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Research Article

RESEARCH ARTICLE

Polyphasic characterization of 10 selected ecologically relevant filamentous cyanobacterial strains from the South Shetland Islands, Maritime Antarctica

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One sentence summary: A polyphasic study of 10 cyanobacterial strains of characteristic species of Antarctic terrestrial environments to link morphological, molecular and ecological features.

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ABSTRACT

The evolutionary relationships of 10 Antarctic cyanobacterial strains of the order Oscillatoriales isolated from King George and Deception Islands, South Shetland Islands were studied by a polyphasic approach (morphology, 16S rRNA and internal transcribed spacer sequences). The studied taxa are characteristic of coastal Antarctic biotopes, where they form distinct populations and ecologically delimited communities. They were isolated from terrestrial habitats: microbial mats in seepages; crusts on soil, rocks, bones and mosses; mud, sometimes close to bird colonies; and from guano. Based on major phenotypic features, the strains were divided into four distinct morphotypes: *Leptolyngbya borchgrevinkii* (A), *Leptolyngbya frigida* (B), *Microcoleus* sp. (C) and *Wilmottia murrayi* (D). This morphological identification was in agreement with the phylogenetic relationships. For the first time, the 16S rRNA gene sequence of a strain corresponding to the *L. borchgrevinkii* morphotype was determined. Morphotype B is most related to sequences assigned to *L. frigida* isolated from microbial mats of coastal lakes in East Antarctica. Morphotype C belongs to a cluster including strains with morphotypes corresponding to *Microcoleus attenuatus*, *Microcoleus favosus* and *Microcoleus* sp., which are from Antarctica and other continents. Morphotype D is grouped with sequences assigned to *W. murrayi* mostly isolated from Antarctica.

Keywords: cyanobacteria; *Leptolyngbya*; *Wilmottia*; *Microcoleus*; rRNA operon; Antarctica

INTRODUCTION

Cyanobacteria are a fascinating and versatile group of bacteria of immense biological importance. This remarkable group of prokaryotic microorganisms has had the opportunity to colonize many habitats during their long evolution on Earth and has retained a large diversity up to the present times. They occur in a wide range of ecological niches including extreme

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environments, like the hostile cold circumpolar areas. A large number of eco- and morphotypes, which probably present ecophysiological adaptations, are observed in Antarctica and their identification is crucial to understand their distribution and ecology.

For the last 40 years, the taxonomy of cyanobacteria has been the subject of substantial controversy. The classical botanical taxonomy, based on morphology (e.g. Anagnostidis and Komárek 1985, 1988), has been challenged by the bacteriological approach. A brief review of the botanical and bacteriological approaches to cyanobacterial taxonomy is outlined by Wilmotte (1994). A preliminary and fragmentary bacteriological classification was given in *Bergey's Manual of Systematic Bacteriology* edited by Castenholz (2001). However, the authors restricted themselves to the description of 'form-genera', because of insufficient knowledge on the whole cyanobacterial diversity. Thus, the taxonomic systems currently used to identify and classify the cyanobacteria are still largely based on morphological characters. However, the simple morphology and the important plasticity of cyanobacteria are problematic, as is discussed in a number of taxonomic works (e.g. Anagnostidis and Komárek 1988; Casamatta *et al.* 2005; Jungblut *et al.* 2005; Taton *et al.* 2006a). The necessity to investigate not only the phenotypic but also the genotypic characters in a so-called 'polyphasic approach' has clearly been demonstrated. More and more molecular data become available and are used now to revise the cyanobacterial taxonomy (e.g. Komárek 2006; Komárek *et al.* 2014), but there are still many taxa for which there are no sequences available.

Antarctic cyanobacteria are still poorly studied despite of their great abundance and importance in Antarctic non-marine ecosystems. The members of the order Oscillatoriales are abundant and widespread in the ice-free regions of Antarctica. Moreover, they dominate the biomass of photosynthetic organisms in a variety of terrestrial and aquatic habitats (Vincent 2000). Cyanobacterial communities play an important role in colonization and succession as well as soil forming processes in Antarctic deglaciated environments (Elster 2002; Komárek and Komárek 2003) but their precise identification is hindered by the uncertainties that are currently plaguing the cyanobacterial taxonomy, as explained above. Many floristic and taxonomic phycological studies in various regions of Antarctica included some morphological and ecological data about cyanobacteria (e.g. West and West 1911; Broady 1979, 1986; Ling and Seppelt 1998). A few works have focused solely on the characterization of Antarctic Oscillatoriales based on morphological investigations (Broady, Garrick and Anderson 1984; Broady and Kibblewhite 1991). The number of publications that have integrated both sources of information, genomic and phenotypic, for taxonomic purposes and phylogenetic reconstruction of Antarctic Oscillatoriales has increased during the last years but is still limited (Nadeau, Milbrandt and Castenholz 2001; Taton *et al.* 2003, 2006a, 2008; Casamatta *et al.* 2005; Jungblut *et al.* 2005; Comte *et al.* 2007; Marquardt and Palinska 2007, 2008; Strunecký, Elster and Komárek 2010). Therefore, as explained by Komárek and Komárek (2010), the Antarctic cyanobacterial biodiversity records hold on one hand many traditional morphospecies characterized only by their morphology and that are recorded in ecological surveys but not identified by molecular methods, and on the other hand inventories of genotypes with little or no morphological and ecological characterizations. However, a polyphasic approach is crucial to determine baseline biodiversity data and correct ecological information for the taxa.

In his review of 2015, Komárek has proposed the existence of Antarctic endemic taxa, forming stable communities in specific habitats. His list of possible endemic species included *Leptolyngbya borchgrevinkii* Komárek 2007, *Pseudanabaena frigida* (Fritsch) Anagnostidis 2001 (= *Leptolyngbya frigida* Anagnostidis et Komárek 1988), and *Wilmottia murrayi* (W. et G.S. West) Strunecký, Elster et Komárek 2011.

The purpose of the present research is to characterize 10 selected ecologically relevant Antarctic strains of Oscillatoriales by a polyphasic approach (morphology, 16S rRNA gene and internal transcribed spacer (ITS) sequence data). These strains were deposited in a public culture collection (BCCM/ULC366-373 and 376) to serve as references. The analysis of the biogeographic distribution of the organisms based on the morpho- and genotypes also contributes to enlarging knowledge of Antarctic cyanobacteria. Together with data from environmental surveys, these baseline data on species' identity and distribution could contribute in future to assessing changes in cyanobacterial ecological ranges, which might be due to the impacts of global climatic and anthropogenic changes.

MATERIAL AND METHODS

Collection of samples

Samples were collected from a variety of non-marine habitats of the South Shetland Islands in January 1990 and during the 21st and 22nd Brazilian Antarctic Expeditions in December 2002–January 2003 and December 2003–January 2004, respectively (Table 1).

Isolation and cultivation of cyanobacteria

Sample material was spread over the surface of BG11 2% agarized medium (Rippka *et al.* 1979) in Petri dishes. Plates were incubated at 15°C under continual artificial illumination with white fluorescent lamps providing an irradiance of 80 μmol photons m^{-2} s^{-1}. Unicyanobacterial stock cultures were obtained by picking off material from the edges of discrete colonies and by subsequent repetitive transfers of single trichomes or hormogonia in tubes of agarized BG11 medium. The clonal cultures were maintained at 10°C and 25°C under the conditions described above.

For DNA isolation, the clonal cultures were cultivated in liquid BG11 medium at 25°C under a 16:8 light:dark photoperiod.

Microscopy and morphometry

Morphological characteristics of the cyanobacterial strains were observed using an Olympus BX51 microscope equipped with differential interference contrast optics (UPlanSApo ×100/1.40 oil immersion objective). The following diacritical quantitative and qualitative features were recorded: width and length of intercalary cells, cell shape for intercalary and terminal cells, presence or absence of constrictions at the cross-wall, necridic cells, attenuation of apical cells and sheath morphology. The lengths and widths of 50–70 cells were measured for each strain on microphotographs with ImageJ 1.33 software. The minimum and maximum values (mean value ± standard deviation) were calculated. Microphotographs for documentation of the cyanobacterial strains were taken with an ARTCAM 300MI 3Mpxl CMOS USB 2.0 Camera, equipped with Quick PHOTO MICRO 2.1 software. The strains were identified according to the literature for temperate regions (Komárek and Anagnostidis 2005) and Antarctic habitats (Komárek 2007; Strunecký, Elster and Komárek 2011).

Table 1. Origin of 10 Antarctic strains and corresponding 16S rRNA and ITS GenBank accession numbers.

Strain designation (isolator, year/collection no.)	BCCM/ULC number	Sampling locality	Habitat	GenBank accession number	
				16S rRNA	ITS
KOVACIK-ANT 1990/4	366	Nelson Island; 62°14′S, 59°01′W	Brown–black cyanobacterial mats in a seepage	JN979956	JN966937
KOVACIK-ANT 2003/73	367	King George Island, Admiralty Bay, Keller Peninsula; 62°04′08.0″S, 58°25′07.7″W	Dry epilithic crusts in a seepage	JN979965	JN966938
KOVACIK-ANT 2003/11a	368	King George Island, Admiralty Bay, Keller Peninsula; 62°04′53.0″S; 58°25′30.1″W	Black epilithic crusts near *Larus dominicanus* nests	JN979962	JN966939
KOVACIK-ANT 2003/15	369	King George Island, Admiralty Bay, Keller Peninsula; 62°04′40.5″S, 58°25′25.5″W	Black skinny crusts on moss near the *Catharacta maccormicki* nests	JN979964	JN966940
KOVACIK-ANT 2003/11b	370	King George Island, Admiralty Bay, Keller Peninsula; 62°04′53.0″S; 58°25′30.1″W	Black epilithic crusts near *Larus dominicanus* nests	JN979957	JN966942
KOVACIK-ANT 2003/43	371	King George Island, Admiralty Bay, Keller Peninsula; 62°05′02.9″S; 58°25′19.0″W	Dark green growth on remainder of whale bone near seashore	JN979958	JN966943
JANCUSOVA-ANT 2004/3	372	King George Island, Admiralty Bay, Crepin Point; 62°05′30.3″S, 58°28′32.3″W	Green powdery soil crust near birds' nests	JN979960	JN966944
JANCUSOVA-ANT 2004/4	—	King George Island, Admiralty Bay, Rakusa Point; 62°09′30.0″S, 58°27′30.0″W	Guano sample from a penguin rookery site	JN979961	JN966945
KOVACIK-ANT 2004/50	373	King George Island, Admiralty Bay, Copacabana; 62°10′45.0″S, 58°26′49.0″W	Green soil powder near a penguin rookery side	JN979959	JN966946
KOVACIK-ANT 2004/75	376	Deception Island, Whalers Bay; 62°59′S, 60°33'W	Mud from a littoral shallow coastal pool	JN979963	JN966941

Molecular analysis

DNA was extracted from clonal cultures of all strains using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Liquid culture was transferred to a microcentrifuge tube and centrifuged for 2 min at 15 000 g. Sedimented biomass was disrupted by repetitive vortexing and freeze-thawing in the presence of glass beads (0.1 g/sample) and Buffer AP1. Gel electrophoresis was performed in 0.5% agarose gel in Tris–acetate–EDTA (TAE) buffer in order to estimate the concentrations of the isolated DNA.

PCR amplification of a cyanobacterial 16S rRNA gene plus the internal transcribed spacer (ITS) was performed in a 50-μl reaction mixture containing 1× Super *Taq* Plus PCR buffer, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 0.5 μM 16S27F and 23S30R primers (Supplementary Table S1), 1 mg ml^{-1} of bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), 2–20 μl of DNA solution, and 0.8 U of Super *Taq* Plus (HT Biotechnology, Cambridge, UK). Amplification was carried out with a Gene Cycler (Bio-Rad, Hercules, CA, USA) as follows: one cycle of 5 min at 94°C, 10 cycles of 45 s at 94°C, 45 s at 57°C and 2 min at 68°C; 25 cycles of 45 s at 92°C, 45 s at 54°C and 2 min at 68°C; and a final elongation step of 7 min at 68°C.

Electrophoresis of 8 μl PCR products was performed in 1.5% agarose gel in TAE buffer. A negative control without DNA was included in each series of PCR and a double volume of its product was loaded to better check for contamination.

For each strain, a partial 16S rRNA and a complete ITS sequence were determined using the sequencing primers 16S359F, 16S1092R, 16S1494R and 23S30R, respectively (Supplementary Table S1). Sequencing was carried out by GenomeExpress (Paris, France) and GIGA (http://www.giga.ulg.ac.be/) (Liège, Belgium). The 16S rRNA and ITS sequences were submitted to GenBank and the accession numbers JN979956 to JN979965 and JN966937 to JN966946, respectively, were obtained.

The received sequences were edited and assembled using GENEIOUS (Kearse *et al.* 2012). For the 16S rRNA sequences, the six most closely related strains and the two most related uncultured sequences were retrieved with the option 'Seqmatch' of the Ribosomal Database Project (RDP) software (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp). Phylogenetic trees with all these sequences were constructed with the neighbor-joining method and the software TREECON (Van De Peer and De Wachter 1997), on an alignment of 1051 positions (*E. coli* 415–1464) that were common to most of our strains. The Kimura model was used to correct for multiple mutations. Indels were not considered. A bootstrap analysis involving 500 resamplings was carried out. In addition, a Maximum Likelihood tree was constructed with the K2+G model (selected on the basis of the Bayesian information criterion (BIC) value) using MEGA6 (Tamura *et al.* 2013). Both trees had the same topology and only the first one is shown. The sequences were grouped by MOTHUR with the average neighbour (Schloss *et al.* 2009) into operational taxonomic units (OTUs), which are groups of partial 16S rRNA sequences with more than 97.7% similarity. This threshold was chosen as close to the minimal value for a prokaryotic species (Stackebrandt and Goebel 1994; Gevers et al. 2005). Moreover, a BLAST analysis was performed to find the most similar sequences in GenBank (14 October 2015). For the ITS sequences, the related sequences were found by a

BLAST analysis but only the sequences that could be meaningfully aligned were kept in the alignment. A phylogenetic analysis was performed as for the 16S rRNA sequences, but with the Jukes and Cantor model. The pairwise similarity percentages were calculated manually, not taking into consideration the ambiguities.

RESULTS

Morphological characteristics

The microscopic observation of 10 Antarctic strains revealed that they belong to the order Oscillatoriales, more specifically to the genera *Leptolyngbya*, *Microcoleus* and *Wilmottia* (Komárek and Anagnostidis 2005; Komárek 2007; Strunecký, Elster and Komárek 2011). The strains were divided into four distinct morphotypes on the basis of major phenotypic features. The morphological characteristics of the strains are summarized in Table 2.

Within *Leptolyngbya*, the strains KOVACIK-ANT 1990/4 and KOVACIK-ANT 2003/73 belong to two morphotypes, A and B, respectively. Morphotype A is distinguished from morphotype B mainly by thinner trichomes and longer cells. It corresponds to *Leptolyngbya borchgrevinkii*, which was described by Komárek (2007) as a new Antarctic species. The presence of spirally coiled filaments inside the colonies and the narrow filaments with polar granules are typical for this species (Fig. 1A; Supplementary Fig. S1, 1–8). Morphotype B corresponds to *Leptolyngbya frigida* described in Komárek and Anagnostidis (2005) and also to the Antarctic strains with a *L. frigida* morphotype described in Taton *et al.* (2006a) (Fig. 1B; Supplementary Fig. S1, 9–16).

Strains KOVACIK-ANT 2003/11b, KOVACIK-ANT 2003/43, JANCUSOVA-ANT 2004/3, JANCUSOVA-ANT 2004/4 and KOVACIK-ANT 2004/50 were designated as morphotype C and correspond to a *Microcoleus* morphotype based on generally straight trichomes (width 4.1–7.4 μm) with frequently curved cell ends that usually contained a calyptra (thickened outer wall at the tip of the end cell). The cell content of the strains cultivated at 25°C was markedly keritomized (Fig. 1C–G; Supplementary Fig. S1, 18, 32, 33, 38–40, 45, 46, 57–58). This net-like appearance of the cytoplasm seems to be due to widened intrathylakoidal spaces (Komarek and Anagnostidis 2005). Within this group, the strains differed in the degree of coiling and colour at 10 and 25°C (Supplementary Figs S2 and S3). The strain KOVACIK-ANT 2004/50 kept a dark blue-green thallus and showed no coiling at both temperatures. Strain JANCUSOVA-ANT 2004/3 similarly kept an olive-green thallus without coiling at 10 and 25°C. In contrast, the dark blue-green thallus of strains KOVACIK-ANT 2003/11b and KOVACIK-ANT 2003/43 at 25°C changed into olive-green at 10°C, with markedly coiled filaments. This coiling was not observed for strain JANCUSOVA-ANT 2004/4 that also changed from blue-green at 25°C to olive-green at 10°C.

Three strains, KOVACIK-ANT 2003/11a, KOVACIK-ANT 2003/15 and KOVACIK- ANT 2004/75, were morphologically distinct from the other strains on the basis of the characteristics of their thallus, trichomes, cell granulation and apical cells. They were designated as Morphotype D and assigned to *Wilmottia murrayi* (Fig. 1H–J; Supplementary Fig. S1, 59–66, 67–78, 79–82). The first two strains are quasi identical and slightly distinct from the third one. The dark blue-green thallus of the strains KOVACIK-ANT 2003/11a and KOVACIK-ANT 2003/15 had a tendency to change to a yellowish-green colour, which was not observed for strain KOVACIK-ANT 2004/75.

Phylogenetic analysis

According to the phylogenetic analysis based on the 16S rRNA sequences (Fig. 2), the 10 strains were divided into two major clades. Clade 1 includes the *Leptolyngbya* morphotypes A and B, whereas sequences of morphotypes C and D are located in clade 2. In the latter, the strains were very closely related on the basis of the 16S rRNA and the higher resolution groupings given by the ITS sequences were necessary to better affiliate our strains with reference strains and determine their taxonomy.

In clade 1, we have obtained for the first time the 16S rRNA gene sequence of a strain (KOVACIK-ANT 1990/4) that corresponds to a *L. borchgrevinkii* morphotype. As shown in Fig. 2, the most similar strains (ca 99% similarity) are Antarctic strains identified as *Leptolyngbya antarctica* and isolated from microbial mats of lakes in the Larsemann Hills (LH) in Eastern Antarctica (ANT.LG2.5 (ULC032), ANT.LWAV6.1 (ULC043), ANT.LWA.1 (ULC036), ANT.L18.1 (ULC031)) and of lake Fryxell in the McMurdo Dry Valleys (MMDV) in Southern Victoria Land (ANT.LFR.1) (Taton *et al.* 2006a). Moreover, this strain is also closely related (ca 99%) to Antarctic clone sequences (RJ102 and Fr397) from lakes in the same regions as the strains (Taton *et al.* 2006b). In addition, the BLAST analysis shows more than 97.5% sequence similarity to Antarctic clones from a lake moss pilar in Syowa Oasis (MPB1-8) and from Lake Bonney ice cover (LB3-46) (Priscu *et al.* 1998) but also to a clone from Nepalese glaciers (B10905G) (Schmidt *et al.* 2011), clones from biofilms on dolomite filters in Scotland (9d7, 2C7, 5g7) (Feder et al. 2015) and one clone from a Swiss biofilm on a wooden wall of a freshwater spring in the central Alps (C10-AlvEE).

The ITS sequence of strain KOVACIK-ANT 1990/4 is most related (ca 94%) to clones (LMM1-20 and Fr005) from microbial mats of lakes Miers and Fryxell (MMDV) (Fig. 3) and share about 87% ITS similarity with the strains of *L. antarctica* from LH cited above.

The 16S rRNA sequence of strain KOVACIK-ANT 2003/73 is most related (only one substitution for 1019 positions) to two sequences identified as *L. frigida* (ANT.LMA.1 (ULC020), ANT.L70.1 (ULC025)) isolated from the LH (Taton *et al.* 2006a). The tree of Fig. 2 also shows that the OTU includes a strain (1T12c) from an Italian fountain (Cuzman *et al.* 2010) and *Leptolyngbya schmidlei* GSE-PSE46-15a from the USA (Bohunická, Johansen and Fučíková 2011) but also clones from lakes in the Pyrenees mountains (GBe_032, GBe_058) (Bartrons, Catalan and Casamayor 2012). The ITS sequence can only be aligned with five others and is most similar to the sequences of the two Antarctic *L. frigida* strains cited above and the clone RJ010 from LH (97.7%). In contrast, the next related ITS sequence, *L. schmidlei* GSE-PSE46-15a, only shows 73% similarity (Fig. 4).

In clade 2 of the 16S rRNA tree (Fig. 2), the morphotypes of strains KOVACIK-ANT 2003/11b, KOVACIK-ANT 2003/43, JANCUSOVA-ANT 2004/3, JANCUSOVA-ANT 2004/4 and KOVACIK-ANT 2004/50 correspond to a cluster of strain sequences generally identified as *Microcoleus* sp. They do not possess the 11 bp insertion that is considered as typical for *Microcoleus vaginatus* (Strunecky *et al.* 2013). However, the five strains could be distinguished into four subgroups based on 11 variable positions. More than 250 related sequences were identified with BLAST at 16S rRNA similarity levels above 98%, which shows the abundance of this genotype in public databases. They included Antarctic and non-Antarctic strains identified by different names, illustrating the need for further study of this group (Fig. 2). As the 16S rRNA similarities were very high, it was necessary to consider the higher resolution groupings given by the

Table 2. Morphological characteristics of 10 Antarctic strains and their arrangement into four morphotypes and taxonomic assignments (sensu Komárek and Anagnostidis 2005; Komárek 2007; Strunecký, Elster and Komárek 2011, 2013).

Strain	Morphological description	Morphotype	Taxon	Figures
KOVACIK-ANT 1990/4	Thallus consisting of densely entangled filaments, blue-green in colour. Filaments nearly straight, ± regularly or irregularly coiled, forming spirally coiled formations inside colonies. Sheaths indistinct, or distinctly developed around individual trichomes, colourless. Trichomes pale blue-green, 0.9–1.1 μm wide, slightly constricted at the cross walls. Cells cylindrical, longer than wide, 1.2–2.6 μm long, with one polar granule near cross walls. Apical cells rounded or conical rounded.	A	*Leptolyngbya borchgrevinkii* Komárek	Fig. 1A; Supplementary Fig. S1, 1–8
KOVACIK-ANT 2003/73	Thallus consisting of densely entangled filaments, blue-green in colour. Filaments ± curved or regularly coiled, entangled, sometimes parallel arranged. Trichomes with colourless sheaths, pale blue-green, 1.3–1.8 μm wide, clearly constricted at the cross walls. Necridic cells present. Cells cylindrical, mostly longer than wide, sometimes shorter than wide (quadratic?), 1.3–2.4 μm long, with one granule in the centre. Apical cells rounded.	B	*Leptolyngbya frigida* (Fritsch) Anagnostidis et Komárek	Fig. 1B; Supplementary Fig. S1, 9–16
KOVACIK-ANT 2003/11b	Thallus a spreading mat of filaments, sometimes markedly coiled, penetrating the agar, dark blue-green to olive-green in colour. Filaments mostly straight. Sheaths firm, distinct, colourless. Trichomes blue-green or greyish blue-green, 4.7–7.0 μm wide, not constricted at the cross walls, abruptly attenuated at the ends, straight or weakly hooked. Necridic cells present. Cells cylindrical, shorter than wide, 1.7–5.6 μm long, granular, with granules especially concentrated at the cross walls. Cell content keritomized. Apical cells frequently elongated, capitate, with calyptra.	C	*Microcoleus attenuatus* (Fritsch) Strunecký, Komárek et Johansen	Fig. 1C; Supplementary Fig. S1, 17–27
KOVACIK-ANT 2003/43	Thallus a spreading mat of filaments, sometimes markedly coiled, penetrating the agar, dark blue-green to olive-green in colour. Filaments mostly straight. Sheaths firm, distinct, colourless. Trichomes blue-green or greyish blue-green, 4.6–7.4 μm wide, not constricted at the cross walls, abruptly attenuated at the ends, straight or weakly hooked. Necridic cells present. Cells cylindrical, shorter than wide, 1.7–5.9 μm long, granular, with granules especially concentrated at the cross walls. Cell content keritomized. Apical cells frequently elongated, capitate, with calyptra.	C	*Microcoleus attenuatus* (Fritsch) Strunecký, Komárek et Johansen	Fig. 1D; Supplementary Fig. S1, 28–33
JANCUSOVA-ANT 2004/3	Thallus a spreading mat of filaments, penetrating the agar, olive-green in colour. Filaments mostly straight, rarely entangled. Sheaths firm, distinct, colourless. Trichomes blue-green or greyish blue-green, 4.6–7.0 μm wide, not constricted at the cross walls, abruptly attenuated at the ends, straight or weakly hooked. Necridic cells present. Cells cylindrical, shorter than wide, 1.7–4.7 μm long, granular, with granules especially concentrated at the cross walls. Cell content keritomized. Apical cells capitate, with calyptra.	C	*Microcoleus* sp. 1	Fig. 1E; Supplementary Fig. S1, 34–40
JANCUSOVA-ANT 2004/4	Thallus a spreading mat of filaments, penetrating the agar, dark blue-green to olive-green in colour. Filaments mostly straight. Sheaths firm, distinct, colourless. Trichomes blue-green or greyish blue-green, 4.4–6.6 μm wide, not constricted at the cross walls, abruptly attenuated at the ends, straight. Necridic cells present. Cells cylindrical, shorter than wide, 1.4–5.3 μm long, granular, with granules especially concentrated at the cross walls. Cell content keritomized. Apical cells capitate, with calyptra.	C	*Microcoleus* sp. 2	Fig. 1F; Supplementary Fig. S1, 41–49
KOVACIK-ANT 2004/50	Thallus a spreading mat of filaments, penetrating the agar, dark blue-green in colour. Filaments mostly straight. Sheaths firm, distinct, colourless. Trichomes blue-green or greyish blue-green, 4.1–6.1 μm wide, not constricted at the cross walls, abruptly attenuated at the ends, straight or weakly hooked. Necridic cells present. Cells cylindrical, shorter than wide, 1.5–5.2 μm long, granular, with granules especially concentrated at the cross walls. Cell content keritomized. Apical cells capitate, with calyptra.	C	*Microcoleus favosus* (Gomont) Strunecký, Komárek et Johansen	Fig. 1G; Supplementary Fig. S1, 50–58

Table 2. *(Continued.)*

Strain	Morphological description	Morphotype	Taxon	Figures
KOVACIK-ANT 2003/11a	Thallus a spreading mat of filaments, penetrating the agar, dark blue-green or yellowish-green in colour. Filaments straight to flexuous. Trichomes with colourless sheaths, blue-green or greyish blue-green, 2.9–5.3 μm wide, not constricted at the cross walls. Necridic cells present. Cells cylindrical, mostly shorter than wide, sometimes longer than wide, 2.2–7.2 μm long, granular, with large granules concentrated near the cross walls but present throughout the cell as well. Cell content keritomized. Apical cells rounded or conical rounded. Calyptra absent.	D	*Wilmottia murrayi* (W. et G. S. West) Strunecký, Elster et Komárek	Fig. 1H; Supplementary Fig. S1, 59–66
KOVACIK-ANT 2003/15	Thallus a spreading mat of filaments, penetrating the agar, dark blue-green or yellowish-green in colour. Filaments straight to flexuous. Trichomes with colourless sheaths, blue-green or greyish blue-green, 2.9–4.5 μm wide, not constricted at the cross walls. Necridic cells present. Cells cylindrical, mostly shorter than wide, sometimes longer than wide, 2.3–8.1 μm long, granular, with large granules concentrated near the cross walls but present throughout the cell as well. Cell content keritomized. Apical cells rounded or conical rounded. Calyptra absent.	D	*Wilmottia murrayi* (W. et G. S. West) Strunecký, Elster et Komárek	Fig. 1I; Supplementary Fig. S1, 67–78
KOVACIK-ANT 2004/75	Thallus a spreading mat of filaments, penetrating the agar, dark blue-green in colour. Filaments straight to flexuous. Trichomes with colourless sheaths, blue-green or greyish blue-green, 3.9–4.4 μm wide, not constricted at the cross walls, rarely abruptly attenuated at the ends. Necridic cells present. Cells cylindrical, mostly shorter than wide, sometimes longer than wide, 2.7–5.8 μm long, granular, with large granules concentrated near the cross walls but present throughout the cell as well. Cell content keritomized. Apical cells rounded or conical rounded. Calyptra absent.	D	*Wilmottia murrayi* (W. et G. S. West) Strunecký, Elster et Komárek	Fig. 1J; Supplementary Fig. S1, 79–82

ITS sequences. The ITS of the five *Microcoleus* strains showed the same four subgroups as obtained with the 16S rRNA, but they could be better differentiated as there was up to 22% dissimilarity between the most distant strains (JANCUSOVA-ANT 2004/3 and KOVACIK-ANT 2004/50) (Fig. 5). The ITS of strains KOVACIK-ANT 2003/11b and KOVACIK-ANT 2003/43 were identical to each other and to the ones of two Antarctic *Microcoleus attenuatus* strains, KI19 and KG29 (later data not shown). The most similar ITS sequence to JANCUSOVA-ANT 2004/3 was LCRCYANT11 (soil of Wright Valley, MMDV), but with only 96.5% similarity. Strain JANCUSOVA-ANT 2004/4 is situated in a lineage with *Phormidium autumnale* CCALA 697 (soil Canadian Arctic; Palinska and Marquardt 2008) and P00 (epipelon Czech pond; Hašler *et al.* 2012), but with only 97.3% and 96.7% similarity, respectively. The ITS of the most divergent strain, KOVACIK-ANT 2004/50, is included in a lineage with the strain *Microcoleus favosus* JR2 (James Ross Island (JR), Antarctica) with 98.9% similarity, and their closest relatives (94%) are sequences from Forlidas Pond, Transantarctic Mountains (Fernandez-Carazo *et al.* 2011).

The 16S rRNA sequences of the strains KOVACIK-ANT 2003/11a, KOVACIK-ANT 2003/15 and KOVACIK-ANT 2004/75 were very similar (Fig. 2). The first two are quasi identical to each other and to Antarctic sequences of strains CCAP 1462/11, KGI28, Ant-Ph58 and 29RL-C and an uncultured *Phormidium* sp. 16S-3 (one or two substitutions on ca. 1000 positions), some of which were assigned to *Phormidium murrayi*. The latter species has been recently revised and renamed as *Wilmottia murrayi* with the strain KOVACIK-ANT 2007/1 (KGI28) from King George Island (KGI) as type strain (Strunecký, Elster and Komárek 2011). This group of seven sequences present four substitutions with the 16S rRNA sequence of KOVACIK-ANT 2004/75. The OTU D also includes other Antarctic strains [ANT.LPE.2 (Taton *et al.* 2006a), A27 (Strunecký, Elster and Komárek 2011)], but also clones from Spanish rivers (MED-16, MSmat.3.54) and a Japanese stromatolite (ust3-103). Due to the very high 16S rRNA similarities, the ITS sequences were needed to better distinguish the strains. The ITS of strains KOVACIK-ANT 2003/11a and KOVACIK-ANT 2003/15 were identical, and showed only 95% similarity to the sequence of strain KOVACIK-ANT 2004/75 (Fig. 6). There were only six related ITS sequences obtained with BLAST that could be aligned with these three strains. The type strain of *W. murrayi* KGI28 (Strunecký, Elster and Komárek 2011) has an intermediate position between our strains with 97.3% similarity to KOVACIK-ANT 2003/15 and 96.1% to KOVACIK-ANT 2004/75. Another intermediately located strain was isolated from soil of South Orkney Islands, Antarctica (CCAP 1462/11) with 99% and 94.7% ITS similarity to KOVACIK-ANT 2003/15 and KOVACIK-ANT 2004/75, respectively. Two strains isolated from Pendant lake (LH) are situated at some distance in the ITS tree (77.3% similarity) though they had 99.3% 16S rRNA similarity.

DISCUSSION

The morphological investigations of the present study have revealed four different morphotypes assigned to the genera *Leptolyngbya*, *Wilmottia* and *Microcoleus* according to current taxonomic literature (Komárek and Anagnostidis 2005). The strains were able to grow at 25°C, furthering the evidence that many Antarctic cyanobacteria are not psychrophilic (Nadeau, Milbrandt and Castenholz 2001; Taton *et al.* 2006a).

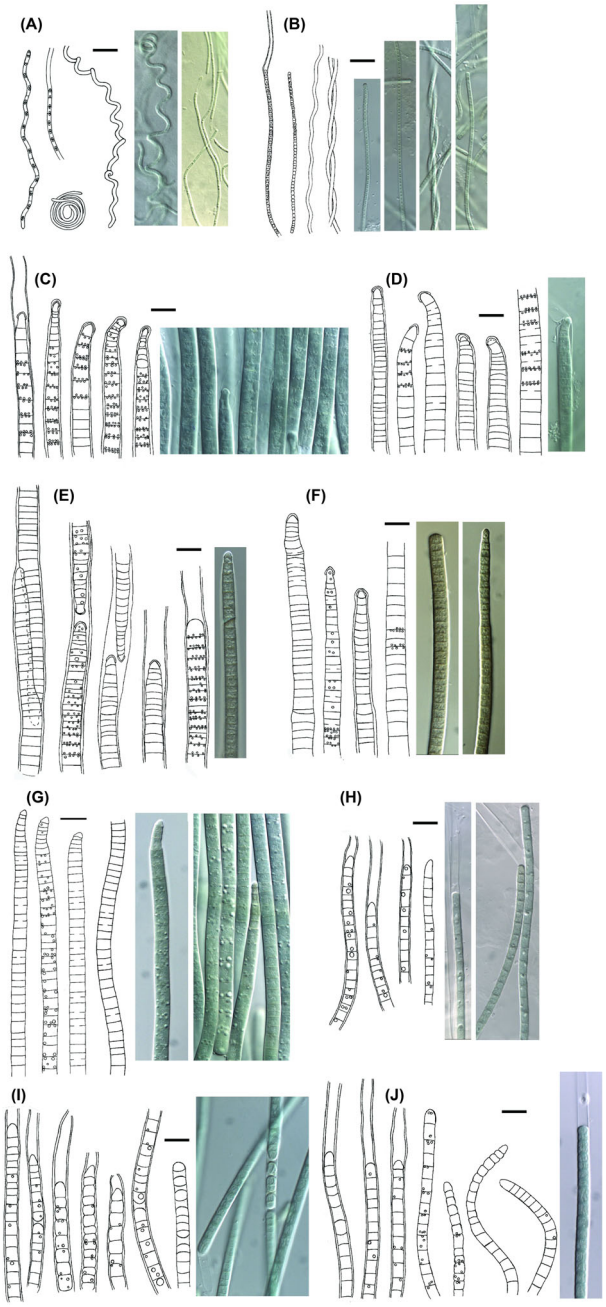

Figure 1. Iconographs and light microscopy photographs of 10 Antarctic strains. (**A**) Strain KOVACIK-ANT 1990/4 (*Leptolyngbya borchgrevinkii*). (**B**) Strain KOVACIK-ANT 2003/73 (*L. frigida*). (**C**) Strain KOVACIK-ANT 2003/11b (*Microcoleus attenuatus*). (**D**) Strain KOVACIK-ANT 2003/43 (*M. attenuatus*). (**E**) Strain JANCUSOVA-ANT 2004/3 (*Microcoleus* sp. 1). (**F**) Strain JANCUSOVA-ANT 2004/4 (*Microcoleus* sp. 2). (**G**) Strain KOVACIK-ANT 2004/50 (*M. favosus*). (**H**) Strain KOVACIK-ANT 2003/11a (*Wilmottia murrayi*). (**I**) Strain KOVACIK-ANT 2003/15 (*W. murrayi*). (**J**) Strain KOVACIK-ANT 2004/75 (*W. murrayi*). Scale bar represents 10 μm.

The genus *Leptolyngbya* is represented by two strains with morphotypes A and B corresponding to *L. borchgrevinkii* and *L. frigida*, respectively.

On the basis of the 16S rRNA and ITS sequences, the strain KOVACIK-ANT 1990/4, identified as *L. borchgrevinkii*, seems to be closely related to but distinct from (13% ITS dissimilarity) a cluster of strains identified as *L. antarctica* by Taton *et al.* (2006a) and collected in three lakes of the LH. However, this OTU does not appear to be endemic to Antarctica. Indeed, the BLAST analysis shows a 99.35% similarity to the unpublished clone C10-AlvEE (Swiss Alps biofilms), 99.27–98.96% similarity to biofilms in UK (9d7, 2c7 and 5g7) and 99.16% similarity to sequences of Annapurna glaciers (Nepal) (B10905G).

The morphotype of *L. borchgrevinkii* has been described as characteristic of seepages and streams in James Ross Island (Komárek, Elster and Komárek 2008) and seepages of KGI (Komárek and Komárek 2010) where it was first described. It is commonly observed in maritime Antarctica in wetlands and sites with a continuous influx of water during summer, but sometimes under a wrong identification (Komárek 2007). Our observation of this morphotype in cyanobacterial mats from a seepage coincides with the described habitats. Its relation with *L. antarctica* is still to be elucidated.

The 16S rRNA sequence of strain *L. frigida* KOVACIK-ANT 2003/73 is quasi identical to the sequences of two strains and one clone isolated from microbial mats of lakes in the LH (Eastern Antarctica) (Taton *et al.* 2006a,b). This is supported by a very high similarity of 98% at the level of ITS between the LH sequences and our strain. The geographic distance between the LH and KGI (ca. 6000 km) might be reflected by the presence of some differences (2.3%) in the ITS sequences.

Leptolyngbya frigida has been observed in freshwaters in Antarctica (Komárek and Anagnostidis 2005; Taton *et al.* 2006a), and documented in seepages and streams in KGI and JR (Komárek and Komárek 2010). However, this morphospecies was also recorded in the Arctic and Europe (Komárek and Anagnostidis 2005) and the OTU (threshold of 97.5% 16S rRNA sequence similarity) is also present on other continents. At the taxonomic level, Komárek and Anagnostidis (2005) noted that the classification of *L. frigida* was not clear, and they described it as *Pseudanabaena frigida* but without giving motivations for this change (Anagnostidis 2001). As the genus *Pseudanabaena* is already quite broadly defined, we would prefer a thorough taxonomic study before changes are made.

The 16S rRNA and ITS sequences of the five strains assigned to the *Microcoleus* genus show that they present a certain genetic diversity and are positioned in four branches of the trees (Figs 2 and 5). If a 98.9% similarity threshold was used to define bacterial species, as advocated by Stackebrandt and Ebers (2006), KOVACIK-ANT 2004/50 would be in a different species from the four others (unpublished data). Due to its genotypic and phenotypic similarity to strain JR2, KOVACIK-ANT 2004/50 is assigned to *Microcoleus favosus*, which was originally described (as *Phormidium favosum*) from mountain oligotrophic springs and aquatic habitats, forming benthic mats attached to submersed substrates (like rocks, sand and plants) and distributed worldwide (Komárek and Anagnostidis 2005). Therefore, the ecological range of this species seems wider than earlier assessed. Based on their ITS identity with KI19 and morphological similarity, KOVACIK-ANT 2003/11b and KOVACIK-ANT 2003/43 can be identified as *M. attenuatus*, which is considered to be typical for wet Antarctic ornithogenic soils and shallow pools in coastal Antarctica and high Canadian Arctic (Komárek and Anagnostidis 2005). This fits well with the origin of the two strains. The two last strains appear related to isolates placed in clade D by Strunecký *et al.* (2013), and therefore they are identified as *Microcoleus* sp. The genetic diversity of the five strains coincides with slight differences in colour and thalli formation at the level of the morphology. A distinct keritomization is observed in strains cultivated at 25°C and may be due to more intense photosynthetic activity of thylakoids in comparison with the strains cultivated

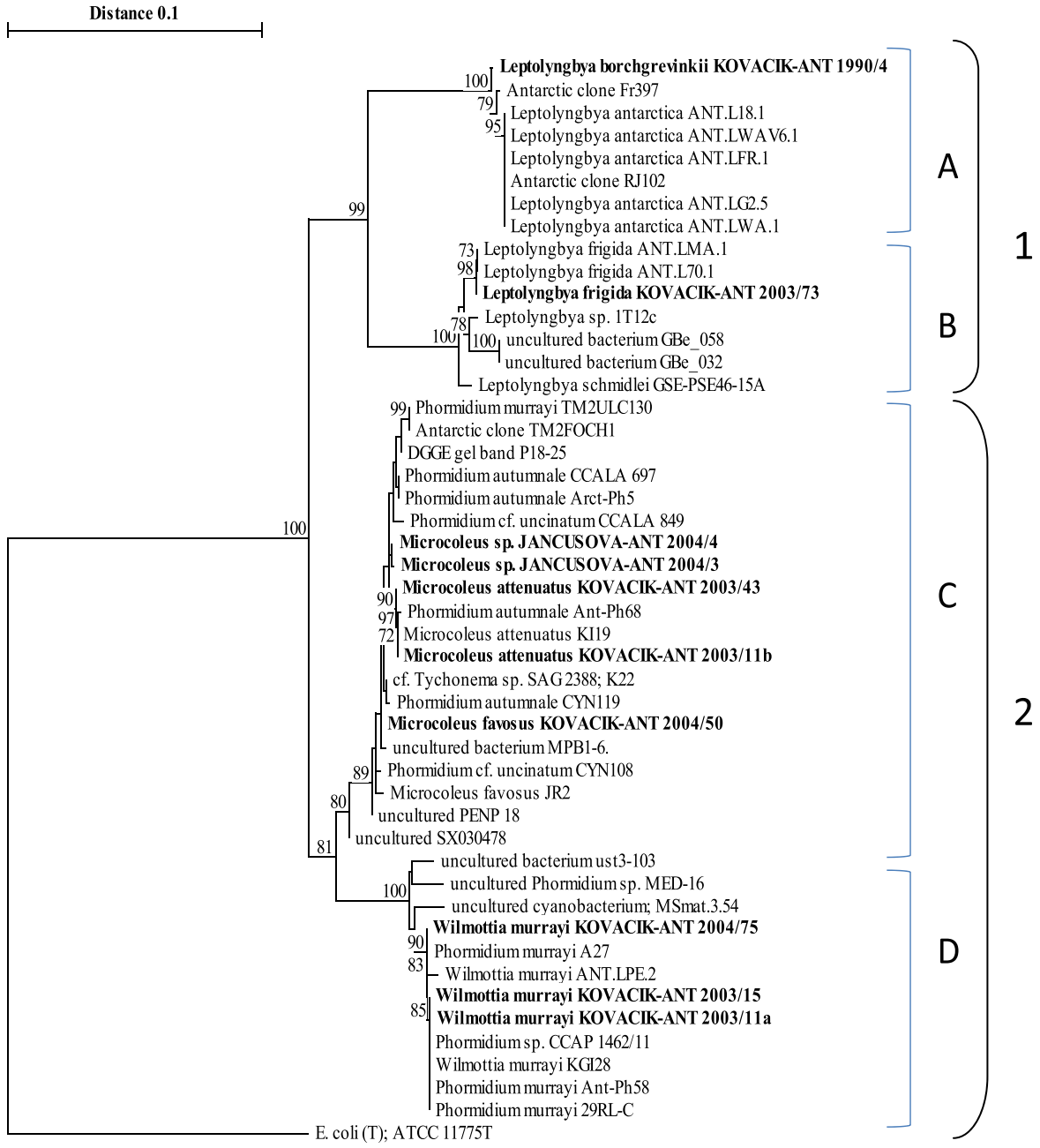

Figure 2. Distance tree inferred from 16S rRNA gene sequences (*Escherichia coli* positions 415–1464) by the neighbor-joining method with TREECON for Windows (Van De Peer and De Wachter 1997). The evolutionary distances were computed using the Kimura correction for multiple mutations. Indels were not taken into account. The bootstrap values inferred from 1000 replicates are shown next to the branches when they are higher than 70%. The letters indicate the location of the 4 morphotypes and the numbers indicate the two major clades.

at 10°C. The keritomization or widening of the intrathylakoidal space is very visible due to the radial arrangement of thylakoids and is common in this genus (Komárek and Anagnostidis 2005; Strunecký *et al.* 2013).

Before the taxonomic revision by Strunecký *et al.* (2013), these strains would have been identified as *Phormidium autumnale* and this species has frequently been reported in the past from a large diversity of habitats distributed worldwide (Komárek and Anagnostidis 2005; Palinska and Marquardt 2008; Strunecký, Elster and Komárek 2010). Accordingly, the number of sequences from this taxonomic group in databases is very large. The ITS tree shows a quite large variation in sequences, even between the Antarctic ones. This radiation might reflect multiple colonization events and possibly further diversification within Antarctica. However, there also exists a lineage of four Maritime Antarctic strains with identical ITS sequences, including the two *M. attenuatus* strains from this study, isolated from samples collected in 2003 from the Keller Peninsula (about 900 m distance) but in slightly different biotopes (black epilithic crusts or green growth on whale bone near a shore), and two strains previously isolated in 1996 from soils in nearby Killingbeck Island (KI19) and in 1978 from the slightly more distant South

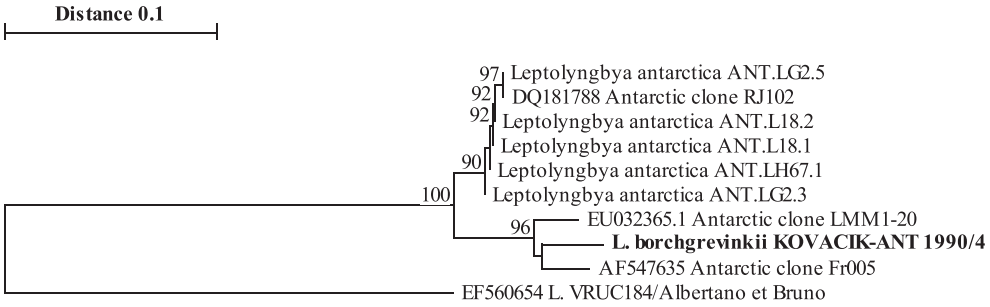

Figure 3. Distance tree inferred from the ITS sequences of *Leptolyngbya borchgrevinkii* and most related sequences (643 bp) by the neighbor-joining method with TREECON for Windows (Van De Peer and De Wachter 1997). The evolutionary distances were computed using the Jukes–Cantor correction for multiple mutations. Indels were not taken into account. The bootstrap values inferred from 1000 replicates are shown next to the branches when they are higher than 70%.

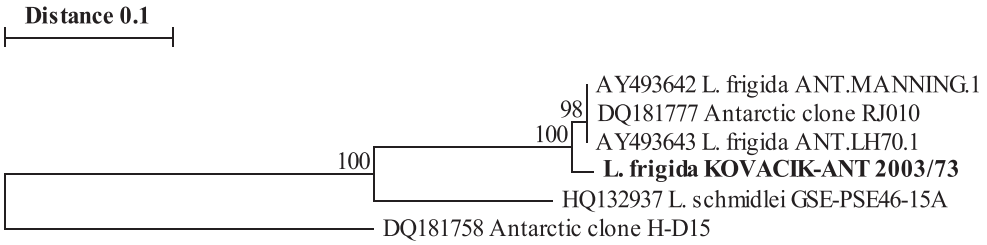

Figure 4. Distance tree inferred from ITS sequences of *Leptolyngbya frigida* and most related sequences (605 bp) by the neighbor-joining method with TREECON for Windows (Van De Peer and De Wachter 1997) as described in Fig. 3.

Orkney Islands (CCAP 1462/11). This could hint at the existence of an ITS genotype endemic for the Maritime Antarctic, but this would need to be confirmed when more ITS sequences are available in public databases.

For *W. murrayi*, at the morphological and molecular level, KOVACIK-ANT 2004/75 is slightly distinct from the two others, which are quasi identical and more closely related to the reference strain of *W. murrayi* (KGI28) and to strain CCAP 1462/11 isolated from the South Orkney Islands. As the five strains were collected in different islands and types of biotopes in Maritime Antarctica, the genetic difference might reflect a certain degree of speciation occurring between the populations of South Orkney Islands, KGI and Deception Island on slightly different substrates. Two strains from Pendant lake (LH) that were identified as *Phormidium murrayi* belonged to the same OTU but their ITS sequence is quite different. Thus, it would be interesting to investigate more molecular taxonomic markers for the *W. murrayi* group. Interestingly, Strunecký, Elster and Komárek (2011) had hypothesized that the new species could be endemic to Antarctica, as it included at least 13 Antarctic strains on the basis of 16S rRNA sequences. However, if an OTU threshold of 97.5% 16S rRNA sequence similarity is used to delimitate the species, strains from other continents are present and the species would appear cosmopolitan.

The morphotype of *W. murrayi* was observed as benthic mats in littoral of lakes, streams or in seepages, but also growing on wet soils in moss communities, in epilithic habitats or cryoconites (Komárek, Elster and Komárek 2008; Strunecký, Elster and Komárek 2011). Komarek and Anagnostidis (2005) noted that this species was originally described from Antarctic soils and stagnant, generally salty, waters, and that it was recently commonly isolated from wet soils in both polar regions. It was observed in the maritime and continental Antarctica (Broady and Kibblewhite 1991; Casamatta *et al.* 2005; Taton *et al.* 2006a; Comte *et al.* 2007).

The cyanobacterial taxonomy aims to define cyanobacterial species that are morphologically and genotypically similar but also ecologically delimited and present in specific habitats (Komárek 2015). For the 10 studied strains, the link could be made between morphological, genotypic and ecological characterizations. The molecular data also give a hint about the biogeographical distribution of the genotypes, though the sequence databases do not cover equally all parts of the world. The frequency of BLAST hits from Antarctic biotopes illustrates that the OTUs assigned to *L. borchgrevinkii*, *Microcoleus* and *Wilmottia* are probably abundant there and/or easily isolated in culture, though they are also found in other continents. However, Konstantinidis and Tiedje (2005) have shown that the standard bacterial genomospecies definition (70% DNA/DNA reassociation) defines very large taxonomic units, where members may share only 65–95% of their gene content. Therefore, they would favour a species definition encompassing more related strains and integrating the information about the ecological distinctiveness. If a new taxonomic standard was applied to our OTUs, smaller taxonomic units could probably be obtained and might show an endemic distribution.

Further research is still needed to elucidate the genetic outline of the cyanobacterial species, its relation with the morphological features, and the usefulness of ITS to complement the quite conserved 16S rRNA sequence. This use has already been advocated by Wilmotte (1994) and applied with success for many taxonomic studies (e.g. Taton *et al.* 2006a; Palinska and Marquardt 2008; Bohunická, Johansen and Fučíková 2011). In this study, the ITS was useful to distinguish the strains at a higher resolution level than enabled with the 16S rRNA. Interestingly, the ITS sequences were identical for two pairs of strains of *P. autumnale* and of *W. murrayi* isolated from samples collected the same year at a small geographic distance from each other (less than 1 km), even if their substrates varied from soil and rock to whale bone. One limitation of the ITS comparisons is the

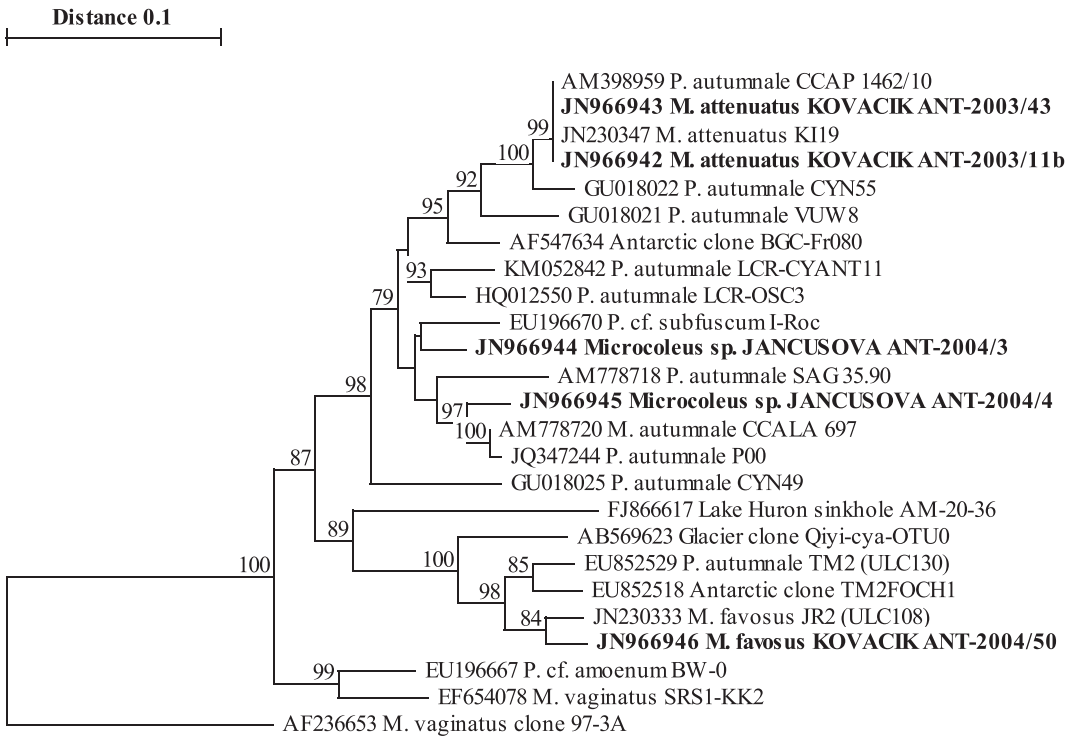

Figure 5. Distance tree inferred from the ITS sequences of *Microcoleus* sp. and most related sequences (602 bp) by the neighbor-joining method with TREECON for Windows (Van De Peer and De Wachter 1997) as described in Fig. 3.

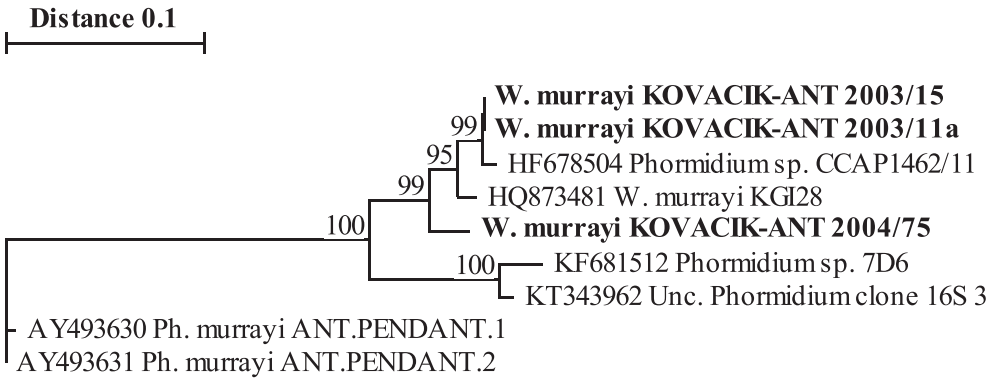

Figure 6. Distance tree inferred from the ITS sequences of *Wilmottia murrayi* and most related sequences (436 bp) by the neighbor-joining method with TREECON for Windows (Van De Peer and De Wachter 1997) as described in Fig. 3.

fact that there are fewer sequences available than for the 16S rRNA, and thus, not all the 16S rRNA lineages can presently be studied in more detail by ITS sequences. However, as the number of ITS sequences grows, this molecular marker becomes more and more useful. The presence of conserved domains (D1–D5), the tRNA genes and the antiterminator (box B and box A) helps to align the sequences (Iteman *et al.* 2000). However, the definition of a quantitative threshold to define genotypic units on the basis of the ITS, in a similar manner to OTUs based on the 16S RNA, has not been reached. Moreover, the large sequence variations of ITS primary structures between distant strains also mean that there is a similarity level where the alignment stops being meaningful. Thus, only rather related organisms can be compared. More data would be needed to determine if it is possible to set some quantitative threshold values.

The polyphasic approach used in this study aimed to maximize the possibility of identifying the strains by morphological and genotypic criteria in their natural environment and determine their ecological relevance. The two *Leptolyngbya* species appear to be characteristic of wetlands, seepages and streams in Antarctica. *Wilmottia murrayi* has a wider ecology as it also grows on wet soils, in epilithic habitats or on cryoconites in both polar regions, and a certain genetic diversity seems present in the Maritime Antarctic, as the strain collected on mud from Deception Island is distinct from the ones isolated from soil and moss crusts in King George Island. The *Microcoleus* strains belong to a very broadly distributed genus. *Microcoleus attenuatus* strains were described as typical for wet soils and pools in both polar regions. The description of *M. favosus* mentioned freshwater habitats, including an alpine distribution, but not the polar regions,

and therefore our identification increases the ecological range of this species.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared.

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