western Greenland Sea (Mergaert et al., 2001). Although information on the distribution of bacteria is fragmentary at present, as more habitats are studied and bacteria are identified to species level with molecular techniques, new information will allow more reliable conclusions on bacterial distribution and endemism.

7. Discussion

The assemblage of regional biota involves a balance between processes that operate over short time scales such as dispersal, colonization and extinction as a result of regional changes in geophysical and climatic conditions and biotic interactions, as well as, over longer time scales, migration of entire biota, speciation and extinction. For many groups of macroorganisms, decades of intensive recording of species' distributions, evidence from the fossil record, phylogenetic studies and extensive sampling of regions worldwide; provide an extensive and solid framework to study the processes affecting the composition and spatial-temporal dynamics of regional floras and faunas. For microbes, in contrast, much basic research remains to be done on all of the above fronts. The enormous diversity of prokaryote and eukaryote microbes makes this a challenging undertaking for most major taxonomic groups.

Extrapolating from the evidence for a strong regionalization and endemism among Antarctic diatoms, green algae and cyanobacteria discussed above, we hypothesize that these are not exceptional cases among the Antarctic microbial flora and fauna. For ciliates, for example, several cases of morphospecies endemic to the Antarctic continent are already known (Petz et al., 2007). Out of a total of 334 species identified in both polar regions, only 44 species were common to both Arctic and Antarctic freshwater bodies. While many morphospecies were classified as cosmopolitans, over 20% of the taxa found in any one of the studied areas were new to science. The low similarity in species composition between both polar areas further suggests that long-distance dispersal of limnetic ciliates is restricted and that some species have a limited geographical distribution.

One of the major flaws of the ubiquity hypothesis is the uncritical application of a (wide) morphospecies concept to eukaryotic microbes. Although we do not intend to review the many fundamental/philosophical and practical issues related to the definition of species concepts, there is abundant evidence that for many if not most eukaryotic microbial groups, morphospecies contain much hidden diversity (e.g. Mann, 1999; Vanormelingen et al., 2008). In the case of diatoms, we are confident that the currently adopted fine-grained morphospecies concept better reflects discontinuities in morphological variation and thus species' boundaries than the broad morphospecies concept used in earlier studies. Indeed, the use of a narrow morphospecies concept is broadly supported by several in-depth case studies on unrelated groups of diatoms which show good agreement between fine-grained morphological classification, molecular-genetic markers and breeding studies. The same problem was highlighted for cyanobacteria by Komarek (1999) who determined that about 60% of cyanobacterial morphospecies in ice-free areas of King George Island, were endemic. He explained the apparent cosmopolitanism of the cyanobacterial diversity based on the morphology by the fact that previous authors used determination keys written for temperate regions without considering the different ecologies.

However, the use of molecular data to define species is not without difficulties itself since marker choice and the level of sequence similarity used in the delimitation of OTUs or formal species will evidently influence conclusions as to whether endemism occurs within a given group. For example, Martiny et al. (2009) found that at the finest taxonomic level geographic structuring occurred within the cosmopolitan marine planktonic cyanobacterium *Prochlorococcus*, while in contrast, at a higher taxonomic level the distribution of phylotypes was correlated with environmental conditions. Rybalka et al. (2009) studied the genotypic diversity of the chloroplast-encoded *psb*A/*rbc*L spacer region within Antarctic Tribonemataceae and compared these to available sequence data from temperate and Arctic strains. Applying the monophyletic species concept, they did not find any evidence for the presence of endemic species. However, none of the Antarctic sequences were identical to sequences of temperate regions. This suggests that at the within-species level significant microdiversity exists that is geographically structured and that even when phylotypes appear to be cosmopolitan, this does not necessarily mean that Antarctic isolates/populations are identical to populations living at lower latitudes, nor that they are connected by current gene flow. In a study of the heterotrophic flagellate genus *Spumella*, Boenigk et al. (2007) found that only a minority of the morphotypes and (18S rRNA) phylotypes isolated from the Antarctic continent followed the worldwide trend of a linear correlation between ambient (air) temperature during strain isolation and heat tolerance of the isolates. A high percentage of the Antarctic isolates were obligate psychrophilic, while isolates from locations on all other continents tolerated higher water temperature, even if some of them were isolated at low ambient temperatures. This drastic deviation of Antarctic representatives of *Spumella* from the global trend of temperature adaptation of these organisms provides strong evidence for the existence of dispersal or colonization barriers, a subtle form of endemism. It would be interesting to know if this ecophysiological differentiation is mirrored in geographical structuring of more variable molecular markers.

One of the mechanisms that could underlie these different degrees of geographical structuring of genetic and ecophysiological variation could be differences in dispersal ability. There should be no surprise to find that different groups of microbes differ in their dispersal ability and level of endemism given their widely divergent evolutionary ages and genome make-up. However, very little is known about microbial dispersal and colonization, which was aptly illustrated in a recent review of this topic by Pearce et al. (2009). For example, recent or continual colonization could explain the small proportion of endemic fungi in Antarctica and the numerous fungi that appear to have a bipolar distribution (e.g. Galloway and Aptroot, 1995; Vishniac and Onofri, 2003). Again, however, different molecular markers for fungi should be tested to assess whether these indeed represent globally dispersed organisms or geographically isolated populations that have diverged allopatrically.

Whereas for eukaryotic microbes, overall diversity within Antarctica appears to be moderate, making it feasible to undertake comprehensive large-scale inventories to obtain a robust view of biogeographical patterns, the huge diversity of prokaryotes makes such analyses much more problematic. In Antarctica, recent studies of bacterial 16S rRNA gene clone libraries and community fingerprinting have discovered that prokaryotic communities are highly diverse, even in the extreme terrestrial systems in the ice-free oases (e.g. Niederberger et al., 2008; Verleyen et al, unpubl.). Culture-dependent approaches are revealing a high degree of new diversity, including numerous new species as well as higher taxa (for example, Mevs et al.,

2000; Hirsch et al., 2004; Van Trappen et al., 2004a,b, 2005; Yi et al., 2005; Lee et al., 2007). Rarefaction analyses based on clone library results further indicate that most microbial habitats and regions remain undersampled. Yet, some evidence indicates that also for these organisms, biogeographical rules of macroorganisms may be applicable. Yergeau et al. (2009) used PhyloChip analyses to study bacteria and archaea along a latitudinal gradient ranging from 51°S (Falkland Ils.) to 72°S (Coal Nunatak). Not only did they observe a lower number of taxa detected in individual samples than typically reported for temperate soil environments, but the number of bacterial and archaeal taxa significantly decreased with increasing latitude, with a large reduction in the southernmost sites (Fossil Bluff and Coal Nunatak). This pattern agrees well with diversity estimates based on 16S rRNA gene libraries (Yergeau et al., 2007). Interestingly, studies of northern hemisphere latitudinal gradients have not shown such latitudinal patterns in bacterial diversity, a situation that is similar to the patterns we described above for diatom diversity. A systematic examination of this pattern in other microbial groups (see also Hodgson et al., 2010) would be interesting and could reveal if these differences in latitudinal patterns of microbial diversity between the Southern and Northern Hemispheres can be generally applied.

8. Conclusion

Microbial biogeography in Antarctica offers many challenges and opportunities to study the nature and rates of adaptation of different groups of microbes to the harsh conditions and geographically isolated habitats on the continent and to investigate how much of the modern microbial diversity evolved *in situ* or is the result of more recent colonization events as the continent changed from a temperate forested state to the current ice-house conditions. High-throughput sequencing of DNA and RNA libraries now permit greatly increased sampling intensity for several phylogenetic groups, but also permit investigation of the geographical distribution of both taxa and functional genes present in microbial genomes. The ubiquity hypothesis has had an important impact on the discipline of microbial biogeography, and will remain to function as a null hypothesis against which to evaluate new data. Evidence is accumulating, however, that at least in Antarctica, the microbial biota is to a large extent the result of geographical isolation, and not just a subset of globally distributed taxa adapted to the extreme environments that characterize the continent.

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NiPR
National Institute of Polar Research

INTERNATIONAL POLAR YEAR 2007-2008

L'API, ANNEE POLAIRE INTERNATIONALE: FOCUS SUR LES POLES

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1. L'Année Polaire Internationale

Le 1er mars 2007 marquera le commencement de la quatrième Année Polaire Internationale (API) 2007-2008. Celle-ci aura lieu exactement 50 ans après l'Année Géophysique Internationale (AGI). Celle-ci avait marqué une avancée majeure dans la coopération pour la recherche polaire et avait vu la création d'une première base belge en Antarctique.

L'AGI est un événement qui reste mal connu en dehors de la communauté scientifique bien qu'elle ait véritablement révolutionné notre perception de la terre, de son fonctionnement et de son évolution.

L'Année Polaire Internationale 2007-2008 est une initiative soutenue par de nombreuses organisations scientifiques et environnementales à travers le monde (voir www.ipy.org). Elle est coordonnée par le Conseil International pour la Science (ICSU) et l'Organisation mondiale de Météorologie (WMO).
Elle a pour but :
- d'étudier et d'aider à mieux connaître les régions arctiques et antarctiques;
- de définir leur importance pour le climat de notre planète;
- d'étudier l'impact des changements climatiques sur leur environnement, leur faune, leur flore, et les êtres vivants qui les habitent.

L'API veut aussi éveiller et stimuler l'intérêt du public et des jeunes grâce à des activités de vulgarisation et de communication et au travers de manifestations artistiques.

Pour mieux coordonner l'effort scientifique international, des appels à idées ont été lancés en 2005. 1100 idées ont été regroupées par thématique, pour obtenir de gros projets internationaux qui devaient répondre à plusieurs critères pour obtenir le «label API» du Comité International API : haute qualité scientifique, multi-disciplinarité, caractère international, efficacité,… Finalement, 228 projets scientifiques et de communication ont été officiellement sélectionnés. Ces projets ne sont pas financés par l'API : chaque partenaire cherche des financements au niveau national. Par contre, le fait d'être fédérés dans un projet API permet d'échanger des échantillons, de réaliser des études multidisciplinaires sur les mêmes biotopes, d'accéder plus facilement aux résultats, d'harmoniser les méthodologies, etc.

Le projet MERGE (n°55) traite de l'impact des changements climatiques sur les communautés microbiennes (Microbiological and Ecological Responses to Global Environmental Changes in Polar Regions) et le projet AMBIO (voir plus loin) en fait partie.

Certains pays (Pays-Bas, Espagne, Canada,…), ont bénéficié de budgets supplémentaires dédiés spécifiquement à l'Année Polaire Internationale, mais malheureusement ce ne fut pas le cas en Belgique. La cérémonie d'ouverture officielle de l'API aura lieu le 1er mars, 11 heures à Paris.

2. L'Antarctique est un continent microbien

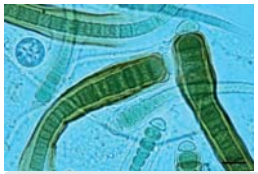
Cette déclaration peut sembler provocatrice et pourtant l'Antarctique est bien un continent microbien, c'est-à-dire, que la majorité de ses habitants permanents sont des microorganismes. En effet, les phoques, pingouins, sternes, skuas, … n'y résident pas de manière permanente et se nourrissent en mer. Par contre, les microorganismes se sont bien adaptés aux conditions extrêmes et colonisent des habitats parfois inattendus.

Cyanobactéries entre les cristaux de quartz, quelques millimètres sous la surface des rochers (Dry Valleys Mc Murdo, Antarctique) Photo : B. Büdel

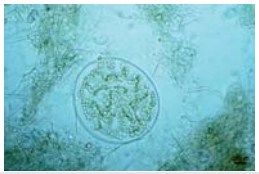
Il s'agit de la face inférieure de roches en quartz, de microfractures entre des cristaux de quartz, de microsillons d'eau liquide dans des masses de glace…

Parmi ces microorganismes : Les cyanobactéries

Les cyanobactéries sont des bactéries photosynthétiques, souvent de couleur bleu-vert, d'où leur nom. On les appelle aussi algues bleues ou cyanophycées, car jusqu'aux années 70, elles étaient considérées comme des algues.



Calothrix



Nostoc

Phormidium

Photos : A. TATON

Les cyanobactéries ont joué un rôle important dans l'évolution de la vie car c'est dans ces organismes qu'est apparu le phénomène de la photosynthèse produisant l'oxygène, dont elles ont plus tard fait bénéficier les algues et les plantes.

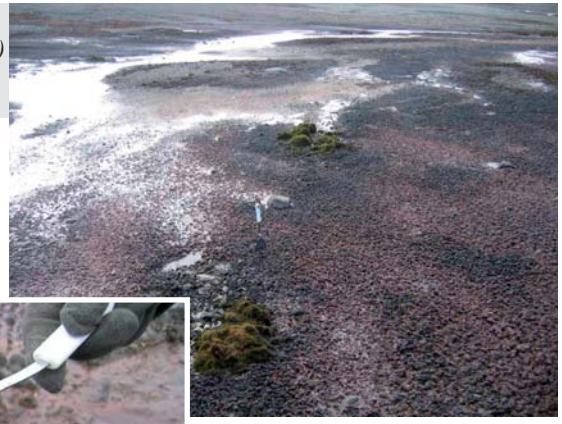
Ce phénomène est apparu il y a environ 3 milliards d'années et a transformé l'atmosphère terrestre qui ne contenait pratiquement pas d'oxygène au début. C'est donc grâce à elles que les organismes comme les algues, les plantes, les champignons, les animaux et nous-mêmes ont pu évoluer.

Les cyanobactéries pouvant vivre «de soleil et d'eau fraîche», elles ont connu à cette époque un «âge d'or» et on retrouve bien des fossiles qui témoignent de leur abondance au Précambrien. Elles ont ensuite régressé à mesure que les algues et les plantes ont envahi leurs biotopes aquatiques et humides et que les prédateurs, par exemple le zooplancton, ont commencé à les consommer.

Sur le continent antarctique, les cyanobactéries peuvent former de grands tapis rose-orange couvrant les zones humides et les fonds des lacs grâce à l'absence de concurrence par les plantes (seulement 2 espèces de plantes à fleurs sur le continent) et de prédation par les animaux (pas de poissons, peu de zooplancton).

Tapis microbien, Livingston Island (Péninsule antarctique)

Photo : A. Wilmotte



Les 2 espèces de plantes à fleurs du continent Antarctique

Deschampsia antarctica et Colobanthus quitensis sur Lagotellerie island

Photos: © Ron Lewis SMITH, British Antarctic Survey

Elles se retrouvent donc un peu dans les conditions de leur «âge d'or» il y a 2 milliards d'années. Une autre ressemblance avec cette époque faste est la croissance en couches, parfois séparées par des dépôts minéraux. La couche supérieure, plus exposée aux intensités lumineuses fortes en été, aux UV, aux vents… est plus riche en pigments protecteurs et contient pas mal d'organismes morts. Sa présence protège les couches internes qui apparaissent plus pauvres en pigments protecteurs et composées d'organismes en bon état.

Ce type de croissance en couches ressemble aux stromatolithes, structures fossiles du Précambrien, mais dont il existe actuellement des exemples vivants, par exemple en Australie (Shark Bay).

1 : Stromatolithe fossile du Barrémien (Crétacé) de Croatie. Photo F. Boulvain (ULg).
2 : Stromatolithe fossile du Protérozoïque (2 milliards d'années) au Québec. Photo A. Bourque (U Laval).
3 : Stromatolithes vivants, Shark Bay, Australie. Photo A. Bourque.

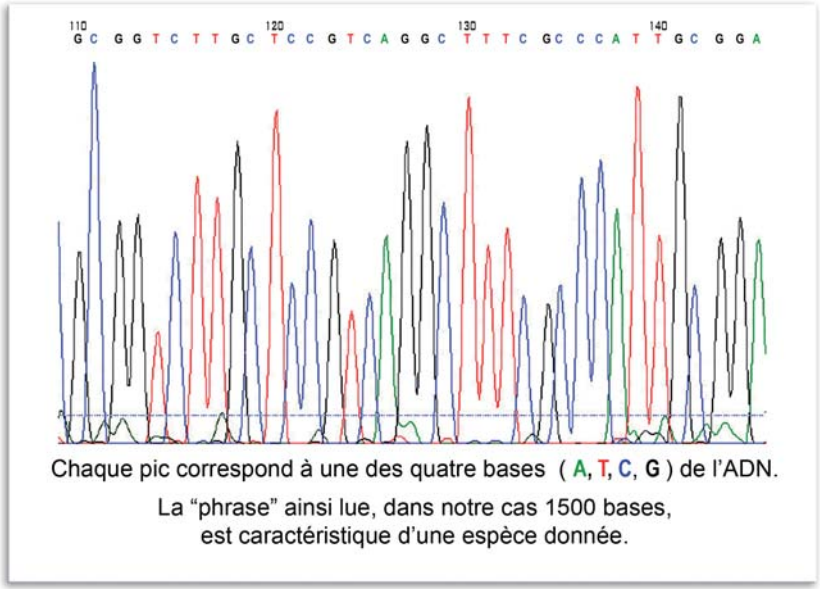
Si les cyanobactéries forment la trame de ces tapis, d'autres microorganismes comme les bactéries, microalgues, champignons participent à la vie de ces communautés et à la production et au recyclage de la matière organique.

Exploration de la biodiversité des microorganismes antarctiques

L'Antarctique est un continent isolé et soumis à des conditions climatiques et écologiques très particulières : extrêmes de températures (en moyenne de -70° à -15°), de lumière (nuit continue en hiver à jour continu en été), vents violents sur le continent, aridité (moins de 5 cm de précipitations à l'intérieur). Il est donc possible que les habitants permanents aient dû développer des adaptations spécifiques pour y vivre et donc, que la biodiversité soit différente de celle des habitats plus tempérés. D'ailleurs, bien des nouvelles espèces et genres de bactéries polaires ont été décrites récemment (notamment par le laboratoire de microbiologie de Gand). Il reste donc de nouvelles espèces à découvrir sur ce continent !

Les microorganismes : comment les reconnaître ?

Ceci nous amène à parler de la façon dont nous identifions les espèces de microorganismes. Alors que pour les plantes et animaux, on dispose facilement de caractères de forme, de couleur,… permettant de les distinguer et de les identifier, ce n'est pas le cas pour les organismes microscopiques. N'oublions pas que le microscope ne date que de 350 ans. C'est pourquoi, pour caractériser de manière fiable un microorganisme, on a recours à des séquences de son matériel génétique, une sorte de «code-barre». C'est d'ailleurs sur l'information contenue dans le matériel génétique que se base la définition de l'espèce bactérienne.



Chaque pic correspond à une des quatre bases (A, T, C, G) de l'ADN.

La "phrase" ainsi lue, dans notre cas 1500 bases,
est caractéristique d'une espèce donnée.

Grâce à la comparaison des séquences «code-barre» provenant des souches en culture à partir des échantillons ou étant directement extraites des échantillons, on peut savoir quelles espèces sont présentes et faire une carte de leur répartition géographique et écologique. Celle-ci peut être interprétée en fonction de ce que l'on sait des écosystèmes et du style de vie des organismes.

Le projet AMBIO

Avec les équipes du Prof. Anne WILLEMS (microbiologie) et Wim VYVERMAN (Protistologie) de l'Université de Gand, je coopère dans le projet BELSPO AMBIO pour :

- explorer et découvrir la diversité des microorganismes des tapis microbiens dans les milieux aquatiques et humides ;

- contribuer à déterminer si les facteurs qui gouvernent la répartition des plantes et des animaux supérieurs, sont aussi ceux qui expliquent la distribution des microorganismes.

Sur base du fait que les microorganismes construisent des populations de taille énorme et peuvent théoriquement se disséminer partout et facilement, des scientifiques ont émis l'idée que les microorganismes sont potentiellement présents partout mais que les conditions de l'environnement déterminent ceux qui vont coloniser un endroit. Baas-Becking a ainsi formulé cette hypothèse «*Tout est partout, mais la nature sélectionne*».
Ceci implique que des environnements similaires à différents endroits de la Terre devraient contenir les mêmes espèces et qu'il est impossible d'avoir des espèces de microorganismes endémiques (limitées à un endroit).

Nos premiers résultats montrent cependant qu'il est possible de trouver des espèces potentiellement locales (ou endémiques) et dont la distribution géographique semble être limitée. A confirmer… en étudiant plus d'échantillons de biotopes différents.

Une meilleure connaissance de la diversité des microorganismes et de leur répartition est aussi utile pour des buts plus appliqués :

- (1) elle peut contribuer à la sélection des sites antarctiques à protéger spécialement (ASPA : aire spécialement protégée de l'Antarctique). Souvent, ces sites sont choisis sur base de la présence de vestiges historiques, d'espèces endémiques d'animaux, lichens ou mousses tandis que l'existence des microorganismes n'est que peu prise en compte par manque d'informations génétiques.

- (2) grâce à l'isolement de souches de microorganismes qui viennent enrichir les collections de BCCM (collections coordonnées belges de microorganismes ; http://bccm.belspo.be/index.php), de nouvelles ressources biologiques deviennent disponibles pour des usages scientifiques ou appliqués, comme la recherche d'enzymes du froid (http://www.ulg.ac.be/biochlab/main/research.html) ou de molécules aux propriétés antibiotiques.
Une collection de cyanobactéries polaires est d'ailleurs en constitution (http://bccm.belspo.be/projects/programme2005-2008/c30014).

- (3) elle peut servir à suivre l'impact des changements climatiques.
Sur la Péninsule, au climat plus doux, les tapis microbiens s'étendent sur de grandes surfaces durant l'été, dans les dépressions où coule l'eau de fonte des glaces.

Si le changement climatique provoque une élévation de température, un allongement de l'été et des changements dans les précipitations, cela pourrait aussi se traduire par des changements des surfaces occupées, de diversité, des invasions de nouveaux organismes, etc...

- (4) dans le cadre de l'évaluation environnementale de la nouvelle base belge Princesse Elisabeth (voir article du Prof. Lejeune), il est aussi demandé par le Comité de Protection Environnementale de l'Antarctique que l'impact sur les organismes vivants et leurs biotopes soit suivi.

- (5) l'ADN fossile provenant de sédiments lacustres qui se sont accumulés au cours du temps et n'ont pas subi de perturbation (par exemple, dans le fonds de lacs couverts de glace) peut permettre d'identifier la diversité passée des microorganismes il y a quelques milliers d'années (http://www.laquan.ugent.be/).

L'Antarctique, un continent fascinant à bien des égards, une Terre de recherche pour les glaciologistes, géologistes, biologistes, et autres chasseurs de météorites ...
A la fois acteur et victime des changements climatiques, il est un témoin de la façon dont nous vivons sur cette planète.

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Dans le cadre du printemps des sciences, Annick Wilmotte présentera l'activité : « Comment les cyanobactéries exploitent la lumière - Les écosystèmes polaires »
http://www.ulg.ac.be/sciences/organisateurs/organis2007/fiche_activit/27_wilmotte.pdf

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