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Lichens from the Utsteinen Nunatak (Sør Rondane Mountains, Antarctica), with the description of one new species and the establishment of permanent plots

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Abstract

In order to establish baseline environmental conditions at the Utsteinen Nunatak (Sør Rondane Mountains, Antarctica) chosen for the installation of the new Belgian Antarctic research station, a detailed survey of the different lichen species was performed in 2007. The establishment of permanent plots will allow the accurate monitoring of possible future impacts of human activities on the biodiversity and, on a long-term scale, to detect future climate changes. A complete survey of the lichen species was made for each of the 23 permanent plots of the Utsteinen Ridge. The abundance of each species within each plot was also evaluated. Exhaustive lists of lichens were also completed for other parts of the Utsteinen Nunatak. Additionally, historic collections from the same area were revised.

A total of twenty-three lichen species and two lichenicolous fungi was detected in the recent samples whereas the historic material yielded three additional species. These results raise the total number of known taxa for the Sør Rondane Mountains from ten to 28, despite the small area investigated. *Trapelia antarctica* is described as new to science. *Buellia bastini* is synonymized with *Buellia nelsonii* and *Lecidea autenboeri* is synonymized with *Carbonea vorticosa*. The lichen flora of Utsteinen is made of a high percentage (48%) of Antarctic endemics.

Lichens were abundantly present on the Utsteinen Ridge. The numbers of species per plot varied from one to 18 with an average of ten. The detailed grid map for the Utsteinen Ridge will be useful in monitoring future changes in lichen population and diversity. Five plots presented the richest lichen flora and need therefore careful protection against any future human activities.

Key words: Dronning Maud Land, monitoring, Princess Elisabeth Station, taxonomy, *Trapelia*

Introduction

The Sør Rondane Mountains (SR Mountains, Figs 1–2, 71°–72° S/20°–30° E) form a typical coastal margin mountainous area, composed of a series of Nunataks, in the eastern part of Dronning Maud Land (East Antarctica). The SR Mountains are part of a series of mountain ranges, running from the Borg Massif (72°45'S/3°30'E) in Western Dronning Maud Land to the Yamato Mountains (also called Queen Fabiola Mountains) (71°30'S/35°40' E) in Eastern Dronning Maud Land. The area follows the Antarctic coastline 200 km land inwards (Van Autenboer 1964), stretching over a distance of 220 km in an east-west direction, culminating at ca. 3000 m (Ohyama *et al.* 1991). The Utsteinen Nunatak (Figs 2, 4 & 5) located a few kilometres north of the SR Mountains, (71°57'S/23°20'E), is composed of granite rocks and consists of two peaks with a maximum elevation of 1564 m. Several blue ice fields and some surface lakes, frozen till the beginning of the summer, surround the nunatak. The south-eastern side of Utsteinen has a large wind scoop (Belgian Science Policy 2007). Climatic data were available from an automatic weather station installed during 2005 at Utsteinen with results analogous to the 1987–91 series from Asuka station, situated 55 km further north-east and 466 m lower in elevation. Average annual temperature at Utsteinen is -18°C, varying between -8°C (December) and

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-25°C (September). The daily maximum does not exceed zero in summer, while the daily minimum reaches -36°C in winter, with the yearly variation of the temperature curve being characteristic of a continental climate. The sun stays permanently below the horizon from May 16 to July 28 (Belgian Science Policy 2007).

Information on biological research in the area is limited. Dodge published in 1962 the only lichen report of the area, listing ten lichen species collected by the Belgian Antarctic Expedition in 1959–1961, three of which were described as new species (*Blastenia autenboeri* C.W.Dodge 1962: 305*, Buellia bastini* C.W.Dodge 1962: 307 and *Lecidea autenboeri* C.W.Dodge 1962: 305). The extreme remoteness of the area and the difficult connections with other research stations are the main reasons for the lack of information. However, lichenological studies were conducted in other areas of the region of Dronning Maud Land, results being reported notably by e.g., Lindsay (1972), Øvstedal (1983a,b, 1986), Thor (1995) and (Nayaka & Upreti 2005).

The past 50 years, human presence in the SR Mountains remained quite limited. The mountains were discovered in 1937 by a Norwegian expedition led by Lars Christensen. Based on photographs taken by the US Navy in 1946–1947, a first map of the area was drawn in 1957 by the Norsk Polarinstitutt. On the occasion of the International Geophysical Year, the Belgian Expedition settled the Base Roi Baudouin (1958–1967), situated at the coast in Breid Bay (Eastern Dronning Maud Land) on the ice shelf. Research activities were concentrated on the coastal zone but also on the SR Mountains, that were extensively studied during that period. In 1986, Japan installed Asuka Station at the foot of the SR Mountains but the base was only active for a period of 6 years before it had to be closed (Ohyama *et al.* 1991, Belgian Science Policy 2007).

Renewed interest in the area, relatively far away from any other research station in Antarctica, revived the idea of a new research station near the SR Mountains. In 2007–2008, a new Belgian base, the Princess Elisabeth Station, was constructed on the Utsteinen Ridge (71°56’59”S/23°20’49”E) next to the Utsteinen Nunatak, 173 km inland from the former Roi Baudouin base and 55 km from the former Asuka station (Belgian Science Policy 2007).

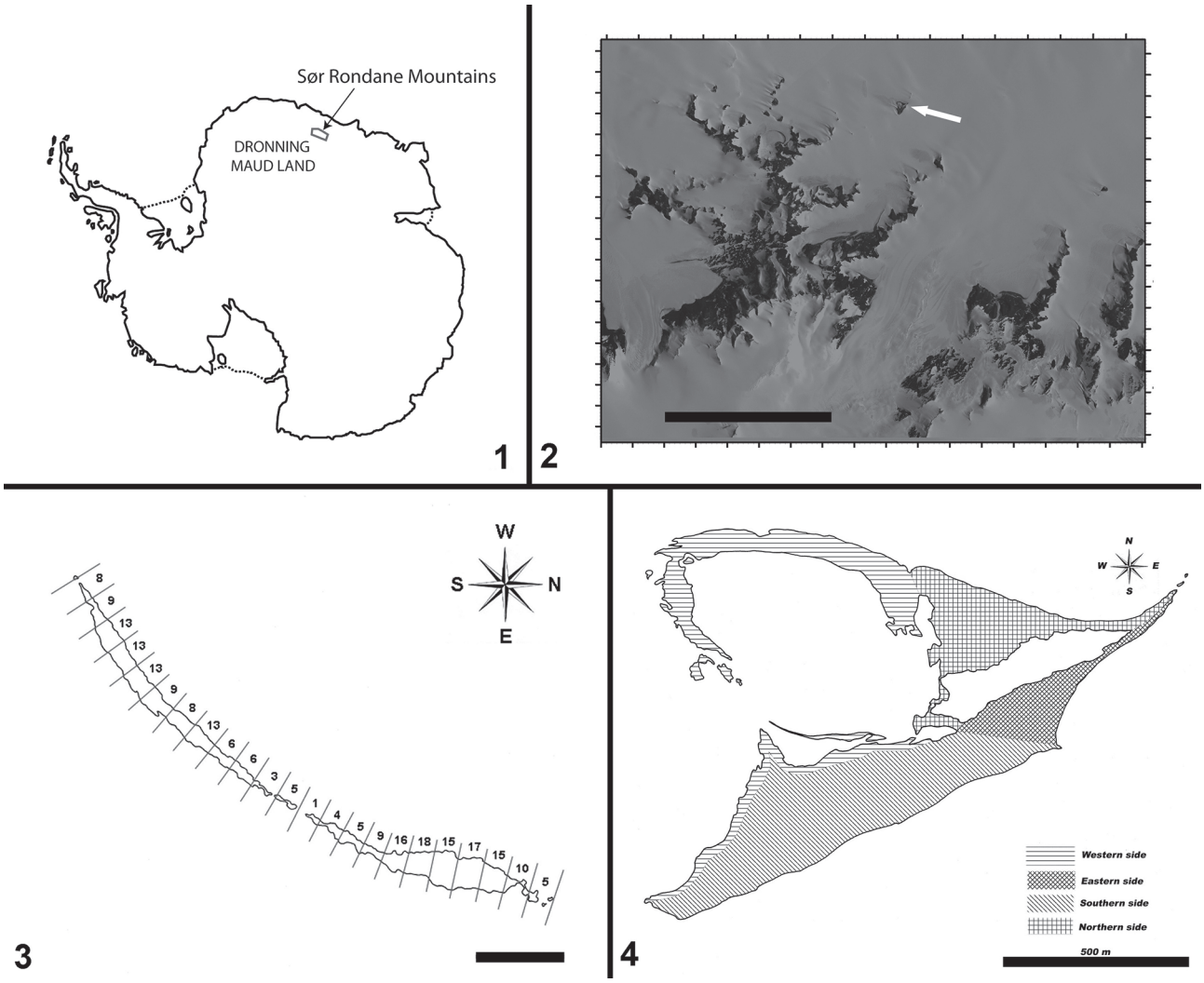
Before the installation of the new Belgian research station, it was essential to achieve a list of the regional fauna and flora, present near the construction area. These baseline data will be used on one hand for the evaluation of future impact of the station and related human activities on the biodiversity of this region and on the other hand to detect, on a long-term scale, future climate change. Results were already published on the microorganisms. Culturable heterotrophic bacterial diversity revealed isolates belonging to numerous potential new taxa, many of them currently restricted to Antarctica (Peeters *et al.* 2011). This may be explained partly by the inland and terrestrial nature of the pristine environment of Utsteinen, before the station was constructed (Peeters *et al.* 2012). An important cyanobacterial diversity with a high proportion of potential endemic taxa (46%) suggested that the study area could have acted as a biological refugium during past glaciations (Fernández-Carazo *et al.* 2012). The present study sampled the vegetation (which consists almost exclusively of lichens and one bryophyte) of the area where the base was to be built. In addition, previously collected lichen material, present in the collection of the Botanic Garden Meise, from the area was re-examined.

Material and Methods

Field studies

During the BELARE expedition in 2007, one of us (DE) visited the study area from 11 January to four February. A detailed study of the lichen vegetation was performed for the Utsteinen Ridge chosen for the construction of the Princess Elisabeth base. The ridge is situated 300 m north of the Utsteinen Nunatak and entirely composed of granite rock. It is 700 m long and a few meters large and at an elevation of 20 m above the surrounding snow surface. Based on a topographic grid provided by the I.P.F (International Polar Foundation), a map was designed for the Utsteinen Ridge to be able to locate precisely the different lichen species, into 23 plots of 30 m long each (Fig. 3). The edges of the plots were permanently marked on the field with metal screws fixed in the rock along the western side. Twenty-four screws were fixed in total, starting with number zero at the southern end of the ridge (71°57’03.8”S/23°20’47.2”E, elev. 1377 m) to 23 at its most northern tip (71°56’42.5”S/23°20’41.2”E, elev.1357 m). For each plot, a complete survey of the lichen species was performed (table 1). The abundance of each species within each plot was evaluated, subdividing them in three categories: a. species covering up to 1 dm^2 (+), b. species covering between 1 dm^2 and 1 m^2 (++) and c. species covering more than 1 m^2 (+++). The indication of the abundance of each species needs to be reflected upon cautiously. A lot of lichen communities were covered by snow during the survey in January–February 2007. Therefore, their abundance may change considerably due to other weather conditions. That’s why the snow cover percentage was

included for each plot. The three abundance categories provide however a reliable idea on the abundance of the species on the different parts of the ridge.



FIGURES 1–4. Maps of the studied sites. Fig. 1. Map of Continental Antarctica. The arrow indicates the position of the Sør Rondane Mountains; Fig. 2. Satellite photo of the study area in the Sør Rondane Mountains. The arrow indicates the position of the Utsteinen Nunatak; Fig. 3. Sketch map of the Utsteinen Ridge with the 23 plots of 30 m long delimited by straight lines. The total numbers of lichen species found per plot is indicated.; Fig. 4. Sketch map of the Utsteinen Nunatak.

The surroundings of the Utsteinen Ridge were also surveyed to provide information for preparation of a future biological scientific program linked to the station. Therefore, exhaustive lists of lichens were completed for the northern, eastern and western part of the Utsteinen Nunatak (Figs 4 & 5). The southern part could unfortunately not be explored due to important wind scoop, i.e. a saucerlike depression in the snow caused by the eddying action of the wind. The survey included also a sampling trip to the Teltet Nunatak (71°59’51.7”S/23°30’56.7”E, elev. 1450 m) and another to a so-called “dry valley” (72°06’59.8”S/23°09’29.5”E, elev. 1700 m) in the Sør Rondane Mountains.

The collecting sites in 1959–1961 during the activities of the Roi Baudouin base were the Petrel Egg Nunatak (72°00’23.8”S, 22°49’44.5”E, elev. 1500 m), Romnes Nunatak (71°28’S/23°58’E, elev. 1000 m), Lagkollane (gravity point, 72°06’20”S/22°00’05”E, elev. 1488 m) and an unknown locality (72°15’S/22–25°E, elev. 1500 m) in the Sør Rondane Mountains.

Samples

All specimens cited here are preserved in BR. All localities are in the Sør Rondane Mountains, Dronning Maud Land, Antarctica.

TABLE 1. Distribution and abundance of the lichens per plot of the grid map of the Utsteinen ridge. Legend—: -: not present, +: up to 1 dm^2, ++: 1 dm^2 to 1 m^2, +++: more than 1 m^2. The % of snow cover is given for each plot. The distinction between *Lecidea cancriformis* and *Lecidella siplei* was not easy on the field because of an important variability of the species, especially in the thallus morphology. Therefore, confusions between both species might have occurred in the field.

Plots	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	total
Maximum width of the rocky plots (m)	8	19.5	21.5	25	22	21	17.5	17.5	12	10	6.5	10.5	6.5	12	13	10.5	20.5	35	39	32.5	29	15	14	
% of the snow cover	60	30	20	20	45	55	40	40	45	60	70	85	70	40	45	30	40	40	40	30	25	45	75	
Bacidia johnstonii	++	++	+++	+++	+++	+++	++	++	+++	++	++	-	-	-	-	+	+	++	+	+	+	-	-	17
Buellia frigida	-	++	-	-	-	-	-	++	-	-	-	-	-	-	++	-	+++	+++	+++	+	+++	-	-	8
Caloplaca tominii	-	-	+	+	++	+	+	++	-	-	-	-	-	-	-	+	+	+	+++	+	++	+++	+	14
Candelariella flava	++	++	+++	+++	++	+++	+	++	+	-	-	+	-	-	+	++	+++	+++	+++	+++	++	++	+	19
Lecanora expectans	-	-	+	-	+	+	-	+	-	+	-	-	-	-	-	++	+	+	+	-	+	+	-	11
Lecanora fuscobrunnea	-	-	++	+	+	-	-	+	-	-	-	-	-	-	++	-	+	+	-	+	+	-	-	9
Lecanora mons-nivis	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-	+	-	-	-	4
Lecidea cancriformis	++	++	+++	+++	+++	++	++	++	++	++	+++	++	+++	+++	++	++	+	++	+++	+++	++	++	++	23
Lecidella siplei	++	++	+++	++	++	+	+	-	++	++	+++	++	-	++	-	++	+	++	++	+	+	+	++	20
Myriospora sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	1
Physcia caesia	-	-	+	++	++	-	-	++	-	-	-	-	-	-	-	-	+	++	+++	+++	+++	++	-	10
Physcia dubia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	+	+	++	++	-	-	5
Pseudephebe minuscula	+	+++	+++	++	+++	++	++	++	-	+	-	+	-	+	+	++	+++	+++	+++	+++	+++	+++	++	20
Rhizocarpon geographicum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	++	-	-	-	-	-	2
Trapelia antarctica	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	7
Umbilicaria aprina	+	+	-	+	+	-	-	+	+	+	-	-	-	-	-	++	+++	+++	+++	++	+++	++	-	14
Umbilicaria decussata	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	2
Usnea sphacelata	+	-	+	++	+	++	+	++	-	-	-	-	-	-	-	-	++	+++	+++	+++	+++	++	-	13
Xanthomendoza borealis	-	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-	++	++	++	+	+	+	-	10
Xanthoria elegans	+	+	+	+	-	-	-	-	+	-	-	+	-	-	-	+	++	+	+	+	+	-	-	12
Total	8	9	13	13	13	9	8	13	6	6	3	5	1	4	5	9	16	18	15	17	15	10	5	

Microscopical and chemical observations

Microscopical examination was done using hand-made sections in water, 5% KOH (K) or Lugol's reagent (1% I_2) without (I) or with KOH pre-treatment (K/I). Measurements and drawings of asci and ascospores all refer to material examined in KOH. Ascospore measurements are indicated as (minimum–) $X–\sigma_x–X+\sigma_x$ (–maximum), all values rounded to the nearest multiple of 0.5 µm, followed by the number of measurements (n); the length/breadth ratio of the ascospores is indicated as l/b and given in the same way. Thin-layer chromatography (TLC) of acetone extracts was performed in solvant systems EA, G and C on silica gel 60 F254 layer glass plates and aluminium plates of 20 × 20 cm. 10% sulphuric acid was used as a reagent for the visualization of the spots (Orange *et al.* 2001).

Results

A total of twenty-three lichen species and two lichenicolous fungi were observed in the recent samples; the old material included three additional species. One lichen species appears to be new to science and is formally described in the genus *Trapelia*; one lichenicolous fungus might be new to science but is left undescribed as the material is very sparse; it should be looked for on its host [*Physcia dubia* (Hoffm. 1796: 156) Lettau (1912: 254)]. In contrast, only one species of bryophyte [*Schistidium antarctici* (Cardot 1906: 15) L.I.Savicz & Smirnova 1965: 252], was found in the study area. The lichen flora in the SR Mountains was dominated by crustose lichens represented by 15 species in the genera *Amandinea* (1), *Bacidia* (1), *Buellia* (3), *Caloplaca* (1), *Candelariella* (1), *Lecanora* (3), *Lecidea* (1), *Lecidella* (1), *Myriospora* (1), *Rhizocarpon* (1) and *Trapelia* (1). The foliose lichens in the area were represented by seven species in the genera *Candelaria* (1), *Physcia* (2), *Umbilicaria* (2), *Xanthomendoza* (1), *Xanthoria* (1), while only three fruticose lichens were present, i.e. genera *Pseudephebe* (1) and *Usnea* (2). The lichenicolous taxa belonged to the genera *Arthonia* (1, on *Xanthomendoza*), *Carbonea* (1, on *Physcia dubia*) and cf. *Toninia* (1, on *Physcia dubia*).

The lichens were abundant on the Utsteinen Ridge with 20 species, especially along the cracks and on the gravel whereas the more exposed rocky surfaces were almost devoid of lichens. The abundance and distribution of the taxa were studied for the ridge using the 23 plots of the grid map (Fig. 3, Table 1). The number of species per plot varied from one to 18 with an average of ten. The plots 17 to 21 presented the richest flora with 15 to 18 species, whereas the plots 11–15 (between 71°56'54.5"S–23°20'50.7"E and 71°56'49.7"S–23°20'48.8"E) connecting the two main parts of the ridge showed the lowest diversity with only one to five species. This can be easily explained by the fact that the plots of the pass are narrower with a more important snow cover. *Lecidea cancriformis* C.W.Dodge & G.E.Baker (1938: 539) was the most abundant and widespread species being present in every plot, followed by *Lecidella siplei* (C.W.Dodge & G.E.Baker 1938: 530) May.Inoue (1991: 282) and *Pseudephebe minuscula* (Nyl. ex Arnold 1978: 293) Brodo & D.Hawksw. (1977: 140) (20 plots each), *Candelariella flava* (C.W.Dodge & G.E.Baker 1938: 605) Castello & Nimis (1994: 6) (19 plots), *Bacidia johnstonii* C.W.Dodge (1948: 109) (17 plots), *Caloplaca tominii* (Sav. 1930: 194) Ahlner (1949: 159) and *Umbilicaria aprina* Nyl. (1863: 12) (14 plots). The rarest species were *Myriospora* sp. (1 plot), *Rhizocarpon geographicum* (L. 1753: 1140) DC. in Lamarck & de Candolle (1805: 365) (2 plots) and *Umbilicaria decussata* (Vill. 1789: 964) Zahlbr. (1932: 490) (2 plots).

The surveyed localities exhibited a certain variation in the occurrence of lichen species. Fewer species (18) were found on the Utsteinen Nunatak, despite being much larger than the Utsteinen Ridge. Moreover, the western side of the nunatak bore only four species of lichens. Although on the northern side, 12 species were recorded, the lichen communities were only very sparsely distributed on the rocky slope. The southern side was not accessible so that no data were available from this rocky slope. Finally, the richest part of the nunatak is the eastern side where 17 species were recorded. The lichens were however abundant only at the base of the rocky slope under the petrel colony, as well as at the northern part of this side where a lichen vegetation, similar to the one existing on the ridge, were observed. Two species, viz. *Amandinea petermannii* (Hue 1915: 96) Matzer, H.Mayrhofer & Scheid. (1994: 39) and *Candelaria murrayi* Poelt (1974: 203) were only found on the Utsteinen Nunatak (eastern side) but in very low quantities (two tiny specimens each). Their presence might be linked to the snow petrel colony.

Lichens were also poorly represented in other ice and snow free areas visited briefly in the SR Mountains S and SW of Utsteinen. *Buellia subfrigida* May.Inoue (1993: 20) was an additional species collected once in a dry valley. Among the older material published by Dodge (1962), three additional species [*Buellia nelsonii* Darb. (1912: 15), *Carbonea vorticosa* (Flörke 1808: 311) Hertel (1983: 442) and *Usnea antarctica* Du Rietz (1926: 93)] were present suggesting that more species might be found in the SR Mountains. Out of the total of 25 taxa that could be identified to the species level, 12 (48%) are endemic to Antarctica (as defined by Øvstedal & Lewis Smith 2001), one (4%) is austral, six (24%) are cosmopolitan whereas another six (24%) are bipolar in distribution.

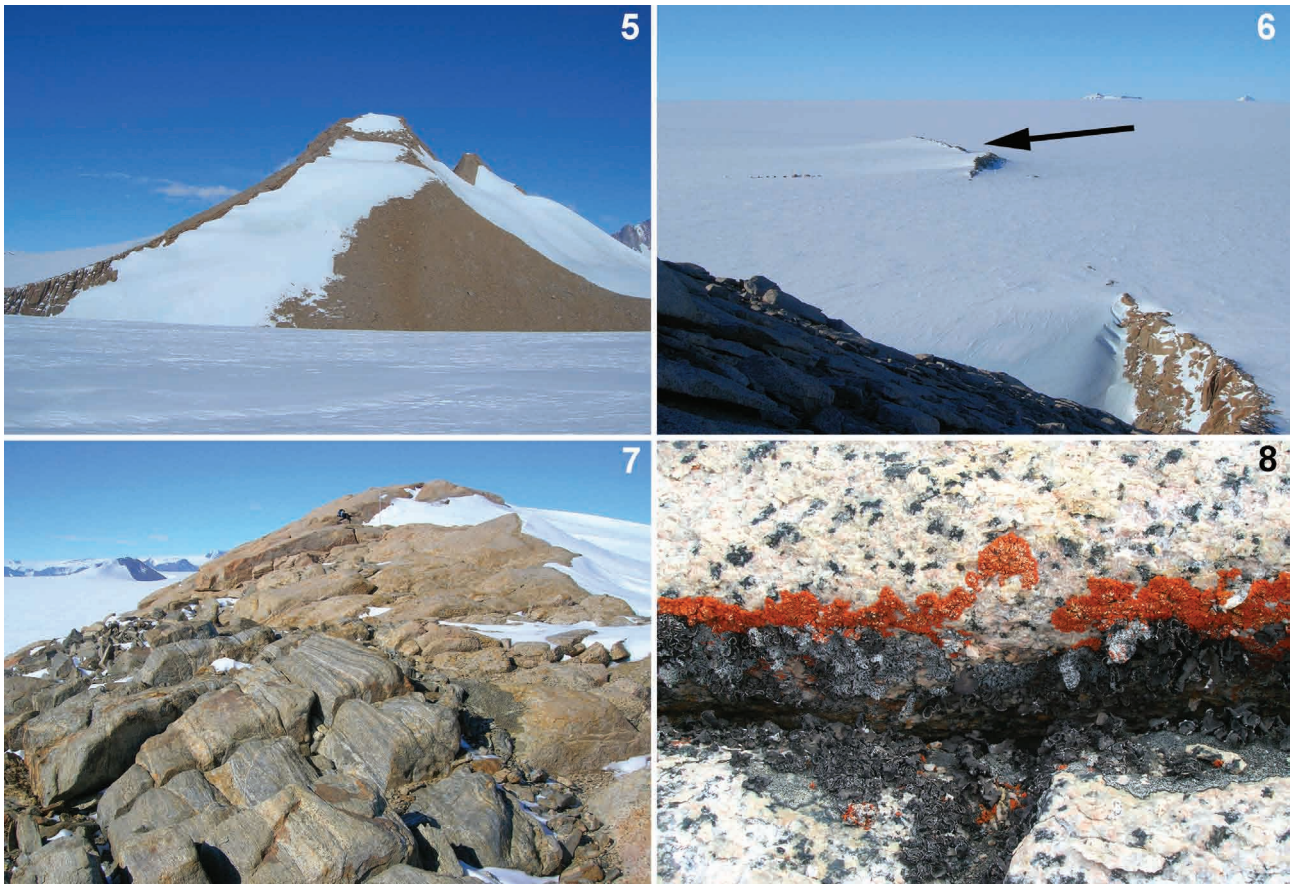
The species

Amandinea petermannii (Hue) Matzer, H.Mayrhofer & Scheid.
Antarctic endemic (Matzer *et al.* 1994, Øvstedal & Lewis Smith 2001). New to Dronning Maud Land. The specimen is small and was collected at the eastern side of the Utsteinen Nunatak, at 1455 m elev., on granite gravel on the slope with a snow petrel colony. The species was previously known from Antarctica at 2–1100 m elev. (Øvstedal & Lewis Smith 2001). The Utsteinen material deviates from typical material by the hymenium inspersion.

Specimen examined:—*Ertz* 10253B.

Bacidia johnstonii C.W.Dodge
Antarctic endemic, so far only known from the type locality at King George V Land (Øvstedal & Lewis Smith 2001). Found in Utsteinen in rock crevices on granite ridge sticking out of the snow at 1375 m elev. The taxon is said to be close and perhaps conspecific to *B. stipata* I.M. Lamb (1954: 126) (Øvstedal & Lewis Smith 2001). *Bacidia johnstonii* differs from the latter by the more reduced thallus, the colour of the upper part of the hymenium and the slightly smaller spores (Øvstedal & Lewis Smith 2001). The Utsteinen material fits better the concept of *B. johnstonii* by the olivaceous brown colour of the uppermost part of the hymenium and the short ascospores [(14.5–)15.5–19.8(–25) × (2–)1.8–2.7(–3) µm (n=52)]. However, the thallus is often clearly minute fruticose as described for *B. stipata*. Because the thallus is likely to be more variable, we decided to use the name *B. johnstonii* for our material. It must be noted that the ascospores of *B. stipata* are probably erroneously mentioned as being 7–10-septate in Øvstedal & Lewis Smith (2001) as only 5-septate ascospores are illustrated by these authors. In the protologue of *Bacidia stipata* 4–7 septate ascospores are described (Lamb 1954).

Specimens examined:—*Ertz* 10211, 10215 (TLC: nil), 10276 (TLC: nil).



FIGURES 5–8. Studied sites in 2007, before the construction of the Belgian Antarctic research station. Fig. 5. Utsteinen Nunatak viewed from the Utsteinen Ridge; Fig. 6. Utsteinen Ridge (arrow) viewed from the eastern side of the Utsteinen Nunatak, with the camp (tents) on the left; Fig. 7. plot 20 of the Utsteinen Ridge; Fig. 8. Granite rock with a crack bordered by a lichen vegetation including *Buellia frigida*, *Physcia caesia*, *Umbilicaria aprina* and *Xanthoria elegans*.

Buellia frigida Darb. (1910: 7) (Fig. 9)

Antarctic endemic; one of the most common and widespread lichen species throughout Continental Antarctica from sea level to high elevations inland (Øvstedal & Lewis Smith 2001). Abundant on the eastern side of the Utsteinen Nunatak and on the Utsteinen Ridge, growing on granite rock, especially along crevices or in cavities, at 1375–1420 m elev.

Specimens examined:—*Ertz* 10219, 10246 p.p., 10230 p.p., 10231, 10308, 10358, 10280.

Buellia nelsonii Darb.

Buellia bastini C.W.Dodge (Dodge 1962: 307), *synon. nov.*—Type: Princess Ragnhild Coast, Romnoes Nunatak summit, near Base Roi Baudouin, 1959, *Bastin* 3a (lectotype!: BR, designated here); 3 p.p. (isolectotype!: BR, designated here).

Antarctic endemic, known from various isles and the Antarctic peninsula, but new to Continental Antarctica including Dronning Maud Land (Øvstedal & Lewis Smith 2001). Found on the summit of Romnoes Nunatak at about 1000 m elev., on gneiss with garnets. According to Øvstedal and Lewis Smith (2001), known from coastal rock at 6–60 m.

There are 3 packets with many rocks in BR with collection number Bastin 3. Only one contains a few, sterile but otherwise well-developed thalli. A collection which was separated off as 3a is fertile but none of the thalli are well-developed.

Morphological observations: Thallus crustose, areolate, up to 1 mm thick, in the centre decorticated, yellowish grey to whitish, medulla soft, thallus margin usually with thick black hypothallus. Apothecia round, sessile to seemingly stipitate, black with evanescent black margin, flat to convex, 0.2–0.4 mm. diam. Hypothecium and excipulum brown, dense. Asci cylindical, with 8 ascospores. Paraphyses with apical brown thickenings of ca. 5 µm diam. Ascospores brown, 1-septate, straight or slightly bent, 16–21 × 7–8 µm. Chemistry: xanthones, indicated by UV+yellow reaction of the thallus.

Specimens examined:—*Bastin* 3, 3a.

Buellia subfrigida May.Inoue

Antarctic endemic, already known from Dronning Maud Land (Øvstedal & Lewis Smith 2001). Found here on rock in a dry valley within the Sør Rondane Mountains at 1700 m elev. The species was only reported at 20–150 m elev. so far.

Specimen examined:—*Ertz* 10347B.

Caloplaca tominii (Sav.) Ahlner

Rather widespread in the northern hemisphere and only recently recorded from Antarctica, Victoria Land (Smykla *et al.* 2011). New to Dronning Maud Land. Found in a dry valley within the Sør Rondane Mountains and at Utsteinen, at respectively 1700 and 1375 m elev., respectively in a narrow crack of a big granite boulder and on a granite ridge sticking out of the snow, on gravel.

Specimens examined:—*Ertz* 10300, 10306, 10310 p.p., 10352.

Candelaria murrayi Poelt

Antarctic endemic. A single very small but characteristic specimen found at the eastern side of the Utsteinen Nunatak, at 1455 m elev., on granite slope with a petrel colony, on gravel. The species was only known at 8–165 m elev. so far (Øvstedal & Lewis Smith 2001).

Specimen examined:—*Ertz* 10253A.

Candelariella flava (C.W.Dodge & G.E.Baker) Castello & Nimis

Blastenia autenboeri C.W.Dodge (Dodge 1962: 305)—Type: Princess Ragnhild Coast, Petrel Egg Nunatak, north of Tanngarden, 8 Jan. 1961, *Van Autenboer* 1a (holotype!: BR). Synonymy already reported by Castello & Nimis (1994), confirmed here.

Antarctic endemic, widespread in Continental Antarctica (Castello & Nimis 1994, Øvstedal & Lewis Smith 2001). Found at the Utsteinen Nunatak and on the Utsteinen Ridge, at 1375–1420 m elev., on gravel, and at the Petrel Egg Nunatak (=SR Mountains) at about 1500 m elev. and at Lagkollane on moss.

Specimens examined:—*Ertz* 10246 p.p., 10297; *Van Autenboer* 1a, 1c p.p., 4d.

Carbonea vorticosa (Flörke) Hertel

Lecidea autenboeri C.W.Dodge (Dodge 1962: 301), *synon. nov.*—Type: Princess Ragnhild Coast, Lagkollane, gravity point, near Roi Baudouin base, 8 Jan. 1961, *Van Autenboer* 4a (holotype!: BR).

Bipolar, widespread in Continental Antarctica (Øvstedal & Lewis Smith 2001). Found at Lagkollane at 1488 m elev. on *Physcia dubia* on moss. *Lecidea autenboeri* is synonymized here with *Carbonea vorticosa* in a wide sense, as used in e.g. Øvstedal & Lewis Smith (2001), thus including apparently lichenicolous specimens such as the present one.

Morphological observations: Thallus unapparent or at most a whitish hue, over or probably lichenicolous on *Physcia dubia*. Apothecia black, ca. 0.2 mm diam., black with thin regular margin. Hymenium blue, with dense paraphyses. Hypothecium brown under thick hyaline subhymenium. Ascospores hyaline, ellipsoid, unicellular, 10–12 × 4.5–5.5 µm.

Specimen examined:—*Van Autenboer* 4a.

Lecanora expectans Darb. (1910: 5) (Fig. 10)

Antarctic endemic; widespread especially in coastal areas (Filson 1974b, Øvstedal & Lewis Smith 2001, Śliwa & Olech 2002). Found on the Utsteinen Nunatak and on the Utsteinen Ridge, at 1336–1381 m elev., on gravel.

Specimens examined:—*Ertz* 10284, 10310 p.p., 10361, 10370, 10394.

Lecanora fuscobrunnea C.W.Dodge & G.E.Baker (1938: 577) (Fig. 11)

Lecanora exsulans (Th. Fr. 1902: 208) C.W.Dodge & G.E.Baker (1938: 570)—Specimens *Van Autenboer* 1a, 1b, 1f and 1j reported under this name by Dodge (1962: 304).

A species of the *Lecanora polytropa* group, endemic to Continental Antarctica (Castello 2003, Ruprecht *et al.* 2012). Found here on the Utsteinen Ridge and in a dry valley within the Sør Rondane Mountains, at respectively 1375 m and 1700 m elev., always on gravel, and at the Petrel Egg Nunatak at 1500 m elev.

Specimens examined:—*Ertz* 10210 (TLC: usnic acid), 10348, 10312, 10293, 10283B; *Van Autenboer* 1a p.p., 1b, 1f.

Lecanora mons-nivis Darb. (1912: 9)

Antarctic endemic reported from the Antarctic Peninsula and Continental Antarctica (Victoria Land) (Øvstedal & Lewis Smith 2001, Śliwa & Olech 2002, Smykla *et al.* 2011). Found on the Utsteinen Ridge, at 1375 m elev., on gravel and exposed rock.

Specimens examined—*Ertz* 10309, 10311, 10357.

Lecidea cancriformis C.W.Dodge & G.E.Baker

Antarctic endemic (Øvstedal & Lewis Smith 2001). Found in Utsteinen where it is abundant on the ridge, at 1375 m elev., on granite (exposed rock, cavity). The ascospores of the Utsteinen material are (7–)7.8–9.8(–11) × (3–)3.9–4.7(–5.5) µm (n=45), thus slightly wider than those mentioned in Ruprecht *et al.* (2010, ascospores of 3–4 µm wide). The hypothecium is dark brown but often lighter than illustrated in Ruprecht *et al.* (2010, Fig. 4e). However, the absence of secondary compounds tested by thin layer chromatography and the I–medulla suggest that our material belongs to *Lecidea cancriformis*.

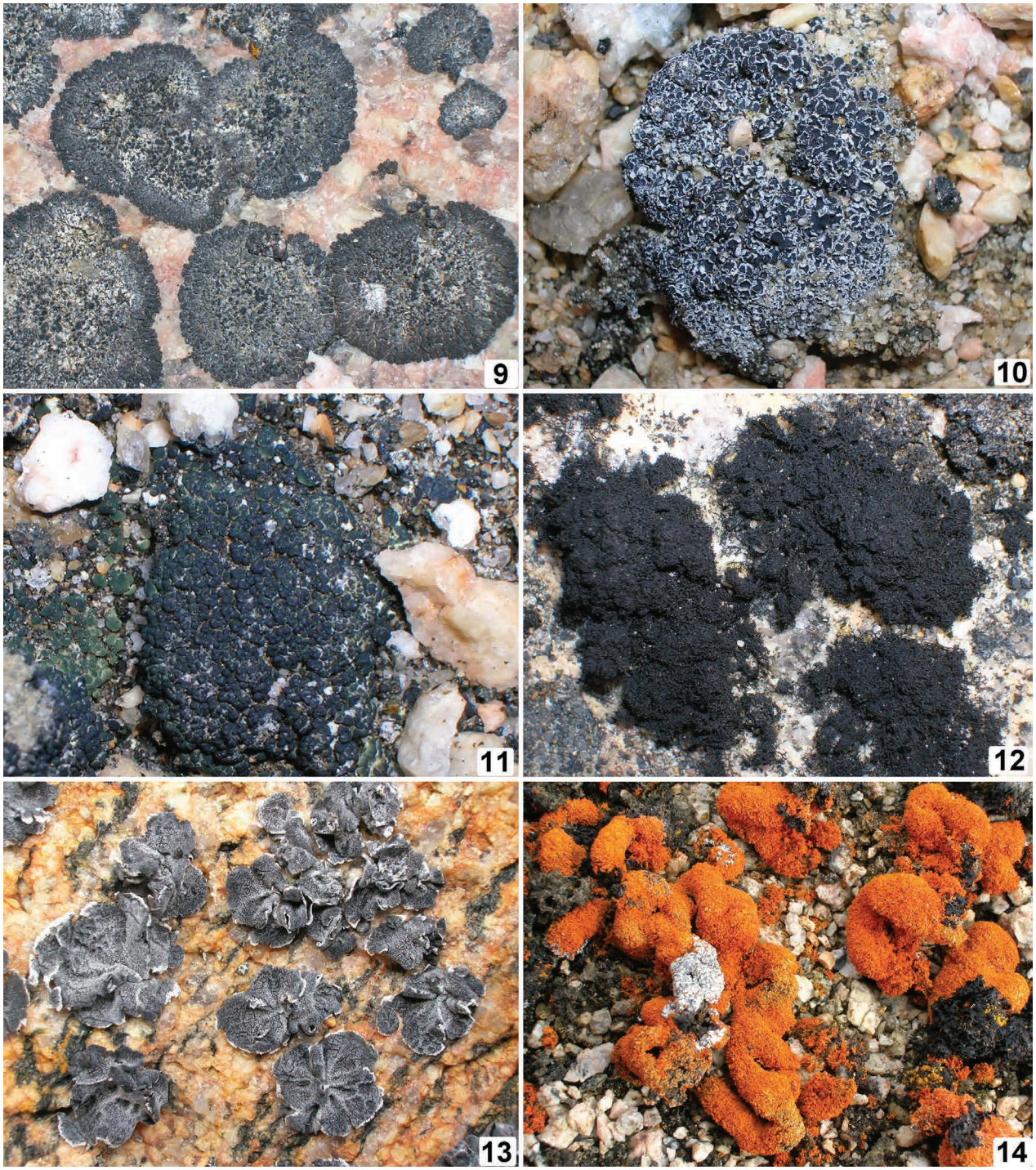
Specimens examined:—*Ertz* 10212 (TLC: nil), 10232, 10277 (TLC: nil), 10282 (TLC: nil), 10288, 10301, 10303, 10386 (TLC: nil), 10402 (TLC: nil).

Lecidella siplei (C.W.Dodge & G.E.Baker) May.Inoue

Bipolar (Ruprecht *et al.* 2012), including Dronning Maud land in Continental Antarctica (Øvstedal & Lewis Smith 2001). Found on the Utsteinen Ridge and the Utsteinen Nunatak on exposed rock or on gravel, at 1352–1420 m elev., and at the base of the Teltet Nunatak, on gravel (gneiss) at 1450 m elev.

Our specimens were first identified as "*Lecidella* sp. E" using the identification key of Øvstedal & Lewis Smith (2001) but eventually as *L. siplei* by U. Ruprecht (comm. pers.) using our ITS sequences. "*Lecidella* sp. E" was described as having wider (7–8 µm) ascospores than *L. siplei* (6–7 µm) (Øvstedal & Lewis Smith 2001). As a consequence, the material named "*Lecidella* sp. E" in Øvstedal & Lewis Smith (2001) belongs to *L. siplei*. The ascospores in the Utsteinen material are (10–)11.7–14.5(–16) × (6–)7–8.5(–9.5) µm, l/w ratio 1.5–2 (n=50), thus wider than mentioned by Ruprecht *et al.* [2012; ascospores (5–)6–7 µm wide] suggesting a highly variable spore size as already highlighted by Castello [2003; ascospores of 10–16 × 6–9(–10) µm].

Specimens examined:—*Ertz* 10278, 10279, 10304, 10307B, 10323, 10330, 10373, 10374, 10375, 10388.



FIGURES 9–14. Diversity of lichens on the Utsteinen Nunatak. Fig. 9. *Buellia frigida*; Fig. 10. *Lecanora expectans*; Fig. 11. *Lecanora fuscobrunnea*; Fig. 12. *Pseudephebe minuscula*; Fig. 13. *Umbilicaria decussata*; Fig. 14. *Xanthomendoza borealis*.

Myriospora sp.

Only a single small specimen with six mature ascomata was found on the Utsteinen Ridge, on granite at 1375 m elev. It is tentatively identified as a species of *Myriospora* but more material is needed to confirm its identification. The thallus of our specimen is very thin and inapparent; apothecia 0.2–0.3 mm in diam.; asci >40-spored; paraphyses slender; ascospore hyaline, ellipsoid, unicellular, 3–3.5 x 1–1.5 µm.

Specimen examined:—*Ertz* 10371B.

Physcia caesia (Hoffm.) Fürnr. (Fig. 8)

Parmelia griseola C.W.Dodge & G.E.Baker (1938: 596)—Specimen *Van Autenboer* 4f reported under this name by Dodge (1962: 305).

Cosmopolitan (Filson 1974a, Øvstedal & Lewis Smith 2001). Found on the Utsteinen Nunatak and on the Utsteinen Ridge, at respectively 1420 m and 1375 m elev., on gravel; and at Lagkollane growing on moss.

Specimens examined:—*Ertz* 10257, 10246 p.p., 10295; *Van Autenboer* 4f.

Physcia dubia (Hoffm.) Lettau

Almost cosmopolitan. Widespread in Antarctica at 2–285 m elev. (Øvstedal & Lewis Smith 2001). Found at the Utsteinen Nunatak and on the Utsteinen Ridge, at respectively 1420 m and 1375 m elev., on gravel; at Princess Ragnhild Coast at Lagkollane at 1488 m elev. on moss. Specimen *Ertz* 10334B is parasitized by a lichenicolous fungus. This might be an undescribed species of *Toninia*, characterized by minute black sessile apothecia, a grey epihymenium, dark red-brown hypothecium and fusiform, consistently 3-septate ascospores of 14–16 × 3–4 µm. The thallus is completely absent and there is no doubt that it is lichenicolous on *Physcia dubia*. It is not described here as only ca. ten apothecia were found, on three host thalli. It should be looked for in Antarctic collections of this host. Specimen *Van Autenboer* 4a is parasitized by *Carbonea vorticosa*.

Specimens examined:—*Ertz* 10334A-B, 10246 p.p., 10371A; *Van Autenboer* 4a p.p. (host).

Pseudephebe minuscula (Nyl. ex Arnold) Brodo & D.Hawksw. (Fig. 12)

Bipolar. One of the most common lichens of ContinentalAntarctica from sea level to high elevations (Øvstedal & Lewis Smith 2001). Abundant on the Utsteinen Ridge, on rock, often along cracks or in cavities. Also observed on the eastern side of the Utsteinen Nunatak.

Specimens examined:—*Ertz* 10229, 10287, 10384, 10336.

Rhizocarpon geographicum (L.) DC.

Cosmopolitan in colder areas. Already known from Continental Antarctica (Huneck *et al.* 1984, Øvstedal & Lewis Smith 2001).

Specimen examined:—*Ertz* 10343

Trapelia antarctica Ertz, Aptroot, G.Thor & Øvstedal, sp. nov. (Fig. 15–20)

MycoBank No. MB810998

Type:—ANTARCTICA. Dronning Maud Land: Sør Rondane mountains, Utsteinen, ridge partition 4, 1375 m, 71°57’02.1”S, 23°20’49.6”E, on gravel on granite ridge sticking out of the snow, 21 January 2007, *Ertz 10299* (holotype!: BR).

Thallus of pale to dark brown or black, mat, smooth, effigurate or subsquamulose, +/– convex, often +/– overlapping, 0.6–0.8(–1) mm thick, 0.5–1.5 × 0.4–1 mm areoles. Hyphae of the medulla covered by numerous crystals of 1–4(–6) µm in diam. not dissolving in K (tested in polarized light). Photobiont unicellular green algae, 4–8(–10) µm in diam. *Cortex* 10–15(–20) µm thick, dark brown sometimes with a purple tinge, K+ purple strongly accentuated. *Prothallus* not observed. *Ascomata* often numerous, sometimes covered large parts of the thallus, sessile, often shortly stipitated, on the centre of areoles, at first globose and appearing perithecia-like, then splitting at apex, rounded, 0.2–1 mm in diam.; thalline margin dark grey, 75–90 µm thick, first pronounced, usually becoming quickly excluded; hymenial disc first concave, then plane to slightly convex, rugose, dark brown to black. *True excipulum* hyaline, 20–35(–55) µm thick, cellular, dense, I–, K/I–, K+ strongly purple (not dissolving). *Hymenium* hyaline to pale pink, 60–80 µm (–140 µm) tall, I+ blue, K/I+ blue. *Epihymenium* dark brown, I+ blue, K/I+ blue, K+ strongly purple (not dissolving), with numerous crystals disappearing in K (tested in polarized light), 15–35 µm high. *Hypothecium* hyaline, 100–125 µm thick at the centre of the apothecia, thinner near the margin, 50–60 µm thick, I+ blue, K/I+ blue to greenish blue, with numerous irregularly shaped and hyaline crystals of 1–6 µm in diam., not dissolving in K (tested in polarized light). *Paraphyses* richly branched, anastomosing, 2 µm wide, slighty enlarged at the apex (2.5–3.5 µm). *Asci* 8-spored, 50–65 × 12–14 µm, wall evenly pale K/I+ blue, no ocular chamber (*Trapelia*-type). *Ascospores* hyaline, simple, narrowly ellipsoid to ovoid, (15–)16.6–19.9(–22.5) × (4.5–) 5.9–7.2(–8) µm, l/w ratio of ascospores still in the asci 2.5–3.1 (n=61), l/w ratio of discharged ascospores 3–4 (n=30), without a distinct perispore.

Chemistry: Thallus C+ red in cross section, due to gyrophoric and 5-O-methylhiascic acids (holotype tested).

Habitat and distribution:—Endemic to the Antarctic continent. In Utsteinen (type locality) the new species grows on a granite ridge sticking out of the snow at 1375 m elev. The species is also known from Vestfjella where it grows on

wet sand, a rare habitat in this region. The lichen was included in Øvstedal & Lewis Smith (2001: 333) as '*Trapelia* sp. A', where it is reported from the southeastern Antarctic Peninsula, Eternity range, in fissures in rock rich in iron and magnesium with *Lecidea cancriformis*, at 2000 m elev., in addition to Dronning Maud Land.

Additional specimens examined:—Antarctica, Dronning Maud Land, Sør Rondane Mountains, Utsteinen Ridge, 2007, *Ertz* 10294, 10209, 10281, 10307C, 10292, 10305 (BR); ibidem, Vestfjella, Fossilryggen nunatak, the hill 50 m N the shale area in the central part of the nunatak, elev. 700 m, 73°23'S, 13°02'W, 1991, *Thor* 10398 (S).

Taxonomic remarks:—The new species is well characterized by the strong K+ purple reaction of the epihymenium and the long-ellipsoid, quite large ascospores. It is the first species of *Trapelia* known to have this reaction. The most similar species is *T. glebulosa* (Sm. in Smith & Sowerby 1809: 1955) J.R. Laundon (2005: 492) (known from Europe, Macaronesia, N. and C. America, Asia, Australia and New Zealand; Purvis *et al.* 2009). It is similar to the new species by the shape of thallus areoles and the production of gyrophoric acid. It differs from it by the lack of K+ purple reaction and by wider ascospore (7–13 µm wide) (Purvis *et al.* 2009, Wirth 1995). The widespread *T. coarctata* (Turner ex Sm. in Smith & Sowerby 1809: 534) M. Choisy (in Werner 1932: 160) differs from the new species by having a crustose thallus (without a differentiated cortex), apothecia with a white thin thalline margin and by the lack of the K+ purple reaction (Øvstedal & Lewis Smith 2001). Among the non-sorediate species of *Trapelia* and in addition to the K+ purple reaction of the epihymenium and the cortex, *T. macrospora* Fryday (2004: 144) and *T. rediviva* Brusse (1991: 154) differ from the new species notably by having much larger ascospores [respectively (25–)27–30 × (12–)15–18(–19) µm and 22–33.5 × 12–20 µm] (Fryday 2004, Brusse 1991); *T. herteliana* Fryday (2004: 143) differs from the new species by the elongate ascomata immersed in thallus and with a red brown to dark brown usually white pruinose disc, and by a pale orange-brown hypothecium (Fryday 2004); *T. chiodectonoides* Brusse (1987: 187) differs from the new species by having a ashy or chalky whitish thallus, brownish red ascomata, a pale reddish brown excipulum and wider ascospores (8–10.5 µm wide) (Brusse 1987); *T. pallidicervina* (Kremp. 1876: 378) Hertel (1970: 181) differs from the new species by the shorter and wider ascospores (11–13 × 8–9 µm) and by a brownish-black hypothecium (Krempelhuber 1876). *T. mayaguez* (Zahlbr. 1930: 75) Hertel (1970: 181) differs from the new species by a chalky white thallus and a taller hymenium (200–210 µm). We also checked the original descriptions of all *Trapelia* species to ensure that no older names were available.

Umbilicaria aprina Nyl. (Fig. 8)

Umbilicaria spongiosa var. subvirginis (Frey & I.M.Lamb 1939: 272) C.W.Dodge (1948: 148). Specimens *Van Autenboer* 1g and 1h reported under this name by Dodge (1962: 303).

Rather cosmopolitan; widespread in Continental Antarctica from low elevation coastal sites to high elevation inland sites (Filson 1987, Hestmark 1990, Øvstedal & Lewis Smith 2001). Found on the Utsteinen Nunatak and Ridge, on granite rock, and at Princess Ragnhild Coast at the Petrel Egg Nunatak.

Specimens examined:—*Ertz* 10214, 10331, 10341 (TLC: gyrophoric or lecanoric acid); *Van Autenboer* 1d, 1g, 1h.

Umbilicaria decussata (Vill.) Zahlbr. (Fig. 13)

Omphalodiscus subcerebriformis (C.W.Dodge 1948: 149) C.W.Dodge (1962: 302). Specimen *Van Autenboer* 2 reported under this name by Dodge (1962: 302).

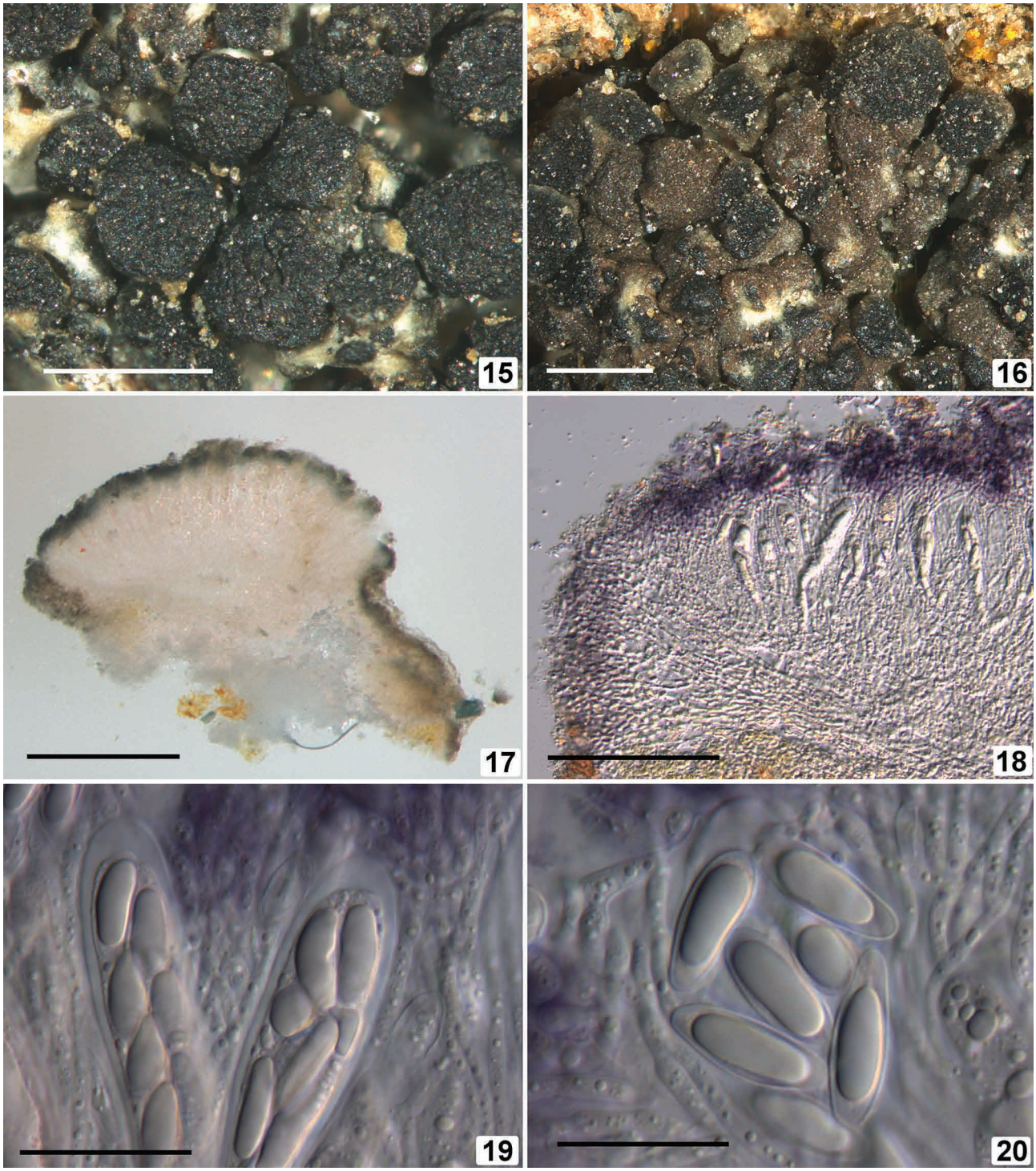
Cosmopolitan in colder regions and widespread in Continental Antarctica from coastal sea level sites to high elevation far inland sites (Filson 1987, Hestmark 1990, Øvstedal & Lewis Smith 2001). Found on the eastern side of the Utsteinen Nunatak, on the Utsteinen Ridge and in a dry valley within the Sør Rondane Mountains on granite rock; and on unknown locality in the Sør Rondane Mountains on metamorphic rock. *Umbilicaria decussata* is said to contain gyrophororic acid (Øvstedal & Lewis Smith 2001), but three specimens examined by TLC all lacked lichen substances. Our results are thus in agreement with Løfall & Timdal (2005) who stated that *Umbillicaria decussata* contains gyrophoric acid in northern Europe and Greenland, but some material lacks the substance in other regions of the world including Antarctica.

Specimens examined:—*Ertz* 10363 (TLC: nil), 10347A (TLC: nil), 10368, 10401 (TLC: nil); *Van Autenboer* 2 (2 packets).

Usnea antarctica Du Rietz

Austral; widespread in Continental Antarctica (Øvstedal & Lewis Smith 2001). Found in Lagkollane, on granite and on tiny gravel in moss.

Specimens examined:—*Van Autenboer* 4b, 4c.



FIGURES 15–20. *Trapelia antarctica sp. nov.* Figs 15–16. Thallus and apothecia; Fig. 17. cross section in apothecia in water; Fig. 18. cross section of apothecia showing the purple epihymenium in K; Fig. 19. Asci in K; Fig. 20. Ascospores in K. Scale bars: A–B = 0.5 mm, C = 200 µm, D = 100 µm, E–F = 20 µm.

Usnea sphacelata R. Br. (1823: 49)

Bipolar; the most common and widely distributed macrolichen in Continental Antarctica from sea level to high elevations (Øvstedal & Lewis Smith 2001). Found on the Utsteinen Ridge at 1369–1375 m elev., growing on granite rock and gravel.

Specimens examined:—*Ertz* 10381, 10388B, 10307A, 10380, 10382.

Xanthomendoza borealis (R.Sant. & Poelt in Poelt & Petutschnig 1992: 15) Søchting, Kärnefelt & S.Kondratyuk (2002: 237) (Fig. 14)

Xanthoria mawsoni C.W.Dodge (1948: 236). Specimen *Van Autenboer* 1c reported under this name by Dodge (1962: 307), although the holotype of *Xanthoria mawsonii* belongs to another species (Lindblom & Søchting 2008).

Apparently bipolar, including Continental Antarctica (Lindblom & Søchting 2008, Øvstedal & Lewis Smith 2001 as *X. mawsonii*). Found on gravel on the Utsteinen Ridge and on the Utsteinen Nunatak, at respectively 1375 m and 1346–1415 m elev., and at Princess Ragnhild Coast at the Petrel Egg Nunatak at 1500 m elev.

Specimen *Ertz* 10246 is parasitized by a lichenicolous fungus (about ten ascomata), *Arthonia* cf. *molendoi* (Frauenf. in Arnold 1864: 462) R.Sant. (1986: 2): ascomata black, convex, 0.08–0.13 mm in diam., not forming galls; epihymenium pale brown, hypothecium hyaline to pale yellow, asci *Arthonia*-type, 8-spored, 25–30 × 15–16 µm; ascospores hyaline, 1-septate, 10.5–13 × 4–5 µm. Our specimen deviates from normal *A. molendoi* by ascomata having a pale hypothecium.

Specimens examined:—*Ertz* 10269, 10246 p.p., 10289, 10399; *Van Autenboer* 1c p.p.

Xanthoria elegans (Link) Th. Fr. (Fig. 8)

Gasparrinia harrissoni C.W.Dodge (1948: 234). Specimen *Van Autenboer* 1e reported under this name by Dodge (1962: 306).

Rather cosmopolitan, including Continental Antarctica (Øvstedal & Lewis Smith 2001). Found on the Utsteinen Nunatak, on the Utsteinen Ridge and in the "dry valley", at respectively 1362–1420 m, 1375–1379 m and 1700 m elev., on granite including gravel and exposed rock, and at Princess Ragnhild Coast at the Petrel Egg Nunatak at 1500 m elev. One of the most abundant species in Utsteinen.

Specimens examined:—*Ertz* 10213, 10228, 10245, 10246 p.p., 10271, 10333, 10342, 10349; *Van Autenboer* 1e.

Conclusions

Despite only a small part of the SR Mountains being investigated during the field trip, 18 species of lichens and lichenicolous fungi were newly collected, raising the total number of taxa known for the SR Mountains to 28. The lichen diversity is similar to a number of other mountain groups investigated in Dronning Maud Land (Vestfjella: Lindsay 1972; Øvstedal 1983a; Heimefrontfjella: Thor 1995; Schirmacher Oasis: Nayaka & Upreti 2005; the Pyramiden-Lenestolen nunatak group: Øvstedal 1986; H.U. Sverdrup Mountains: Øvstedal 1983b), where the number of species varies between 16 and 35. Other continental localities at similar latitudes and with comparable bedrock have sometimes a higher diversity (e.g. 43 species at Cape Hallett and 28 species at Ebony Ridge in the Ross Sea region; Green *et al.* 2011, Colesie *et al.* 2014) but these localities are closer to the sea and located at a lower elevation. The high percentage of Antarctic endemism in the lichen flora (48%) is similar to data obtained for the microorganisms of Utsteinen (Peeters *et al.* 2011, Fernández-Carazo *et al.* 2012).

The data and the detailed grid map, available now for the Utsteinen Ridge, will be useful in monitoring the future changes in lichen population and diversity in the region. We recommend that the plots 17 to 21 (from 71°56'48.6"S, 23°20'47.9''E to 71°56'44.5"S, 23°20'41.8"E) get official protection from any human activities in the near and far future. The plots 1 to 15 will be subject to a strong influence following the construction of the research station and the related human activities, whereas the other plots are expected to be less influenced as there are more distantly situated from the construction site. Therefore, it will be highly likely that the lichen communities of the parcels 16 to 23 will not change significantly in the near future and should thus be chosen as reference plots for comparison with the plots 1 to 15 in order to evaluate future human activities on the biodiversity of the ridge.

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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

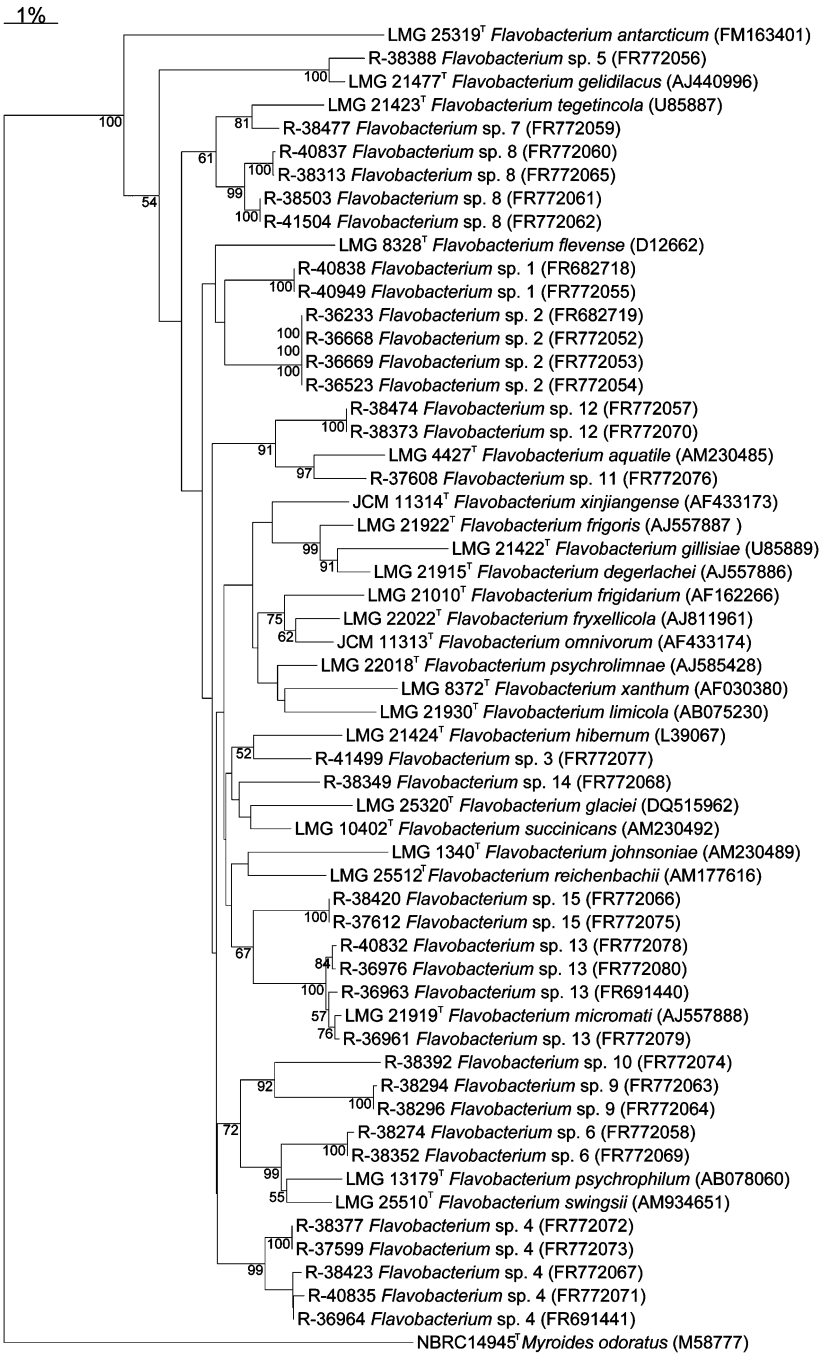

Fig. 1. Phylogenetic tree based on neighbour-joining analysis of the 16S rRNA gene sequence similarities of the *Flavobacterium* strains and closely related species. New *Flavobacterium* isolates are marked as *Flavobacterium* sp., followed by a number. The numbers at branch nodes are bootstrap values 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

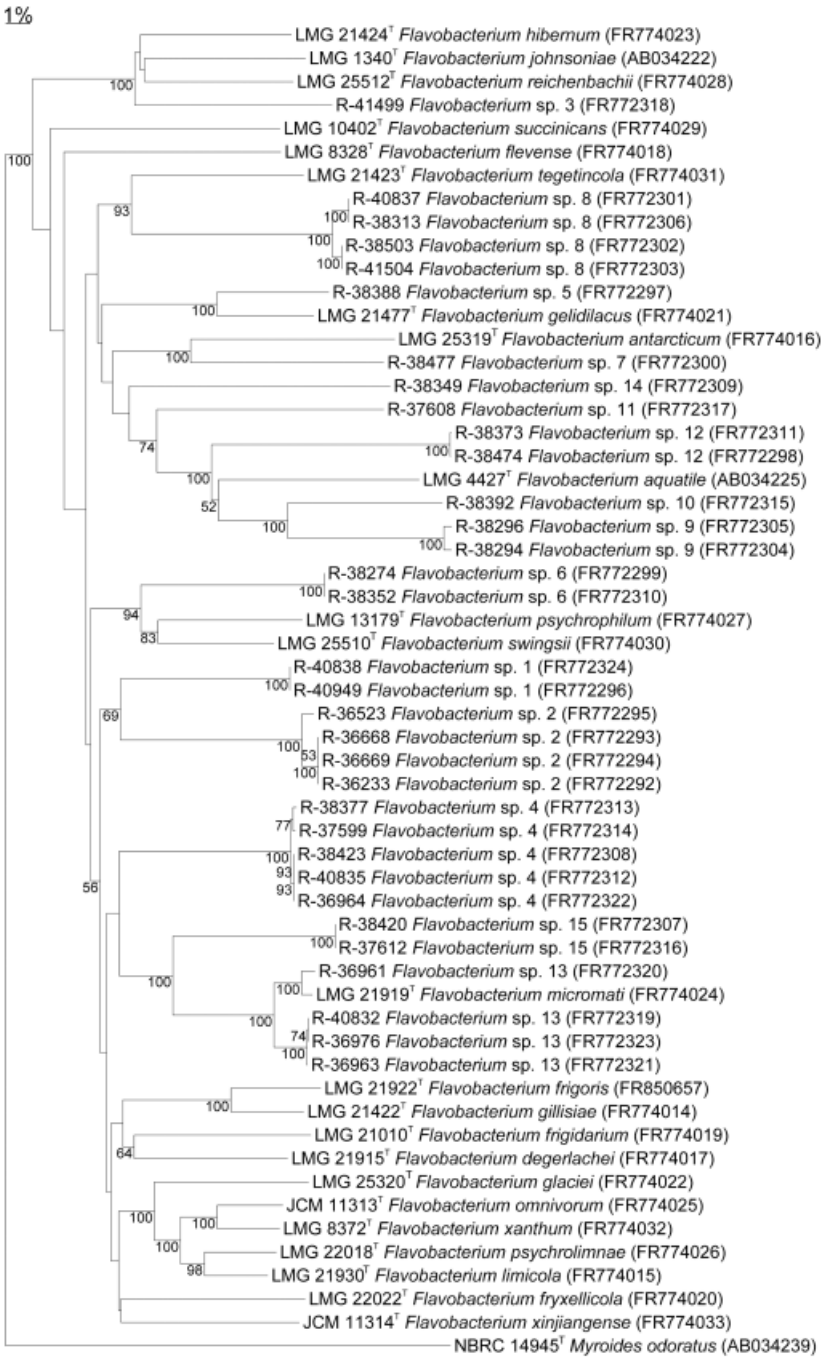

Fig. 2. Phylogenetic tree based on neighbour-joining analysis of the *gyrB* gene sequence similarities of the *Flavobacterium* strains and closely related species. New *Flavobacterium* isolates are marked as *Flavobacterium* sp., followed by a number. The numbers at branch nodes are bootstrap values 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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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

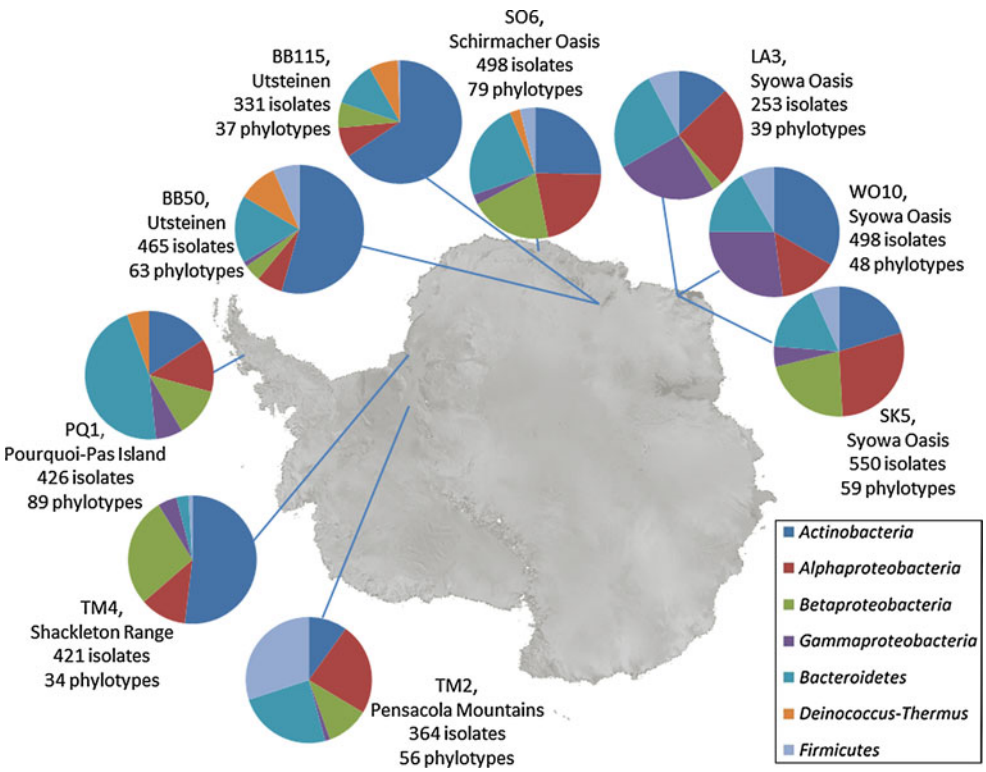

Fig. 1 Division of the phylotypes over the different phylogenetic groups. The number of obtained isolates and phylotypes are mentioned for the different samples. Information for samples BB50, BB115, TM2 and TM4 was based on Peeters et al. 2011a, b

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

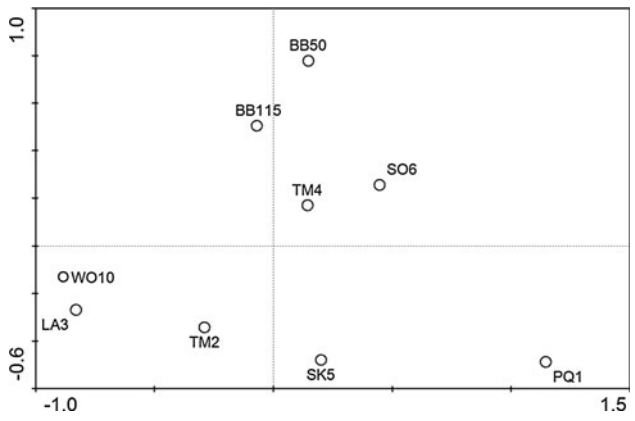

Fig. 2 Principal component analysis (PCA) of the samples showing the differences in bacterial diversity (at genus level) based on the number of rep-types. Information for samples BB50, BB115, TM2 and TM4 was based on Peeters et al. 2011a, b

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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Bacterial Diversity Assessment in Antarctic Terrestrial and Aquatic Microbial Mats: A Comparison between Bidirectional Pyrosequencing and Cultivation

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Abstract

The application of high-throughput sequencing of the 16S rRNA gene has increased the size of microbial diversity datasets by several orders of magnitude, providing improved access to the rare biosphere compared with cultivation-based approaches and more established cultivation-independent techniques. By contrast, cultivation-based approaches allow the retrieval of both common and uncommon bacteria that can grow in the conditions used and provide access to strains for biotechnological applications. We performed bidirectional pyrosequencing of the bacterial 16S rRNA gene diversity in two terrestrial and seven aquatic Antarctic microbial mat samples previously studied by heterotrophic cultivation. While, not unexpectedly, 77.5% of genera recovered by pyrosequencing were not among the isolates, 25.6% of the genera picked up by cultivation were not detected by pyrosequencing. To allow comparison between both techniques, we focused on the five phyla (*Proteobacteria*, *Actinobacteria*, *Bacteroidetes, Firmicutes* and *Deinococcus-Thermus*) recovered by heterotrophic cultivation. Four of these phyla were among the most abundantly recovered by pyrosequencing. Strikingly, there was relatively little overlap between cultivation and the forward and reverse pyrosequencing-based datasets at the genus (17.1–22.2%) and OTU (3.5–3.6%) level (defined on a 97% similarity cut-off level). Comparison of the V1–V2 and V3–V2 datasets of the 16S rRNA gene revealed remarkable differences in number of OTUs and genera recovered. The forward dataset missed 33% of the genera from the reverse dataset despite comprising 50% more OTUs, while the reverse dataset did not contain 40% of the genera of the forward dataset. Similar observations were evident when comparing the forward and reverse cultivation datasets. Our results indicate that the region under consideration can have a large impact on perceived diversity, and should be considered when comparing different datasets. Finally, a high number of OTUs could not be classified using the RDP reference database, suggesting the presence of a large amount of novel diversity.

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Introduction

With its severe physical, chemical, and climatic conditions [1], Antarctica is characterized by harsh environmental settings and hosts communities of well-adapted microbiota that are capable of withstanding selective pressures, such as high UV-radiation, drought, light limitation and extremely low temperatures. These adaptations may therefore be potentially of biotechnological and economical value [2,3]. Until now, studies have mainly used culturing approaches [4,5] and a number of culture-independent techniques such as Denaturing Gradient Gel Electrophoresis (DGGE) [6], Terminal Restriction Fragment Length Polymorphism (t-RFLP) [7,8], Automated Ribosomal Interspacer Analysis (ARISA) [9] and clone libraries [8,10,11,12] to shed light on Antarctic bacterial diversity. These studies reported taxa that are new to science [4,5,13] and/or revealed that – as in other regions and environments [14] – Antarctic microbial diversity is much larger than previously thought.

Whereas Next Generation Sequencing (NGS) techniques have now found their way to nearly every environment, ranging from the deep sea [15] to tropical forest soils [16] and the human microbiome [17], the Antarctic region remains relatively under-represented in these microbial diversity studies. This is surprising, given the fact that the diversity reported with NGS is orders of magnitude higher than that recovered with traditional culturing and Sanger sequencing, and at least one order of magnitude higher than recovered from large clone libraries [18]. More recently NGS has been used to study Antarctic samples, including

McMurdo Dry Valley soils [19,20], soils from Alexander Island [21], rhizosphere bacteria of the only two vascular plants in the Antarctic Peninsula [22], a study of community turnover due to global warming [23], a survey of cyanobacterial diversity in microbial mats [24] and a comparison of seasonal variation in coastal marine bacterioplankton [6]. The relative paucity of Antarctic studies is largely due to the remoteness and vastness of the continent, the harsh environmental conditions and the costs associated with expeditions. Yet, exactly these limitations have kept the environment relatively pristine, thus providing excellent conditions to investigate several questions of particular interest to microbiologists such as to which extent historical processes shape microbial biogeography patterns and the degree of endemism. Moreover, polar regions with their uniquely adapted microbiota are particularly prone to the impact of global warming [25,26,27,28] and microbial diversity data are therefore urgently needed as baseline data for tracking this impact.

Microbial communities typically consist of few high-abundant taxa, with the majority of taxa belonging to the so called rare biosphere [18,29,30]. Although it was shown that cultivation is able to pick up some of these rare community members [31], it is generally thought that only through the deep sequencing that NGS offers, this vast diversity can be detected [18,32]. In turn, this also implies that cultured strains are generally expected to be recovered by pyrosequencing. Here we aimed to test this hypothesis by comparing the diversity of heterotrophic bacterial groups previously recovered from Antarctic microbial mat samples by cultivation with the diversity of the corresponding groups as revealed by 454 pyrosequencing. An additional objective was to assess the impact of the region of the 16S rRNA gene on the diversity data obtained. This was done by comparing forward and reverse pyrosequencing datasets and contrasted with a comparison of forward and reverse data from the cultured strains, where no effects of the pyrosequencing process could be at work.

Materials and Methods

Samples Used

Details of the study sites have been described previously [4,33,34]. Briefly, two terrestrial and seven limnetic microbial mat samples were collected aseptically during different field campaigns in December/January 2003 (PQ1, TM2 and TM4) and in January 2007 (BB50, BB115, LA3, SK5, WO10 and SO6). One sample (PQ1) was collected on Pourquoi-Pas Island off the west coast of Graham Land (Antarctic Peninsula). All other samples were collected from Eastern Antarctic habitats. The two terrestrial microbial mat samples (BB50 and BB115) were taken near the Utsteinen nunatak in the Sør Rondane Mountains (Dronning Maud Land). Three samples were from Lützow-Holm Bay (Dronning Maud Land), namely from a small saline lake in Langhovde (LA3), from Naka-Tempyo Lake (SK5) in Skarvsnes, and from a small saline pond (WO10) in West Ongul Island. One sample (SO6) was taken from Lake Melkoye (unofficial name) in Schirmacher Oasis (Dronning Maud Land). The two remaining samples were collected in the Transantarctic Mountains. Sample TM2 was taken from Forlidas Pond (Dufek Massif, Pensacola Mountains), while sample TM4 was taken from Lundström Lake (Shackleton Range). All samples were kept frozen during transport and stored at -20°C.

Processing of 16S rRNA Gene Sequences of Cultures

The cultured heterotrophic bacterial diversity of these samples was reported earlier [4,33,34,35]. From these, we selected 1,666 high quality sequences for comparison with pyrosequencing. To allow this comparison, the sequences from bacterial cultures were aligned to the Silva reference database [36], and trimmed so as to cover the alignment of the sequences obtained using pyrosequencing (see below). They were further processed together with both forward and reverse pyrosequencing datasets.

Pyrosequencing

To allow direct comparison, DNA was extracted from the same frozen samples previously used for the cultivation experiments using 5 g per sample. Extracellular DNA was first removed as described by Corinaldesi *et al.* [37] and DNA extraction was subsequently performed according to Zwart *et al.* [38]. Sequencing of the 16S rRNA V1–V3 regions was performed using forward primer pA (AGAGTTTGATCCTGGCTCAG 8–27) [39] and reverse primer BKL1 (GTATTACCGCGGCTGCTGGCA 536–516). Because it proved impossible to concatenate the complementary reads due to insufficient overlap, the forward and reverse sequences were analyzed separately. The forward reads hence cover the complete V1 and V2 regions, whereas the reverse reads cover the V3 and part of the V2 region for the longest sequences [40].

Multiplexing was done with barcodes proposed by Parameswaran *et al.* [41]. Each PCR mixture contained 1–2 µl of template DNA, 2 µl of fusion primers and barcodes (10 µM), 2.5 µl dNTPs (10 mM), 1.5 µl of 10x buffer, 0.25 µl of 5 U/µl FastStart High Fidelity Polymerase (Roche) and was adjusted to a final volume of 25 µl with sterile HPLC-water. PCR cycling included 3 min at 94°C, followed by 35 cycles of 94°C for 30 s, 55°C for 60 s and 72°C for 90 s and finally 8 min at 72°C. PCR products were purified using a High Pure PCR Product Purification Kit (Roche). Finally, pyrosequencing was performed on a Roche 454 GS FLX Titanium machine at NXTGNT (Ghent, Belgium) after quality control of the DNA with a Qubit 2.0 Fluorometer (Life Technologies) and a Bioanalyzer (Agilent Technologies).

Raw sequences are available from the NCBI Sequence Read Archive under accession numbers SRR1146576 and SRR1146579.

Processing of Pyrosequences

The obtained reads were processed using Mothur [42] version 1.27.0, generally following Schloss *et al.* [43] and the Mothur SOP (http://www.mothur.org/wiki/454_SOP; version of 6 November 2012). The data were denoized using Mothur's PyroNoise [44] implementation with 450 flows as the minimal flow length and trimming of the longer sequences to this length [43]. Overall, the minimal required sequence length was set at 200 nucleotides (nt). To avoid poor sequence quality, no ambiguous bases (N) were allowed [45] and sequences with homopolymers longer than 8 nt were culled, as it is known that long homopolymers are problematic for 454 pyrosequencing [43,46,47]. The sequences were aligned using Mothur's alignment command, based on the GreenGenes NAST aligner [48] with default parameters and the Silva reference database [36], which takes into account the secondary structure of the 16S SSU rRNA. The starting and ending positions of the alignment were checked to ensure that sequences were overlapping the same alignment space. Sequences not starting at the correct position or ending before 95% of all the sequences were removed from the analysis. To increase computational speed and decrease data size, duplicate (identical) sequences were temporarily removed using the unique.seqs command. Further correction for erroneous base calls was done using single linkage preclustering according to Huse *et al.* [49]. Next, we used Uchime [50] with default parameters for intra-

sample *de novo* chimera checking. Positively identified chimeric sequences were removed from further analyses.

Sequence Identification and OTU Clustering

Sequences were identified using Mothur's implementation of the RDP classifier [51] by means of the modified RDP training-set release 9 (available at http://www.mothur.org/wiki/RDP_reference_files) at an 80% bootstrap value. The RDP database was chosen so that a comparison with the original cultivation data was possible, despite its known limitations because of its small size [52,53], possibly overestimating the number of unclassified OTUs. This training set too was first aligned and trimmed to the alignment space of the query sequences, increasing confidence values and reducing the number of unclassified sequences [52]. Non-cyanobacterial "chloroplast" sequences were removed from the dataset. Distances were calculated (dist.seqs command, default settings), after which the sequences were clustered using the average neighbor joining algorithm to generate OTUs at a 97% cutoff level [54].

SIMPROF Analysis

In order to compare the community composition obtained using culturing versus pyrosequencing a SIMPROF analysis [55] was performed using Primer 6 [56]. SIMPROF is a permutation-based procedure that ranks the pairwise similarities in each group and tests the null hypothesis that samples were all drawn from the same species assemblage. Because the number of sequences is consistently higher in the pyrosequencing dataset, we standardized the number of sequences in each sample to the lowest number of sequences obtained in all of the samples (i.e. 119 forward and 116 reverse sequences in sample LA3). To achieve this, we randomly sampled this number of sequences from each sample with replacement. This procedure was done 5 times, which resulted in 5 subsets for each sample. First, a Jaccard similarity matrix was constructed and subsequently used to undertake a group-average cluster analysis. Second, to ascertain the level of structure present in the groups formed by each dendrogram, a SIMPROF test with 10,000 simulations and the stopping rule specified at the 5% significance level was run. This was done for both forward and reverse datasets.

Results

Sequence Data of Bacterial Isolates

Of the initial 1,666 sequences, 1,578 remained after the forward processing together with the pyrosequences. This was mainly due to the removal of sequences that did not match the correct starting or ending positions of the alignment space. A total of 342 OTUs in 76 genera from five different phyla were obtained (Figure 1). Most of the OTUs belonged to the phyla *Bacteroidetes* and *Proteobacteria*, with 107 and 106 members respectively. *Actinobacteria* was the third best represented phylum with 78 OTUs, followed by *Firmicutes* and *Deinococcus-Thermus* with 31 and 20 OTUs respectively.

The initial 1,666 sequences were also subjected to the reverse processing pipeline. In contrast to the 1,578 forward sequences, this yielded only 1,519 sequences divided over 214 OTUs in 61 genera. The relative proportion of the phyla did not differ drastically when processed through the forward or reverse pipeline (Figures 1 and 2), although only 51 genera were shared between the forward and reverse dataset of the isolates. In total, we identified 86 genera for the combined processed cultivation results, while some sequences remained unclassified. Of these 86 genera, 20 (23% of cultivated genera) were not picked up by pyrosequencing.

Heatmaps showing the distribution of the most frequently recovered OTUs based on the forward (Figure S1) and reverse (Figure S2) cultivation sequences, revealed that many of these OTUs were shared between samples.

Pyrosequencing Data

Forward dataset. After processing the forward pyrosequencing data, 23,510 high quality sequences were left (on average 2,612±829 per sample); they were on average 243±14 nt long. The chimera content per sample in the forward dataset ranged from 0.1% (TM2) to 5.8% (SK5) of sequences (Table S1). For eight samples, in the non-redundant dataset (i.e. dataset filtered for duplicate sequences), the percentage of chimeras was higher than when considering the complete dataset, indicating that many chimeras were singletons or low-abundant sequences.

We observed 2,940 OTUs of which 947 remained unclassified at the phylum level (represented by 7,659 sequences) and 2,066 (15,271 sequences) at the genus level. Per sample, the number of OTUs unclassified at the phylum level varied between 40 (TM4) and 274 (WO10). The identified OTUs belonged to 220 genera in 19 phyla (Tables S2 and S3 respectively). *Proteobacteria*, *Cyanobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Acidobacteria* and *Planctomycetes* were present in every sample (Figure 1), although relative number and OTU richness could differ drastically. Cyanobacteria were well represented in most samples, but less so in SK5 and BB115. *Deinococcus-Thermus* was relatively well recovered and showed a high richness in the terrestrial samples (BB50 and BB115).

A total of 2,693 (84.9%) of the OTUs were restricted to one sample (Figure 3), and 1,464 (46.2%) were effectively singletons (i.e. represented by only one sequence).

The most abundant OTU (OTU3056) was represented by 2,216 sequences, nearly three times as many as the second most abundant OTU (OTU0858, 871 sequences), and was found in six out of the nine samples (BB115, BB50, PQ1, TM2, TM4 and WO10). It was not picked up through cultivation and was not identified using our RDP training set. A separate blast against the GreenGenes database [57], however, revealed that it was identical to *Phormidium autumnale* str. Arct-Ph5 (*Cyanobacteria*, a group not targeted by the cultivation experiments). None of the OTUs was found in every sample through pyrosequencing in the forward dataset. One OTU (OTU2885; *Rhizobacter*, *Gammaproteobacteria*) was found in seven samples. Five OTUs were recovered by pyrosequencing from six samples, including the aforementioned cyanobacterial OTU3056, a *Polaromonas* (OTU2491, which was also cultured) and a *Herbaspirillum* species (both *Betaproteobacteria*), and a *Methylobacterium* species (*Alphaproteobacteria*) and finally OTU2399, identified as *Brevundimonas* sp. (*Alphaproteobacteria*), which was actually retrieved from every sample (i.e. it was recovered through either cultivation, pyrosequencing or in some samples by both). All other OTUs were only recovered from five samples or less.

The combined number of OTUs from forward pyrosequencing and cultivation was 3,172 (totaling 25,088 sequences). Only 110 OTUs were shared between both approaches and 232 were restricted to the cultivation data. A heatmap (Figure S3) showing the distribution of the most frequently recovered pyrotag OTUs, revealed that few of these OTUs were shared between samples. In fact, most of these high-abundant OTUs were merely recovered from one or two samples. The SIMPROF analysis revealed that the community structure in all samples assessed using pyrosequencing is significantly different from that analyzed using culturing (Figure S4). Not unexpectedly, given cultivation bias, the similarity between samples analyzed with the culturing approach is higher.

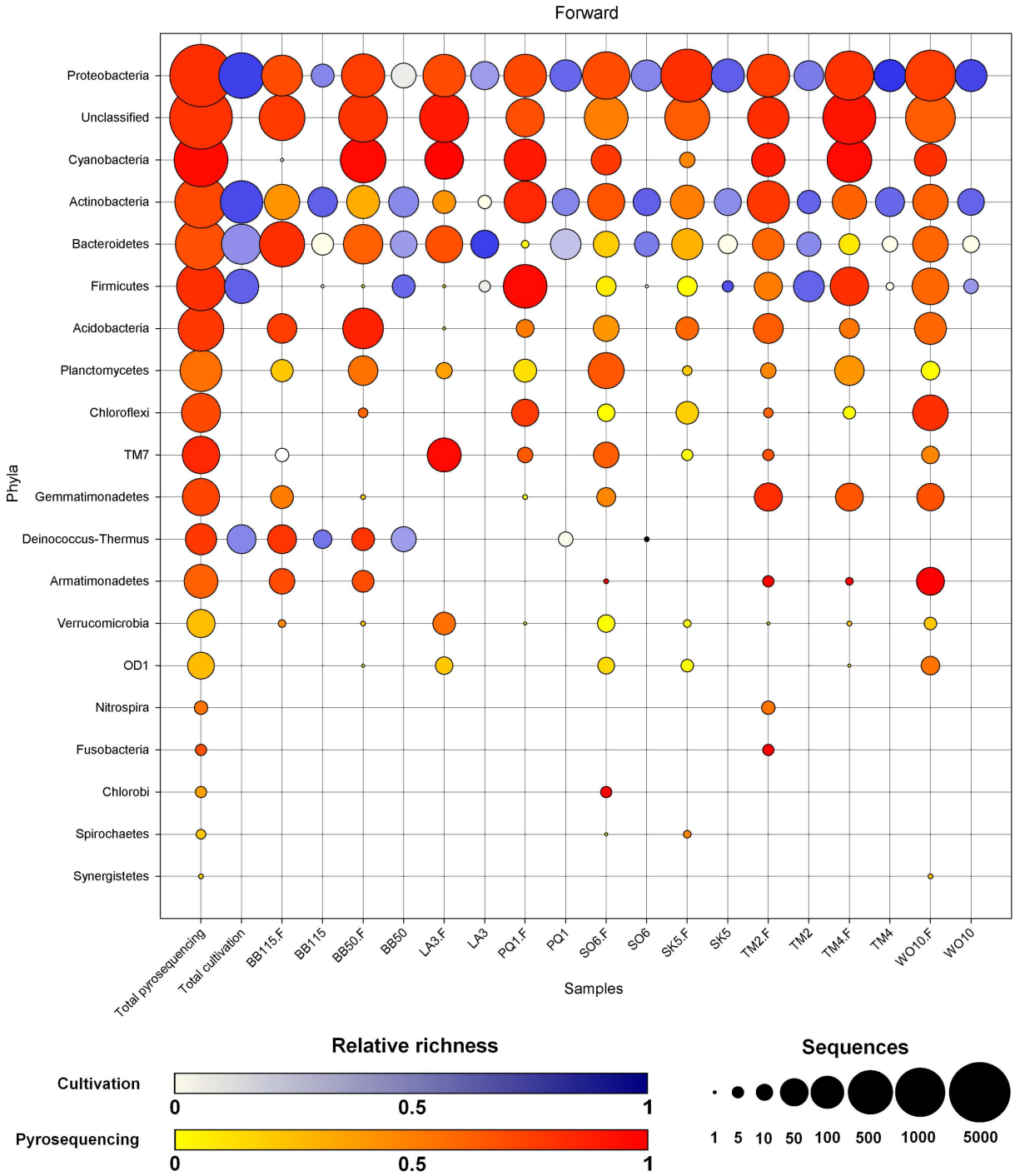

Figure 1. Overview of the distribution of the phyla per sample for the forward sequencing dataset. Circle area is a $\log_2$ transformation of the number of sequences ([$\log_2$(N)*5/Pl], with N the number of sequences in that phylum). Color intensity reflects the number of OTUs per phylum (total OTUs/total sequences), with a darker hue indicating a higher relative richness. The first two columns show the total number of sequences and diversity of each phylum for pyrosequencing and cultivation separately. The phyla are ordered according to decreasing total number of sequences. The yellow to red scale shows pyrosequencing data, the blue-purple scale the cultivation data.
doi:10.1371/journal.pone.0097564.g001

However, these observations were consistent when taking into account only the five phyla that were recovered by both approaches (data not shown).

Reverse dataset. Reverse pyrosequencing starting from the end of the V3 region resulted in 22,778 high quality sequences after processing. The chimera content was generally higher than for the forward pyrotags for all samples (Table S1). Particularly in sample SK5, up to 43.4% of the non-redundant sequences were identified as chimeras by Uchime, resulting in the removal of 23% of all sequences in that sample. Also for sample PQ1 23% of all sequences were removed, while only 19.6% of the unique sequences were flagged as chimeras, indicating a substantial

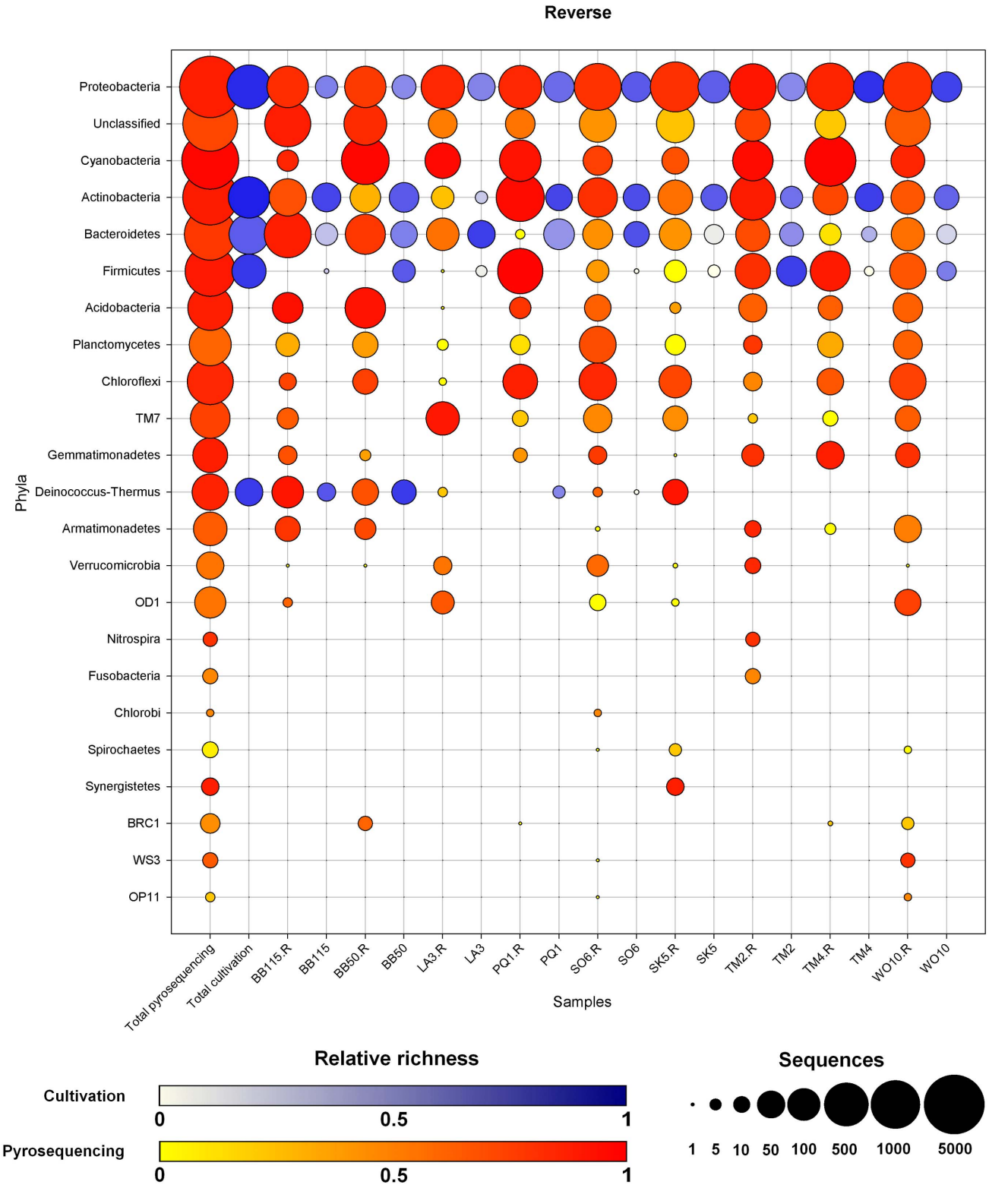

Figure 2. Overview of the distribution of the phyla per sample for the reverse sequencing dataset. Circle area is a $\log_2$ transformation of the number of sequences ([$\log_2$(N)*5/Pl], with N the number of sequences in that phylum). Color intensity is an approximation for the number of OTUs per sequence (total OTUs/total sequences). The first two columns show the total number of sequences and diversity of each phylum for pyrosequencing and cultivation separately. The order of the phyla is as in Figure 1 and additional phyla were added at the bottom. The yellow to red scale shows pyrosequencing data, the blue-purple scale the cultivation data.
doi:10.1371/journal.pone.0097564.g002

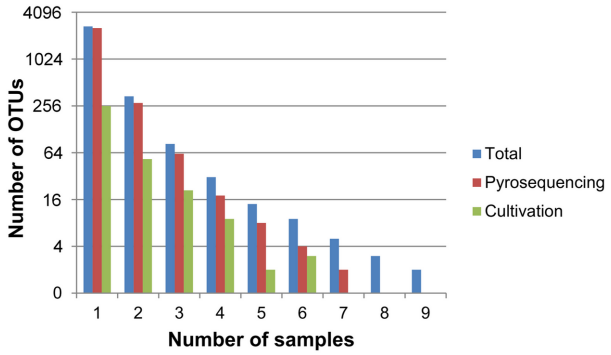

Figure 3. Bar chart illustrating the number of OTUs picked up from one or more samples for the forward dataset. The number of OTUs is $\log_2$ transformed. Blue bars, total sequences (pyrosequences plus cultivated sequences); red bars, pyrosequences only; green bars, cultivation sequences only.
doi:10.1371/journal.pone.0097564.g003

proportion of chimeras in this sample. We obtained only 1,983 OTUs overall, of which 485 remained unclassified at phylum level (2,776 sequences) while the rest belonged to 22 phyla (Figure 2, Table S3). We were able to identify 197 genera in the reverse dataset (Table S2). The taxonomy at genus level remained unresolved for 1,376 OTUs (12,295 sequences). Although considerably fewer OTUs were observed in the reverse dataset, the distribution over phyla were similar to these observed for the forward pyrosequences (Figure 2, Table S3). The number of sequences unclassified at phylum level (485 OTUs, 2,776 sequences) was much smaller than in the forward sequencing (947 OTUs, 7,659 sequences) and represented 24% versus 32% of the OTUs, respectively. Compared to the forward dataset, *Deinococcus-Thermus* was additionally picked up from samples LA3, SO6 and especially SK5 (Figure 2). Also *Cyanobacteria* and *Chloroflexi* were generally more abundantly picked up by the reverse sequencing, and additionally, three extra bacterial phyla were recovered: WS3, OP11 and BRC1. Phylum BRC1 was obtained from four different samples (BB50, PQ1, TM4 and WO10) with six OTUs in total; WRC3 was represented by two OTUs, one from SO6 and a second one from WO10; OP11 was also found in these two latter samples. The number of singleton OTUs was lower for the reverse dataset: 476 (24%) here vs. 897 (31%) in the forward dataset. This discrepancy equals 44% of the difference in the total number of OTUs obtained between both datasets (1,983 in the reverse dataset compared to 2,940).

Heatmaps showing the distribution of the OTUs most abundantly recovered in the reverse pyrosequencing data (Figure S5) and in the reverse cultivation dataset (Figure S2) reveal generally similar trends as for the forward sequencing (Figures S1 and S3). However, nine OTUs (1942, 1956, 1959, 2036, 2043, 2044, 2064, 2109 and 2115) in the high-abundant reverse pyrosequencing selection were also found in the cultivated dataset, which is considerably more than for the forward dataset. Especially OTU2109 (*Sphingomonadaceae* sp., *Alphaproteobacteria*) was recovered well through cultivation (not found in sample TM4), and pyrosequencing (not found in sample BB115). OTU1849 (*Methylobacterium*, *Alphaproteobacteria*) was recovered from all pyrosequencing samples. Four unclassified OTUs were recovered from eight samples (three alphaproteobacteria and one actinobacterium). The most abundant OTU (OTU1804) with 1,226 sequences was found in five samples. It was classified as an unknown cyanobacterial order by the RDP training set. Again, a blast against the Greengenes database resulted in *P. autumnale* (strains Ant-Ph68 and Arct-Ph5, both with an identity score of 100). Similar to the forward dataset, both techniques resulted in significantly different clusters and the variability between the different samples is higher in the datasets obtained through pyrosequencing (Figure S6).

Discussion

Comparison of Forward and Reverse Datasets

Two terrestrial and seven aquatic Antarctic microbial mat samples were subjected to bidirectional pyrosequencing of the V1 to V3 variable region of the 16S rRNA gene. After processing, the forward dataset spanned the V1 and V2 variable regions, while the reverse dataset covered the V3 and part of the V2 variable regions. The comparison of bidirectional sequencing revealed large differences in the number of OTUs recovered, although the number of sequences and genera was generally comparable. More in particular, the number of OTUs was about 50% higher for the forward dataset compared to the reverse dataset. This is in part likely due to the V1 region being more variable than the more conserved V3 region [58,59,60]. Hence, the traditionally used cut-off values (e.g. 95% as a proxy for genus level, or 97% for species level) which have proven to be insufficient or inadequate for all taxa [61], might additionally require modification for different regions of the 16S rRNA gene. Highly variable regions such as V1 could be clustered using lower values (for example 97%) than more conserved regions (e.g. V3 or V6), which might require a higher (e.g. 99%) identity cut-off. These considerations should be taken into account when selecting the region to analyze, but also when comparing studies and diversity data based on different variable regions [62]. Not only did the number of OTUs differ between both regions, identification was affected too. For example, although the number of genera identified from the forward and the reverse dataset was broadly similar (220 vs. 197), only 132 or 67% of the genera identified from the reverse dataset were also present in the forward dataset, corresponding to 60% of the genera in the forward dataset. The combined number of genera based on the RDP training set was 285. Similarly, for the Sanger sequences of the cultures, comparison of forward and reverse trimmed dataset revealed 76 and 61 genera respectively, of which 51 were in common. As pyrosequencing artifacts cannot have been introduced in the cultivation dataset, these differences highlight the impact of the variable zones covered on the outcome of the genus identifications. With the continuous development of NGS techniques, the significance of this problem can be expected to reduce with increasing read length.

Another striking difference between the sequencing directions was that the number of chimeras was higher in the reverse dataset (Table S1). This is probably also due to the differences in variability of the regions targeted; the more conserved V3 region might be more likely to function as a template for annealing than V1, especially between closely related taxa [63,64]. Furthermore, not only do PCR conditions (such as extension times and the number of PCR cycles) or conserved regions affect chimera formation [43,64,65], it has been shown that certain positions in the 16S rRNA gene are more prone to chimera formation [63]. This implies that chimeras are not necessarily restricted to low-abundant sequences, questioning the removal of only OTUs with a low abundance, a common practice to reduce artifacts.

Contrast between Diversity Data from Pyrosequencing and Cultivation

The comparison of the bacterial diversity estimate obtained by bidirectional 454 pyrosequencing with the results from previous

cultivation studies [4,33,34] unsurprisingly confirmed that pyrosequencing results in a higher diversity (in total 22 phyla, 285 genera) than obtained through culturing (5 phyla, 86 genera). Indeed, we observed a striking and significant difference in taxonomic composition and abundance of groups recovered using both methods, with communities standardized to the lowest number of sequences (Figures S4 and S6). This likely results from the obvious bias related to the specific cultivation conditions used, which were set to target mostly heterotrophic, aerobic and psychrophilic or psychrotolerant bacteria [4]. Some of the phyla that were detected by pyrosequencing but not picked up through cultivation included groups that were not targeted such as anaerobes (e.g. *Clostridium* which was frequently recovered in sample PQ1), phototrophic *Cyanobacteria* and *Chloroflexi*, or groups for which cultivation is not yet optimized and that have very few or even no cultured representatives (e.g. *Acidobacteria*, *Planctomycetes*, *Verrucomicrobia*, *Armatimonadetes*, TM7; see Table S3). Given that only heterotrophic bacteria had been targeted in the isolation campaigns and a limited set of cultivation conditions was tested, a comparison with pyrosequencing is only possible to a very limited extent. We tried to take this into account by further focusing this part of the discussion on the OTUs and named genera of the five phyla picked up by both techniques (*Actinobacteria*, *Bacteroidetes*, *Deinococcus-Thermus*, *Firmicutes* and *Proteobacteria*). This restricted comparison confirmed the general observation that pyrosequencing can detect more diversity at all taxonomic levels. Nevertheless, particularly at lower taxonomic and phylogenetic levels (OTUs, genera), we found extremely little overlap in the diversity between both datasets. For example, in the forward sequencing datasets, of the 342 OTUs recovered using culturing, 232 (67.8%) were not picked-up by pyrosequencing. For these five phyla, a total of 204 genera were identified, of which 51 were in common, 131 were unique for pyrosequencing and 22 unique for cultivation. Thus about 30% of cultured genera were not detected in our pyrosequencing data (e.g. the *Firmicutes* genus *Paenibacillus*; see Table S2). Reverse sequencing showed generally analogous results.

In addition to the above mentioned cultivation bias, at least three other non-mutually exclusive processes might underlie the significant differences between the cultivation and pyrosequencing datasets. Firstly, manual picking of individual colonies for further characterization in culture-based approaches introduces an additional bias. The sheer quantity of isolates makes it nearly impossible to select and cultivate every colony separately, especially when the number of samples is high. Phenotypic (morphological) selection may thus lead to an underestimation of the genotypic diversity, because macroscopically identical colonies might in fact represent different OTUs, whether closely related or not. Secondly, the failure of pyrosequencing to detect the majority of the cultured organisms could indicate that our sequencing depth was not large enough (Figures S7 and S8), which is often the case for large scale surveys [66], or that low-abundant organisms were missed because they were below the detection limit of the technique [67]. Thirdly, while sequencing depth is one aspect, PCR-related biases (e.g. GC-content) and sequencing errors (e.g. homopolymers) may also contribute to the observed differences [68,69,70]. A GC-content deviating strongly from 50% may induce a PCR-bias and this could explain why certain OTUs were not detected through pyrosequencing. However, calculation of the %GC of the cultivation-only sequences, in combination with the high number of such OTUs (67.8% of the cultivation OTUs), dismissed this hypothesis in our case (Table 1). Although our preprocessing was done rigorously, e.g. [43,45,50], we cannot exclude the possibility that some erroneous sequences have slipped through [32]. Nevertheless, the limited overlap between culturing and pyrosequencing data is in line with observations from comparisons of cultivation and other culture-independent techniques (e.g. clone libraries) in other ecosystems [71]. High-throughput culturing [72] and the use of more diverse growth conditions [73,74] would probably show that the actual overlap is (much) larger than our results currently suggest. Indeed, extending the incubation time (e.g. up to three months) might reveal additional rare community members [75]. Moreover, cultivation is even able to detect novel organisms where culture-independent techniques fail [74]. It has been proposed that 5000 denoised reads may be needed to describe 90% of the alpha-diversity of 15–20.000 reads and that because of the huge bacterial diversity, almost an infinite number of individuals might need to be identified to accurately describe communities [76].

Our comparison further confirmed that even low-abundant but widely distributed organisms can be picked up by both techniques. As an example, Figure 4 shows the distribution of genera in sample BB115 where, typically, the majority of genera are represented by only one or two sequences, some are moderately abundant and a few are very abundant taxa. That cultivation can pick up low-abundant bacteria may often be the result of cultivation conditions allowing enrichment of these taxa. For example, OTU 2399 (*Brevundimonas* sp., *Alphaproteobacteria*) was recovered from sample SK5 six times through pyrosequencing, while 38 times through cultivation. The ability of cultivation to pick up organisms from the rare biosphere was also demonstrated by Shade *et al.* [31], and these and our results show that the nutritional or cultivation requirements of these rare organisms are not necessarily extensive [72]. In fact, *Escherichia coli* is probably the best example to demonstrate this fact. While readily cultured and even functioning as a Gram-negative model organism, it is not a very abundant organism in the human gut [77].

Of the high-abundant OTUs (i.e. having more than 80 sequences) obtained by forward pyrosequencing, only three were also retrieved through cultivation (Figure S3). OTU2742 (*Porphyrobacter*, *Alphaproteobacteria*) was detected through pyrosequencing in samples LA3, PQ1, TM4, SO6 and WO10, and recovered by cultivation from SK5 and PQ1. A second OTU (OTU1961; unclassified alphaproteobacterium) found in BB115, BB50 and PQ1 was also found in two culture samples (SK5 and BB115). Strikingly, neither of these was found in the pyrosequence data of sample SK5. Finally, OTU2229 (*Sphingopyxis*, *Alphaproteobacteria*) was recovered from samples TM2 and WO10 through cultivation, and from samples SK5, SO6 and LA3 by pyrosequencing. In contrast, most of the OTUs frequently obtained via culturing (more than 10 sequences) were also picked up from the same sample by pyrosequencing, although generally at a lower relative abundance than through cultivation (Figure S1). Moreover, no OTU was shared and present at a high relative abundance in both datasets. In the reverse pyrosequencing dataset nine of the frequently recovered OTUs were also picked up by cultivation. One of these (OTU2043, unclassified alphaproteobacterium) was among the high-abundant OTUs in both techniques (Figures S2 and S5).

Notable Diversity Observations

While *Cyanobacteria* was the dominant phylum of photosynthetic bacteria in all samples, also the phylum *Chloroflexi* was present in all samples. Remarkably, diversity was considerably less in the forward dataset (47 OTUs including genera *Leptolinea* and *Chloroflexus*) than in the reverse dataset (75 OTUs including *Leptolinea*, *Levilinea*, *Caldilinea*, *Heliothrix*, *Herpetosiphon*, *Dehalogenimonas*, *Sphaerobacter*). The genus *Caldilinea*, originally described for thermophilic filamentous bacteria [78,79], was present in all

Table 1. Comparison of the GC content of the cultivation-only sequences with the overall values.

	Forward sequencing (V1–V2 regions)			Reverse sequencing (V3–V2 regions)		
	Average (%)	**Minimum (%)**	**Maximum (%)**	**Average (%)**	**Minimum (%)**	**Maximum (%)**
All sequences	54	28	70	56	34	67
Cultivation-only[a]	52	43	63	55	51	66

[a]Sequences from cultivation that were not picked up by pyrosequencing.
doi:10.1371/journal.pone.0097564.t001

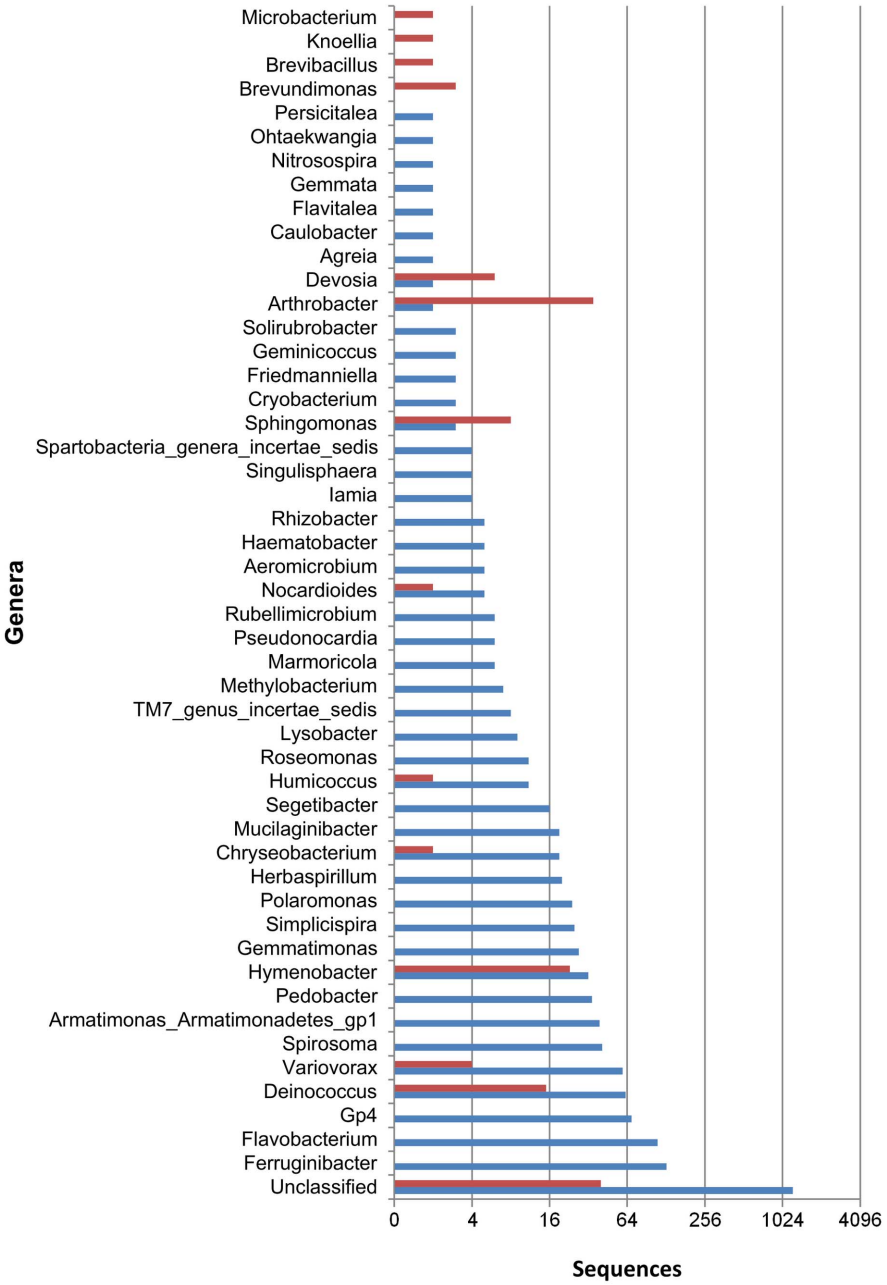

Figure 4. Rank-abundance plot showing the distribution of genera in a sample, illustrating the difference between techniques. Sequence numbers are plotted on a log scale. Blue bars are pyrosequencing based, red bars are cultivation based.
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samples (Table S2). The phylum *Chlorobi* was much less well represented (2 OTUs in one sample).

The phylum *Planctomycetes* was also well represented in all samples: eight genera were detected, although the diversity differed between samples. Notable is the relatively frequent presence of the unusual freshwater genus *Gemmata* [80] with 29 OTUs found in seven of the nine samples in the forward dataset (Table S2).

The genus *Deinococcus* was frequently recovered in the terrestrial samples, which was also especially obvious through cultivation (BB50). Among limnetic mat samples, this genus was only recovered from PQ1 and SO6 by cultivation and was not picked up by pyrosequencing (Table S2). The more exposed nature of terrestrial sites may provide habitats that are particularly suited to *Deinococcus* species which are known for their resistance to radiation and desiccation [81].

A small number of genera were relatively frequently detected in the pyrosequencing data of both terrestrial samples (BB50 and BB115) but rarely in the seven aquatic samples: *Hymenobacter* (30 OTUs terrestrial vs. 5 OTUs in one aquatic sample), *Spirosoma* (17 OTUs terrestrial vs. 1 OTU in one aquatic sample) and *Deinococcus* (12 OTUs terrestrial samples only). Conversely, a considerable diversity of the aquatic and clinical genus *Legionella* was picked up from the aquatic mat samples (62 OTUs in the forward and 39 in the reverse dataset from 6 or 5 of the samples) while no *Legionella* was detected in the terrestrial mat samples.

Pyrosequencing allowed us to obtain a considerable number of OTUs which are as yet unidentified at the genus level (e.g. 70.27% in the forward dataset) in addition to the potentially new taxa already detected through cultivation [4,33,34]. These might represent novel diversity adapted to the pristine and unique environment of Antarctica. This high number of novel sequences is comparable to other NGS studies in extreme and as yet understudied habitats. For example, 46% of the sequences from an acidic Andean hot spring remained unclassified at the phylum level [82]. However, the high number of novel sequences might in part also be related to (i) the database used (RDP) which contains a relatively low number of sequences, but is of high quality, and (ii) the presence of artifacts that could inflate the diversity. Indeed, in view of the many possible factors that can increase the sequence diversity, pyrosequencing data are often extensively filtered to remove flawed and chimeric sequences [43,44,45,49,50,83]. The sequence processing pipeline used here might reduce the error rate down to 0.02% [43]. We therefore assumed that the remaining sequences are of considerable quality, and that most remaining sequencing errors would be masked by clustering. Clustering of the OTUs at 95% similarity did not result in a large reduction of the number of OTUs or singletons (data not shown), indicating considerable diversity among the OTUs left. Our chimera filtering removed 2.5% and 5.7% of the total sequences in the forward and reverse data respectively. We opted not to remove the singletons and low-abundant sequences because (i) our approach already eliminated 16.6% (forward data) and 43.4% (reverse data) of the non-redundant sequences, and (ii) removing singletons may eradicate not only low quality sequences, but also biologically relevant sequences and novel taxa. In fact, 26 out of the 110 OTUs (23.6%) shared by both pyrosequencing and cultivation were singletons in the forward pyrosequencing data that were thus readily picked up from one or more samples through cultivation. In the reverse dataset the singletons comprised 8 of the 77 overlapping OTUs (10%). These high levels indicate that indiscriminate removal of all singletons would eliminate a considerable portion of the actual diversity.

Conclusions

Next Generation Sequencing techniques such as 454 pyrosequencing allow a much deeper sampling of microbial communities compared to the more traditional techniques. Our study revealed many unidentified OTUs and showed that the terrestrial and lacustrine bacterial diversity in Antarctica is orders of magnitude larger than previously believed. The comparison between NGS and culturing revealed that both techniques are complimentary and that only a limited number of OTUs is shared between both datasets. Although only a small number of these organisms were cultured, cultivation was able to pick up organisms from the rare biosphere, including organisms that were not recovered from pyrosequencing. With more sequencing depth and increasing read length, this may improve. It is clear that despite the ongoing technological developments, cultivation remains a useful method to uncover unknown diversity, and is currently certainly still needed for the physiological characterization and unambiguous identification of these organisms. Our comparison of forward (covering V1 and V2) and reverse sequences (covering V3 and part of V2) also revealed considerable differences in diversity obtained between variable regions and differences in the number of chimeras present. These aspects should be considered when comparing different studies.

Supporting Information

Figure S1 Heatmap showing the distribution of the most abundant OTUs based on the forward cultivation sequences**.** These high abundant OTUs are represented by at least 10 sequences. Pyrosequenced samples have the suffix.*F.*
(TIFF)

Figure S2 Heatmap showing the distribution of the most abundant OTUs based on the reverse cultivation sequences. These high abundant OTUs are represented by at least 10 sequences. Pyrosequenced samples have the suffix.*R.*
(TIFF)

Figure S3 Heatmap showing the distribution of the most abundant OTUs based on forward pyrosequencing. These high abundant OTUs are represented by at least 80 sequences. Pyrosequenced samples have the suffix.*F.*
(TIFF)

Figure S4 SIMPROF showing the clustering of the forward dataset. Each sample was subsampled 5 times with replacement to the lowest number of sequences (119 in cultured sample LA3). Full (black) lines are significant, dashed (red) lines are not.
(TIF)

Figure S5 Heatmap showing the distribution of the most abundant OTUs based on reverse pyrosequencing. These high abundant OTUs are represented by at least 100 sequences. Pyrosequenced samples have the suffix.*R.*
(TIFF)

Figure S6 SIMPROF showing the clustering of the reverse dataset. Each sample was subsampled 5 times with replacement to the lowest number of sequences (116 in cultured sample LA3). Full (black) lines are significant, dashed (red) lines are not.
(TIF)

Figure S7 Rarefaction of the forward sequenced samples.
(TIF)

Figure S8 Rarefaction of the reverse sequenced samples.
(TIF)

Table S1 Per sample chimera content for both sequencing directions.
(XLSX)

Table S2 Overview of the genera recovered. The number of OTUs within each genus is shown per sample for both pyrosequencing and cultivation.
(XLSX)

Table S3 Summary of the number of sequences and OTUs at the phylum level.
(XLSX)

Author Contributions

Conceived and designed the experiments: BT AW ADW EV DO WV. Performed the experiments: KP SD DO. Analyzed the data: BT EV TDM WVC. Contributed reagents/materials/analysis tools: KP SD DO WV WVC AW. Wrote the paper: BT EV DO TDM WVC WV AW.

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