POLISH POLAR RESEARCH
vol. 33, no. 4, pp. 369–382, 2012 doi: 10.2478/v10183-012-0020-5

Biogeography of *Phormidium autumnale* (Oscillatoriales, Cyanobacteria) in western and central Spitsbergen

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Abstract: Filamentous types from the order Oscillatoriales, particularly the species *Phormidium autumnale*, have widely diverse morphotypes, which dominate in Arctic aquatic microbial mats and wet soils. We cultivated 25 strains of *Ph. autumnale* from Svalbard and compared them with available strains from surrounding regions. The comparison of strains, based on 16S rDNA and 16S-23S rDNA intergenic spacer sequences, revealed the similarity of strains from Ellesmere Island, the Canadian Arctic and Abisko, Sweden with strains from Svalbard. The rate of colonization of *Ph. atumnale* from aquatic habitats is relatively high and we suggest geese as a main transmission vector from surrounding lands. Strains of *Ph. autumnale* were positioned in the phylogenetic tree according to their occurrence in similar habitats. An apparent clustering factor is the duration of availability of water in lakes and long-lasting streams in contrast to rapid and repeated desiccation in soil and on wetted rock in the spray zone of waterfalls. Strains that grow in very cold waters just above the melting point of snow or ice form a distinct genetic group. The strains investigated in this study show morphological similarity in the shape of the trichomes of the studied specimens. Overall, the cell diameter, except for terminal cells, of our strains varied between 3 and 10 µm. Comparison of 16S rDNA sequences of the genus *Ph. autumnale* with the previously published definition of the species *Microcoleus vaginatus* revealed the identity of these two species.

Key words: Arctic, Svalbard, blue-green algae, ecology.

Introduction

Cyanobacteria play a key role in polar ecosystems as primary producers (Friedmann 1993; Elster 2002; Seckbach 2007). They are well-adapted for life in low temperatures. For instance, a *Phormidium* – dominated community was found growing within liquid water inclusions in lake ice (Priscu *et al.* 1998) of the McMurdo Dry Valleys, Antarctica. They are able to survive prolonged freezing

and desiccation (Šabacká and Elster 2006), and have the ability to re-establish metabolic activity quickly after rehydration (Seckbach 2007). Filamentous types from the order Oscillatoriales, especially populations from the genus *Phormidium* Kützing ex Gomont, 1892, have widely diverse morphotypes and often dominate in aquatic microbial mats and soils (Broady 1996; Elster *et al.* 1999; Elster 2002; Komárek *et al.* 2008). After the retreat of glaciers, cyanobacteria stabilize soil surfaces, raise nutrient status and form the dominant ground cover (up to 34%) (Hodkinson *et al.* 2003). Despite the evident importance of oscillatorialean cyanobacteria in polar areas, there are surprisingly few taxonomic studies focusing on genetic and phytogeographic comparisons of Arctic cyanobacteria belonging to this order (Casamatta *et al.* 2005; Comte *et al.* 2007; Strunecký *et al.* 2010).

Species of the *Ph. autumnale* Kützing ex Gomont, 1892 complex have a high morphological diversity. Freshwater types are generally characterized by simple, cylindrical, isopolar, unbranched filaments lacking heterocytes and akinetes, and forming irregular clusters or colonies with more or less parallel trichomes. Sheaths are thin, hyaline, with the mucous partly diffluent or completely dissolved causing the filaments to adhere in mat-like layers. *Ph. autumnale* can be found commonly in freshwater streams, spray zones of waterfalls, river and lake shores, springs, soils temporarily exposed to water, wet rocks, and other similar freshwater habitats.

There is now excellent molecular and phytogeographical evidence to support Hultén's original hypothesis that Beringia was a major northern refuge for Arctic plants throughout the Quaternary and the spread of vascular plants to the remainder of the Arctic including Svalbard occurred during the Holocene (Alsos *et al.* 2005; Alsos *et al.* 2007). However, recent studies on *Saxifraga oppositifolia* and *Dryas octopetala* (Tremblay and Schoen 1999; Abbott *et al.* 2000) did not exclude the possibility that the northern refuge extended eastward to unglaciated parts of the Canadian high Arctic and north to Greenland. Eidesen et al. (2007) compared the genetic structure of *Cassiope tetragona* in the Arctic and found a strong east-west trend with a Beringian population in the intermediate position, while the strongest differentiation was found between the populations from the Siberian Arctic, west of Beringia, and the remainder. Alsos et al. (2007) stated that plant propagules were likely carried repeatedly from several source regions to Svalbard by wind and drifting sea ice, with the predominant source being the most distant region, northwestern Russia. Colonization from Scandinavia was rare; only *Salix herbacea* appears to have derived mainly from this region (Alsos *et al.* 2007).

The purpose of the present research is to enhance knowledge about the diversity of cyanobacteria on Svalbard and generally on remote island locations in comparison with adjacent regions. Little is known about the biogeography of genetically-defined single species of cyanobacteria. Our results assist in understanding the distribution pathways and survival of cyanobacteria in the Arctic. There is now a need to merge new information about the ecology and evolution of *Ph. autumnale* lineages and to apply a similar approach to other Oscillatoriales in the Arctic.

Material and methods

Phormidium specimens were collected from various localities in Svalbard, Europe and on Ellesmere Island, the Canadian Arctic. 25 strains of *Ph. autumnale* were isolated from Svalbard. The strains were mostly placed onto microtitration plates containing solidified BG11 medium within a few hours of sampling and then transported to the laboratory. Trichomes of *Ph. autumnale* were repeatedly subsampled and transferred to clean microtitration plates to obtain unialgal strains. Strains were cultured on solidified BG11(Rippka *et al.* 1979) at 12°C and under continuous illumination of 24 $\mu mol.m^{-2}.s^{-1}$.

Cultivated strains were identified according to Anagnostidis and Komárek (1990) and Komárek and Anagnostidis (2005). Strain morphologies were analyzed using an Olympus BX51 light microscope; microphotographs were taken with an Olympus DP61 camera. The width and length of at least 50 cells, excluding the end cells, were measured for each *Phormidium* strain under 1000x magnification. Statistical analysis of the measured parameters was conducted using Statistica 9.

DNA from the unialgal strains was extracted using the modified method of Yilmaz et al. (2009). The available amount of cultivated cells was suspended in 750 µl of XS buffer (1% potassium ethyl xanthogenate; 100 mM Tris-HCl, pH 7.4; 20 mM EDTA, pH 8; 800 mM amonium acetate and 1% SDS) in an Eppendorf tube with a mixture of glass beads of 0.1 and 1 mm diameter at a ratio of 1:5. Tubes were shaken for 2 h at 70°C at 1400 rpm in a Thermomixer (Eppendorf). After incubation, the tubes were frozen at -70°C for 30 min. Each sample was thawed and shaken for 10 min in the Thermomixer, centrifuged for 30 min at 15,000g and the supernatant was transferred to a clean microcentrifuge tube. The DNA was precipitated overnight in a 1:3 volume of 100% ethanol with the addition of a 1:20 volume of sodium acetate (3 M, pH 5.2) followed by centrifugation for 60 min at 15,000g. The supernatant was discarded and the pellet was washed with 100 µl of 70% ethanol followed by centrifugation for 15 min. After discarding the supernatant, the pellet was dried and dissolved in 100 µl of miliQ water.

The 16S rRNA gene with the 16S-23S intergenic segment was amplified using the primers 359F (GGGGAATYTTCCGCAATGGG; Nübel *et al.* 1997), and 23S30R (Wilmotte *et al.* 1992) with the following settings: a starting denaturalization step (94°C, 5 min); 40 cycles of 30 s at 94°C, 30 s at 53°C, and 3 minutes at 72°C; final extension for 7 minutes at 72°C and cooling to 4°C. A successful PCR was confirmed by running a sub-sample on a 1% agarose gel stained with ethidium bromide. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen). Sequencing of the 16S rRNA gene fragment was performed on an ABI 3130XL sequencer, using BD3.1 chemistry (Applied Biosystems), with six primers (359F, 23S30R, CYA_1064R (Strunecký *et al.* 2010), S17*-GGCTACCTTGTTACGAC and ILE23F-ATTAGCTCAGGTGGTTAG (Wilmotte and Herdman 2001) to obtain complementary sequences. For constructing the molecular

phylogeny, sequenced strains were grouped together with the sequences of strains studied by previous authors or collected in the frame of our different projects on the basis of geographic proximity, as for example *Ph. autumnale* A23 from Abisko, Sweden or strains E17 and E18_1 from southern Sweden (Table 1).

Sequences were aligned in MAFFT (mafft.cbrc.jp) (Katoh and Toh 2010) considering the secondary structure. Minor changes were made manually with BioEdit 7.0.1 (Hall 1999). A fragment of 1097 nt was used (corresponding to *E. coli* ATCC 11775 16S rRNA residues 302–1,412) for the phylogenetic analysis of 16S rDNA; a

Table 1

Taxonomic assignment and origin of the cyanobacterial strains examined in this study

Name of strain	GPS position	Locality	Habitat	Year	Isolator
CCALA845	77°00'N 15°20'E	Arctic, Svalbard, Hornsund	stream	2008	Šnokhousová and Elster
CCALA846	77°00'N 15°20'E	Arctic, Svalbard, Hornsund	stream	2008	Šnokhousová and Elster
S33	77°00'N 15°20'E	Svalbard, Hornsund	moss stream	2008	Šnokhousová and Elster
CCALA848	77°00'N 15°20'E	Svalbard, Hornsund	shallow lake on sea terrace	2008	Šnokhousová and Elster
sv28	78° 41'N 16° 25'E	Svalbard, Petuniabukta, West coast	drying crust in stream	2009	Strunecký
A28	79°08' N, 80°30' E	North America, Canada, Ellesmere Island	soil	1994	Lukešová
S36	77°00'N 15°20'E	Svalbard, Hornsund	littoral of lake Pikedamen	2008	Šnokhousová and Elster
CCALA697	79°08' N, 80°30' E	North America, Canada, Ellesmere Island	glacial stream	2008	Šnokhousová and Elster
CCALA726	77°00'N 15°20'E	Svalbard, Hornsund, Rabben	wetland vegetation	2002	Kaštovská
CCALA847	77°00'N 15°20'E	Svalbard, Hornsund	little lake on sea terrace	2004	Šnokhousová and Elster
sv31	78° 41'N 16° 36'E	Svalbard, Petuniabukta, West coast	stream	2009	Strunecký
svv10	78° 50'N 16° 5'E	Svalbard, Alanddalen	stream	2009	Strunecký
svs1	78° 35'N 16° 19'E	Svalbard, Adolfbukta	cliff	2009	Strunecký
svv11	78° 51'N 16° 2'E	Svalbard, Raudvatnet	stream	2009	Strunecký
sv30	78° 41'N 16° 36'E	Svalbard, Petuniabukta, West coast	spray zone of waterfall	2009	Strunecký
sv25	78° 40'N 16° 24'E	Svalbard, Petuniabukta, West coast	periphyton in stream	2009	Strunecký
sv11	78° 40'N 16° 37'E	Svalbard, Petuniabukta, West coast	mat in moss	2009	Strunecký
sv26	78° 41'N 16° 24'E	Svalbard, Petuniabukta, East coast	mud in small stream under snow field	2009	Strunecký
sv22	78° 42'N 16° 17'E	Svalbard, Petuniabukta, Herbybreen	mud in small stream under the glacier	2009	Strunecký

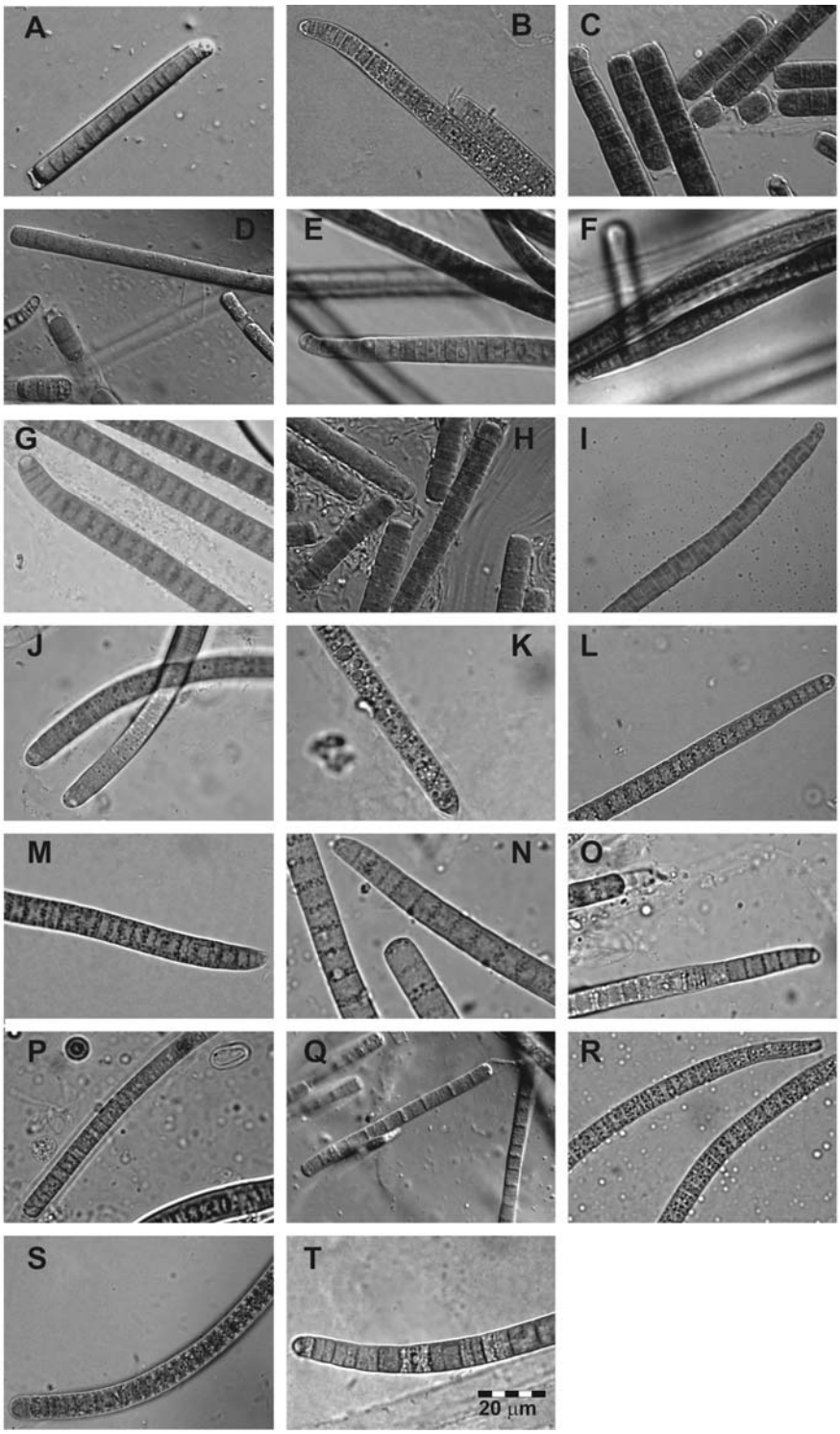
Table 1 – *continued*.

Name of strain	GPS position	Locality	Habitat	Year	Isolator
sv09	78° 40'N 16° 36'E	Svalbard, Petuniabukta, East coast	cliff under snow field	2009	Strunecký
svv4	78° 54'N 17° 33'E	Svalbard, Austenfjorden	small stream in snow field	2009	Strunecký
sv17	78° 40'N 16° 38'E	Svalbard, Petuniabukta, East coast	soil under snow field	2009	Strunecký
sv12	78° 40'N 16° 37'E	Svalbard, Petuniabukta, East coast	small stream under snow field	2009	Strunecký
sv27	78° 41'N 16° 25'E	Svalbard, Petuniabukta, West coast	mud in dry stream	2009	Strunecký
A6	49° 11'N 13° 30'E	Europe, Czech Republic, South Bohemia	soil	1996	Lukešová
E17	56° 30'N 13° 0'E	Europe, Sweden, Laholm	periphyton on mud at the dam shoreline	2010	Strunecký
E18_1	58° 23' N 12° 18' E	Europe, Sweden, Vanern	periphyton on rock at lake bottom	2010	Strunecký
A10	49° 11'N 13° 30'E	Europe, Czech Republic, South Bohemia	soil	1986	Lukešová
A25	NA	North America, USA, Tenessee	soil	2008	Lukešová

fragment of 1455 nt was used for the 16S rRNA gene with the 16S–23S intergenetic segment. Phylogenetic analyses were conducted in Mega 4 (Tamura *et al.* 2007) using the Maximum Likelihood method based on the Tamura-Nei model. The tree topology was validated by MrBayes 3.1 at <www.metacentrum.cz>. For the Bayesian analysis, two runs of four Markov chains over 8 000 000 generations, sampling every 1000 generations with default settings, were employed. The initial 2 500 generations were discarded as burn-in. Phylogenetic classification of the obtained sequences to the species *Ph. autumnale* was cross-checked in a separate phylogenetic validation with a set of sequences previously used in Strunecky *et al*. (2010, 2011).

Results

We isolated and sequenced 25 strains from Hornsund Bay, an area of Petuniabukta, and from a transect running from Petuniabukta to Austenfjorden, all in Svalbard: (Table 1). The morphology of these strains (Fig. 1) confirmed their classification in *Ph. autumnale* (Oscillatoriales, Cyanobacteria). Nucleotide sequences have been deposited in the Genbank database, under the accession numbers JQ769113-JQ769139. Trichome width measurements were made on 13 strains . ANOVA analysis of cell width among the strains showed no relationship with the position of these strains in the phylogenetic tree (Fig. 2) (results not shown). Generally, trichome width was 3–10 µm.



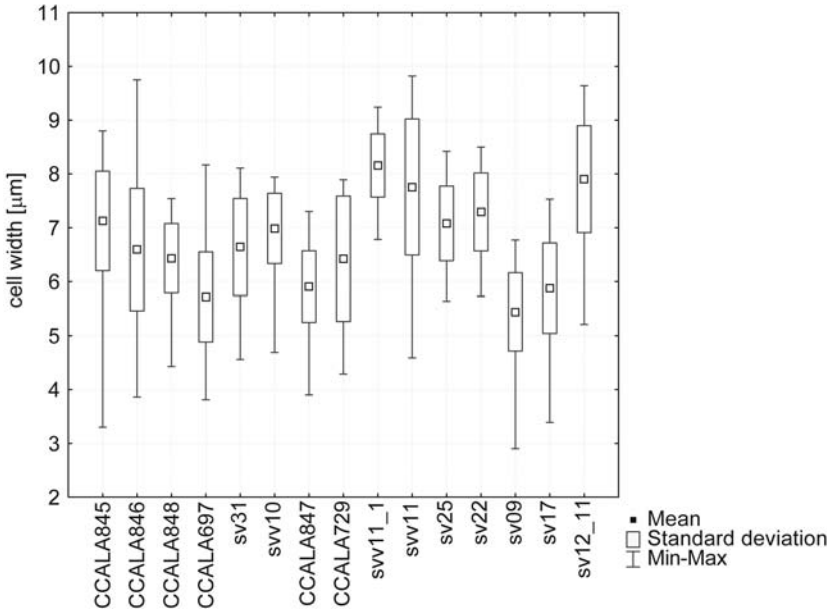


Fig. 2. Trichome widths of cyanobacterial strains from Svalbard examined in this study.

We analyzed two phylogenetic trees containing 16S rDNA (Fig. 3) and 16S rDNA with the 16S–23S rDNA internal transcribed spacer (ITS) (Fig. 4). Phylogenetic analysis of the 16S rRNA gene (Fig. 3) revealed the existence of two more closely related clusters, I and II. The sequences of strains in cluster III contain an 11 bp insert at loop 6 of 16S rRNA that has previously been defined as a characteristic marker of *Microcoleus vaginatus* morphospecies, i.e. these strains should probably be combined into a single species. Cluster I contains the Svalbard strains from Hornsund, Petuniabukta and Alansdalen . This cluster also includes two different strains from Ellesmere Island, northern Canada, in which the 16S rDNA composition is 100% identical to other strains from Svalbard. Based on the 16S rDNA composition, cluster II contains two identical strains, A23, from Abisko, northern Sweden, and sv26, from Petuniabukta, Svalbard . Cluster III includes the type strain (SAG 2211) of *Microcoleus vaginatus*.

The phylogenetic tree of the 16S rDNA gene with ITS fragment (Fig 4) confirmed the similarity of the Svalbard strains with those from Ellesmere Island in

← Fig. 1. Diversity of cyanobacterial morphospecies of the studied strains. All strains belong to *Phormidium autumnale*. Trichome morphology is generally comparable, however differences in cell shape and structure between the presented strains are shown. Strain a28 (A), strain sv28 (B), strain CCALA848 (C), strain CCALA845 (D), strain CCALA846 (E), strain s33 (F), strain s36 (G), strain CCALA697 (H), strain 726 (I), strain sv31 (J), strain svv10 (K), strain svS1 (L), strain sv11 (M), strain sv25 (N), strain sv22 (O), strain sv09 (P), strain svv4 (Q), strain sv 17 (R), strain sv27 (S), strain sv 12_11 (T). Scale 20 mm for all strains.

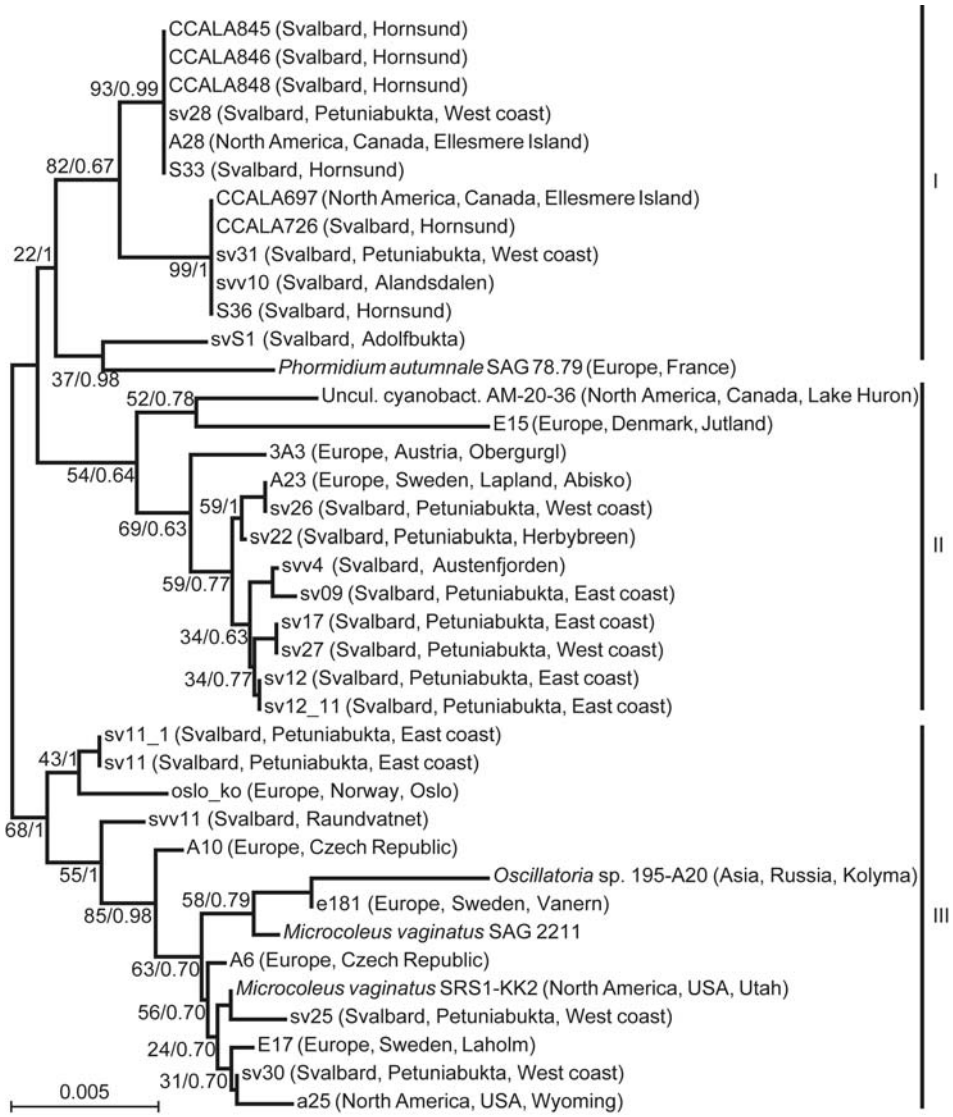


Fig. 3. Phylogenetic relationships of *Phormidium autumnale* of the 16S rRNA gene (a fragment of 1097 nt was used corresponding to *E. coli* ATCC 11775 16S rRNA residues 302–1,412). Origins of the strains are indicated in parentheses. The evolutionary distances were computed using the maximum likelihood method, values at nodes indicate bootstrap values for the ML/ posterior probabilities from Bayesian analyses.

cluster I. However, greater variability in ITS in two sub-clusters of cluster I indicates long-term evolutionary diversification of strains in the Arctic. Cluster II includes strains from both Svalbard and the Alps that were either found directly under snowfields or in the uppermost parts of streams fed by melt from snow or glaciers. The only one exception was of the uncultured cyanobacterium strain AM-20-36 found at

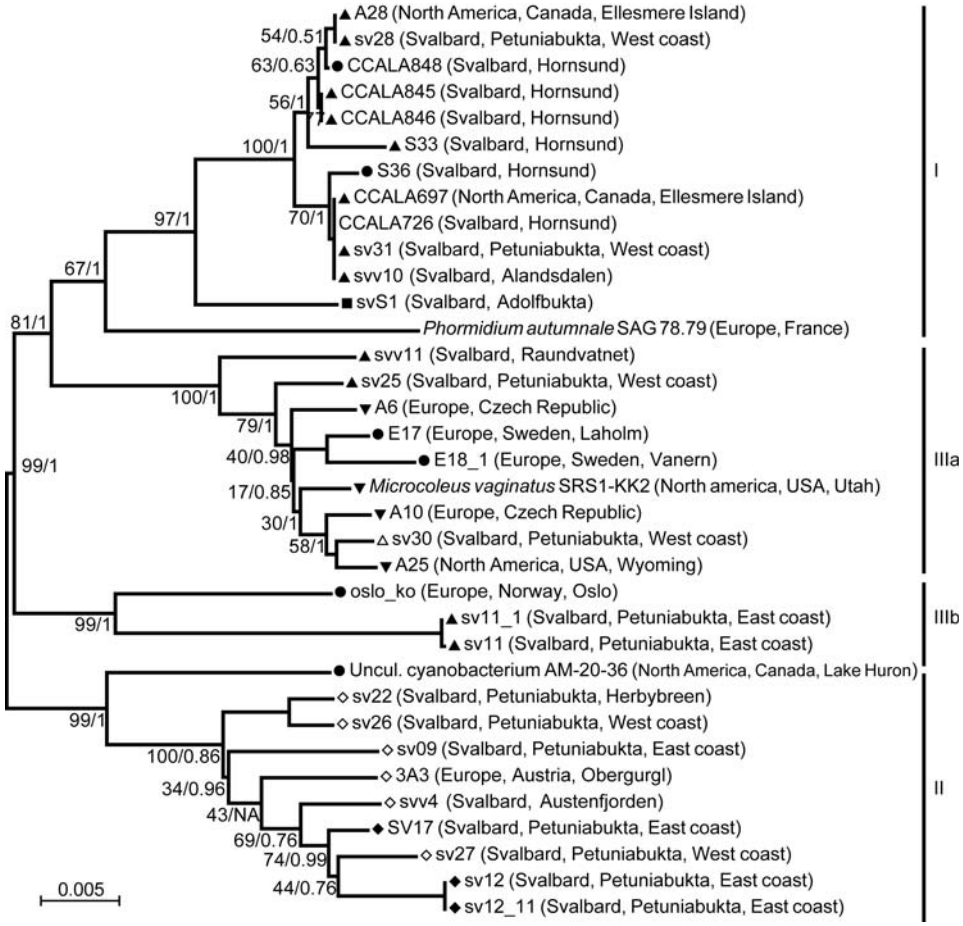


Fig. 4. Phylogenetic relationships of *Phormidium autumnale* estimated by the maximum likelihood method of the 16S rRNA gene with 16S–23S rDNA ITS (a fragment of 1455 nt was used starting at *E. coli* ATCC 11775 16S rRNA position 302). Values at nodes indicate bootstrap values for ML/ posterior probabilities from Bayesian analyses. Symbols denote the following: *circle* lake, *diamond* snowfield, *empty diamond* under snowfield or glacier, *filled triangle* stream, *turned triangle* soil, *square* rock and *empty triangle* spray zone. The origin of species without a symbol is unknown.

Huron Lake. The highest ITS variability was found in cluster III. A subcluster IIIa contains strains of wide geographical origin from the temperate zones of Europe and North America. The Norwegian strain oslo_ko was most related to the Svalbard strain from Petuniabukta in cluster IIIb.

In all groups, strains tend to be clustered according to the habitats in which they were found. Availability of water appears to be an important factor with strains from lakes and streams clustering separately from those from soils and the spray zone of waterfalls where there is a frequent desiccation followed by rehydration. However, the physical proximity of strains did not guarantee their genetic similarity. Strain

sv27 from cluster II (Fig. 4) was collected in the upper part of a stream fed by the permanent snowfield of Mount Pyramiden whereas strain sv28 from cluster I was located close to the sea in the lower part of the same stream. Analogously, strain sv30 from cluster IIIa was found in the spray zone of a waterfall whereas strain sv31 from cluster I was collected in the same stream, but only about thirty meters downstream.

Discussion

Specimens belonging to the *Ph. autumnale* complex are important components of algal communities in periphyton and soil. The majority of morphotypes belonging to Oscillatoriales are supposed to be restricted both ecologically and morphologically (Komárek and Anagnostidis 2005). The variability of *Ph. autumnale* under unique ecological situations has been previously studied (Bosli-Pavoni 1970; Komárek 1972). However, the majority of cyanobacterial genotypes are restricted both ecologically and morphologically (Nübel *et al.* 2000; Rejmánková *et al.* 2004; Richert *et al.* 2006; Taton *et al.* 2006; Flechtner *et al.* 2008). The genotypic diversity of the cosmopolitan and widely distributed *Phormidium* morphotype was expected to be observed in molecular studies. However, such studies (Nübel *et al.* 2000; Nadeau *et al.* 2001; Taton *et al.* 2006) have shown that variation exists in 16S rDNA throughout the complex, but this cannot yet be correlated with the phenotypes described from field-collected specimens.

The study of genetic and phenotypic microbial diversity is also relevant to biogeography (Miller *et al.* 2006; Miller *et al.* 2007). The original idea that the occurrence of microorganisms is driven by environmental factors only is rather old (Baas-Becking 1934). Desiccated propagules of *Ph. autumnale* are able to be dispersed widely. They are also able to survive long term freezing and desiccation and, after rehydration, rapidly become metabolically active (Harel *et al.* 2004; Seckbach 2007). In our previous study (Strunecký *et al.* 2010), we found two closely related genotypes of *Ph. autumnale* on Svalbard. However, sampling was limited to aquatic habitats. Comparison of this wider set of strains from a broader range of habitats has revealed a new view on the diversity of closely related cyanobacteria belonging to one broadly defined ecospecies. The strains of *Ph. autumnale* form clusters in the phylogenetic tree on the basis of growing in similar habitats. The importance of physical factors, such as water availability and temperature or possibly nutrient availability or other chemical properties of the environment, is substantial. An apparent group factor is the long term availability of water in lakes and permanent streams contrasted with ephemeral water availability on soil and wetted rocks as was already suggested for other groups of cyanobacteria in Antarctica (Novis and Smissen 2006). The phylogenetic properties of *Ph. autumnale* strains derived from microscopically consistent mats may significantly differ within streams in the range of tens or a hundred meters as for the two pairs of

strains collected on the west coast of Petuniabukta and which belong to different clusters.

Our results of 16S rDNA and 16S–23S ITS correspond with the classical theory of colonization of Svalbard from different distant locations. The similarity of 16S rDNA and 16S–23S ITS in two sets of strains from Ellesmere Island, the Canadian Arctic and various locations from Svalbard within two subclusters of cluster I (Fig. 4) reflects a relatively present and frequent transfer of *Ph. autumnale* strains within the Arctic. However, the variability of ITS in both subclusters suggets also a further diversification of strains directly in the Svalbard habitats. The subclusters (Fig. 4) are more or less delimited in distinct habitats. For example, we have found also similarity in the 16S rDNA at cluster II of strains A23 from Abisko, Sweden and sv26 from Petuniabukta (Fig. 3).

We obtained provisional estimates of the divergence times of *Ph. autumnale* lineages by assuming a 16S rDNA divergence rate of 1 to 2% per 50 million years (Ma) inferred for both the aphid symbiont *Buchnera* (Moran *et al.* 1993) and the domain *Bacteria* in general (Ochman *et al.* 1999). A lower boundary on the age of the most recent common ancestor of *Ph. autumnale* from the two subclusters of cluster I is estimated to be approximately 13 Ma ago. The speciation events within the subclusters of cluster I, considering two groups of strains with absolutely identical sequences in 16S rDNA, should have occurred in the last 2.5 Ma. The divergence times in cluster II reflected the evolution of Svalbard's strains of *Ph. autumnale* between 7.5 Ma years for the most different strains and 2.5 Ma for the majority of closely related strains. The Svalbard strains of cluster III diverged between 7.5 and 5 Ma from the European and North American strains.

Available evidence largely extracted from marine sediment cores in the Greenland–Iceland–Norwegian Seas suggests that Northern Hemisphere glaciations began between 10 and 5 Ma, with the onset of large-scale glaciations between 3.6–2.4 Ma (Shackleton 1997; Thiede *et al.* 1998; Knies *et al.* 2009; Matthiessen *et al.* 2009; Sejrup *et al.* 2009). The majority of diversifications between Arctic strains of *Ph. autumnale* were estimated to have occurred in the glaciations in the lower Pleistocene and upper Neogene. Provisional divergence times suggested the natural selection of *Ph. autumnale* strains together with their acclimatization to the cold environment. The relatively small diversity found within 16S rDNA in comparison with the high diversity of non-Arctic strains suggests diversification in the available refuge, or a very high rate of transport from nearby non-glaciated parts of the Arctic. We suggest that a combination of both possibilities is probable. Some lineages should have survived the last glaciations directly on Svalbard while some lineages were more likely to be transported to Svalbard after deglaciation by means of natural transport like pack-ice, driftwood, birds such as geese or insects driven by wind. However, further explanations of the introduction of *Ph. autumnale* strains to Svalbard requires more information about the diversity of *Ph. autumnale* strains in other parts of the Arctic, such as Beringia and north Siberia.

Comparison of 16S rDNA and ITS sequences of *Ph. autumnale* with the previously published molecular genetic definition of the species *Microcoleus vaginatus* (Boyer *et al.* 2002) revealed the identity of these two species. A taxonomic revision of this issue should emerge from further studies.

Acknowledgment. — This study was supported by grants INGO LA 341, LM2010009 CzechPolar, KONTAKT ME 934, AV0Z60050516 and the long-term research development project no. RVO 67985939. The authors thank Jana Šnokhousová for cultivation of strains, Dana Švehlová for technical help, Alena Lukešová for Arctic *Ph. autumnale* strains and MetaCentrum v.o. for provision of the supercomputing facilities under the research intent MSM6383917201.

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Received 30 June 2011
Accepted 9 May 2012