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POLYPHASIC CHARACTERIZATION OF *DOLICHOSPERMUM* SPP. AND *SPHAEROSPERMOPSIS* SPP. (NOSTOCALES, CYANOBACTERIA): MORPHOLOGY, 16S rRNA GENE SEQUENCES AND FATTY ACID AND SECONDARY METABOLITE PROFILES[1]

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The genera *Dolichospermum* (Ralfs ex Bornet et Flahault) Wacklin, L. Hoffm. et Komárek and *Sphaerospermopsis* Zapomělová, Jezberová, Hrouzek, Hisem, K. Řeháková et Komárk.-Legn. represent a highly diversified group of planktonic cyanobacteria that have been recently separated from the traditional genus *Anabaena* Bory ex Bornet et Flahault. In this study, morphological diversity, phylogeny of the 16S rRNA gene, production of fatty acids, and secondary metabolite profiles were evaluated in 33 strains of 14 morphospecies isolated from the Czech Republic. Clustering of the strains based on 16S rRNA gene sequences corresponded to wider groups of species in terms of morphology. The overall secondary metabolite and fatty acid profiles, however, were not correlated to each other and neither were they correlated to the 16S rRNA phylogeny nor the morphology of the strains. Nevertheless, a minor part of the detected secondary metabolites (19% of all compounds) was present only in close relatives and can be thus considered as autapomorphic features.

Key index words: **16S rRNA gene; *Anabaena*; cyanobacteria; *Dolichospermum*; fatty acids; morphology; phytoplankton; secondary metabolites; *Sphaerospermopsis*; taxonomy**

Abbreviations: **GC, gas chromatography; HPLC–MS, high-performance liquid chromatography–mass spectrometry; *m/z*, mass divided by charge; MS, mass spectrometry; MSD, mass selective detector; NJ, neighbor joining; RT, retention time**

Traditional taxonomy has, in general, been based on morphology; however, the routine use of molecular and biochemical characters has provided new insights into the taxonomic classification, with a combination of all character types often being used to describe and delimit new taxa. These data are

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especially useful in groups of organisms where morphological characters are insufficient, for example, in the case of cryptic species and organisms with a simple morphology.

In prokaryotes, their extremely simple morphology requires the use of chemotaxonomic traits, such as biochemical markers (fatty acids, secondary metabolites, or enzymes), to characterize taxonomic units, especially at the generic level (Skerratt et al. 1991, Romano et al. 2000, Gugger et al. 2002b). Inclusion of this chemotaxonomic approach is suggested by some authors to be obligatory for characterization of new bacterial taxa (Kämpfer et al. 2003).

However, the potential for lateral gene transfer among cyanobacteria and prokaryotes in general (Schouls et al. 2003, Green 2005, Nesbø et al. 2006, Bolhuis et al. 2010) complicates the application and interpretation of such data. Genes for metabolic pathways (including ''housekeeping genes'') display a higher probability of being transferred than informational genes like the 16S rDNA (Jain et al. 1999).

The genera *Dolichospermum* and *Sphaerospermopsis* are groups of planktonic cyanobacteria that have been recently separated from the traditional nostocacean genus *Anabaena* (Wacklin et al. 2009, Zapomělová et al. 2009, 2010a).

Traditional taxonomy within the genus *Anabaena* sensu lato was based on morphometric characters (Komárek and Zapomělová 2007, 2008). Many previous studies, however, noted the morphological, ecological, and genetic heterogeneity within this genus, especially the pronounced differences between planktonic and benthic representatives (Rajaniemi et al. 2005a,b, Halinen et al. 2008). The main molecular cluster of planktonic *Anabaena* morphospecies (Rajaniemi et al. 2005a,b) was therefore reclassified into the new genus *Dolichospermum* (Wacklin et al. 2009), with the generic name *Anabaena* being retained for benthic, periphytic, and soil morphospecies. Moreover, some other morphospecies traditionally classified as *Anabaena*, form separate genetic clusters, for example, *Sphaerospermopsis* (Zapomělová et al. 2009, 2010a).

It seems clear that the traditional genus *Anabaena* is complex and requires a polyphasic approach (Castenholz 1992) to produce a robust taxonomic system. This cyanobacterial complex was evaluated many times using molecular methods (Li and Watanabe 2001, 2004, Gugger et al. 2002a, Rajaniemi et al. 2005a, Halinen et al. 2008, Stüken et al. 2009), as well as fatty acids (Li and Watanabe 2001, 2004, Gugger et al. 2002b) or toxin analyses (Gugger et al. 2002b, Halinen et al. 2008, Stüken et al. 2009). Nevertheless, the interconnection and discussion of all these approaches has been still missing.

In this study, we have evaluated the morphology, 16S rRNA gene phylogeny, fatty acid production, and secondary metabolite profiles of 33 selected strains of *Dolichospermum* and *Sphaerospermopsis* from the Czech Republic, representing a wide spectrum of various morphospecies. We compared the diversity revealed using these different methods and discuss implications for the delimitation of taxonomic units at the subgeneric level.

MATERIALS AND METHODS

Sampling, strain isolation, and cultivation. Samples of water blooms were collected between 2000 and 2008 from various fishponds and reservoirs in the Czech Republic using a 20 μm mesh plankton net. Morphology of fresh material was evaluated immediately. Single trichomes were isolated from the phytoplankton samples as described by Zapomělová et al. (2007). Clonal cultures were grown in WC medium (Guillard and Lorenzen 1972) at 21°C and an incident irradiance of 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (16:8 light:dark [L:D] cycle). Thirty-three strains representing 14 different morphospecies of the genera *Dolichospermum* and *Sphaerospermopsis* were studied (Table 1). The strains are deposited in the cyanobacterial culture collection of Biology Centre of AS CR, Institute of Hydrobiology, Czech Republic. The biovolume of bacterial contaminants was evaluated prior to the analyses of metabolites using the fluorescent microscopy. It was <0.5% in all cases.

Morphometry. Photomicrographs of at least 30 fresh trichomes were obtained for each natural population (Olympus DP 70, magnification × 400; Olympus America Inc., Melville, NY, USA). Population in this context is taken to mean a set of trichomes of the same morphospecies observed in a natural sample collected from a single location at the same time. Dimensions of all cell types were measured (five vegetative cells per trichome measured in 30 trichomes and as many heterocytes and akinetes as was possible to find in each sample). Length:width ratios of vegetative cells, heterocytes, and akinetes were computed to roughly characterize the cell shapes. All size measurements were performed using the Olympus DP Soft image analysis system.

Statistical evaluation of morphological data. Basic statistical characteristics such as mean values, 25% and 75% percentiles, and extreme values were computed for the morphological features of the field populations. To describe the variability within all morphological data, principal component analysis (PCA) was carried out in the program CANOCO (Ter Braak and Šmilauer 1998), where the mean values, 25% and 75% percentiles, and extreme values of all morphological features were the input data. The data were centered and standardized. CanoDraw software (Šmilauer 1992) was used for construction of the PCA plot.

16S rDNA sequencing. Cultures were harvested in the exponential phase of growth by repeated centrifugation, during which the trichomes were washed several times in NaCl ($1 g \cdot L^{-1}$) to remove or reduce mucilaginous substances. The harvested biomass was stored at −20°C prior to DNA extraction. The DNA was extracted using UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA). The 16S rRNA gene and the rDNA internal transcribed spacer (ITS) region were amplified with primers 16S27F and 23S30R (Taton et al. 2003) 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 72°C; 25 cycles of 45 s at 94°C, 45 s at 54°C, and 2 min at 72°C; and a final elongation step of 7 min at 72°C. PCR products were cleaned using NucleoSpin ExtractII kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). PCR products were then sequenced commercially (Laboratory of Genomics, Biology Centre of AS CR, České Budějovice, Czech Republic) with primers 16S27F, 23S30R (Taton et al. 2003), primer cAlaR (Wilmotte et al. 1994), primer WAW1486R and primer 14 (Wilmotte et al. 1993), and primer CYA781F(a) (Nübel et al. 1997).

TABLE 1. Cyanobacterial strains used in this study and the summary of the analyses performed (*D., Dolichospermum*; *S., Sphaerospermopsis*; FA, fatty acid composition; SM, secondary metabolite content; 16S rRNA, sequencing of 16S rRNA gene; morph., analysis of morphological characteristics). First two numbers of the strain code indicate the year of isolation (all strains isolated in 2000 or later).

Taxa	Strain code	Locality of isolation	FA	SM	16S rRNA	Morph.
Anabaena sp.	08-05	Máchovo fishpond, Czech Republic	+		+	+
D. affine	04-44	Svět fishpond, Czech Republic	+	+	+	+
D. affine	05-03	Staňkovský fishpond, Czech Republic	+		+	+
D. flos-aquae	04-10	Byňovský fishpond, Czech Republic	+		+	+
D. flos-aquae	04-40	Skalka reservoir, Czech Republic	+	+	+	+
D. flos-aquae	04-57	Vajgar fishpond, Czech Republic		+	+	+
D. circinale and *D. crassum* complex	04-22	Husinec reservoir, Czech Republic		+	+	+
D. circinale and *D. crassum* complex	04-26	Jesenice reservoir, Czech Republic		+	+	+
D. circinale and *D. crassum* complex	04-28	Hodějovický fishpond, Czech Republic	+	+	+	+
D. circinale and *D. crassum* complex	04-59	Valcha fishpond, Czech Republic		+	+	+
D. compactum	04-17	Dubnenský fishpond, Czech Republic		+	+	+
D. compactum	06-02	Pěšák fishpond, Czech Republic	+		+	+
D. compactum	06-03	Svět fishpond, Czech Republic	+		+	+
D. curvum	04-19	Hejtman fishpond, Czech Republic	+	+	+	+
D. lemmermannii	04-24	Husinec reservoir, Czech Republic	+	+	+	+
D. lemmermannii	04-38	Senecký fishpond, Czech Republic		+	+	+
D. lemmermannii	04-42	Svět fishpond, Czech Republic		+	+	+
D. mendotae and *D. sigmoideum* complex	04-06	Březová reservoir, Czech Republic		+	+	+
D. mendotae and *D. sigmoideum* complex	04-11	Černiš fishpond, Czech Republic		+	+	+
D. mendotae and *D. sigmoideum* complex	04-33	Orlík reservoir, Czech Republic	+	+	+	+
D. mendotae and *D. sigmoideum* complex	04-45	Svět fishpond, Czech Republic	+	+	+	+
D. mendotae and *D. sigmoideum* complex	05-01	Lipno reservoir, Czech Republic	+		+	+
D. mucosum	06-04	Horák fishpond, Czech Republic	+		+	+
D. mucosum	08-03	Mařka fishpond, Czech Republic	+		+	+
D. planctonicum	00-05	Nechranice reservoir, Czech Republic	+		+	+
D. planctonicum	03-02	Lipno reservoir, Czech Republic	+		+	+
D. smithii	05-05	Dubnenský fishpond, Czech Republic	+		+	+
D. smithii	08-02	Mařka fishpond, Czech Republic	+		+	+
D. spiroides	04-51	Svět fishpond, Czech Republic		+	+	+
D. viguieri	08-04	Mařka fispond, Czech Republic	+		+	+
S. aphanizomenoides	04-43	Svět fishpond, Czech Republic	+		+	+
S. reniformis	06-01	Pěšák fishpond, Czech Republic	+	+	+	+
S. reniformis	07-01	Vyšehrad fishpond, Czech Republic	+		+	+

Phylogenetic analyses. Sequences were aligned in the program ARB (http://www.arb-home.de), and the alignment was edited manually. For the phylogenetic analysis, trees were built with the neighbor-joining method (NJ) (Saitou and Nei 1987) using 500 bootstrap replicates. Nucleotide sequences were deposited at GenBank under the accession numbers AM940218, AM940219, FM161348–FM161350, FM242083–FM242088, and FN691905–FN691926.

Fatty acid profiles. A Hewlett Packard 6890 (series II) gas chromatograph modified for glass-capillaries and an HP-GC mass selective detector (5973B MSD) were used to analyze fatty acid production of the harvested cultures. Fatty acid methyl esters were prepared according to Metcalfe and Wang (1981) and analyzed by gas chromatography (GC) on a capillary column, that is, RTX 1701, 30 m, 0.32 mm, 0.25 mm film (Restek, USA) (Řezanka and Mareš 1987, Řezanka 1990). The GC oven was programmed for 40°C for 2 min and then an increase of 2°C min^{-1} to 300°C followed by 20 min at 300°C. The injector temperature was kept at 180°C. The flow rate of the carrier gas (helium) was 0.5 $mL \cdot min^{-1}$. The mass spectrometry (MS) detector operated at 200°C, and the ionization energy was 70 eV. The scan range was from 30 to 700 *m/z* at 0.9 scans $\cdot s^{-1}$. The solvent delay was 9 min. Fatty acid methyl esters were identified using mass spectral library searches (Wiley 7th, and NIST-98). Statistica 9.0 for Windows (Stat Soft Inc., Tulsa, OK, USA) was used for the evaluation of similarities among the fatty acid profiles of the strains studied (cluster analysis – Euclidean distance, single linkage).

Secondary metabolite content—extract preparation and HPLC–mass spectrometry (HPLC–MS) analysis. Lyophilized biomass of cyanobacterial cultures (~40 mg) was disintegrated by grinding and extracted in 2 mL of 70% (v/v) methanol (MeOH) in microtubes for 30 min. The microtubes were centrifuged at 3,170*g* at 4°C for 15 min (centrifuge Universal 320, Hettich, Smithfield, Australia). The supernatant was concentrated 10 times using a rotary vacuum drier ROTAVAPOR B-840 and WATERBATH R-114 (BÜCHI, Flawil Switzerland). HPLC–MS analysis was performed in order to determine the content of secondary metabolites. The extracts were analyzed on a reversed phase column (Zorbax XBD C8, 4.6 × 150 mm, 5 μm; Agilent Technologies Inc., Santa Clara, CA, USA) using a MeOH/H_2O gradient and a flow rate of 0.6 $mL \cdot min^{-1}$. For a more effective ionization, 0.1% (v/v) formic acid was added into the eluents. The extract composition was analyzed using a HP 1100 Agilent mass spectrometer HP 100 MSD SL-Ion trap (Agilent Technologies Inc.) in positive mode. The settings were selected to cover the mass range between 50 and 2,000 Da, and the ion trap was targeted to molecular masses near 900 Da. Automatic fragmentation of the most intensive peak was applied. The molecular ions were determined based on the presence of sodium and potassium adducts and based on the distribution of isotopologues. Only metabolites found in at least two of the strains studied were taken into consideration. Photosynthetic pigments, identified based on absorption spectra, were eliminated from the analysis. The similarities of whole secondary metabolite profiles among the strains studied were

visualized by cluster analysis (Euclidean distance, complete linkage: Statistica for Windows, Stat Soft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

The present study provides a unique chemotaxonomic data set for the genera *Dolichospermum* and *Sphaerospermopsis*. These data are comparable among all of the strains studied, since they were grown, harvested, and analyzed under identical conditions and time. This is extremely important because both the fatty acid composition and secondary metabolite production were previously shown to be influenced by environmental conditions and growth phase (Sivonen 1996, Rapala and Sivonen 1998, Dembitsky et al. 2003, Dembitsky and Rezanka 2005, Temina et al. 2007), which hampers the overall comparison of the results obtained in the past by different authors (Li and Watanabe 2001, 2004, Gugger et al. 2002b). We assume that the contaminating bacterial communities were insignificant (<0.5% of the total strain biovolume) and similar in all of our closely related strains, in line with previous studies that show specific bacterial phylotypes associated with different microalgae (Jasti et al. 2005, Shi et al. 2009). The effect of bacteria associated with our strains on the results of metabolite analyses should be negligible and more or less similar for all strains.

Morphological characteristics. General morphologies for all of the studied strains are shown in photomicrographs (Figs. S1–S3 in the supplementary material). PCA was employed to compare morphometric parameters of 33 natural populations representing 12 different morphospecies of the genus *Dolichospermum* and two morphospecies of the genus *Sphaerospermopsis*. The analysis has demonstrated a continuum of morphological variability within this cyanobacterial group where no distinctly delimited groups representing the traditional species can be recognized (Fig. 1). The exceptions are *D. compactum* and *Sphaerospermopsis* morphospecies (*S. aphanizomenoides* and *S. reniformis*), which were clearly defined by the shape and position of the akinetes and the morphology of trichome coiling, consistent with the morphological diversity among natural populations of various *Dolichospermum* morphospecies (Zapomělová et al. 2007).

In several cases, wider groups of morphospecies were only recognizable, with the borderlines between single morphospecies being unclear (*D. circinale* and *D. crassum,* and *D. mendotae* and *D. sigmoideum*). This finding is consistent with the findings of Zapomělová (2008) and Zapomělová et al. (2008, 2010b) who demonstrated morphological plasticity in selected *Dolichospermum* strains, supporting the existence of wider complexes of morphospecies within this cyanobacterial group: single strains grown under a range of conditions (temperature, light, concentration of N and P) were shown to span the variability of both the species *D. circinale* and *D. crassum,* or *D. mendotae* and *D. sigmoideum,* respectively, as originally described. Their results

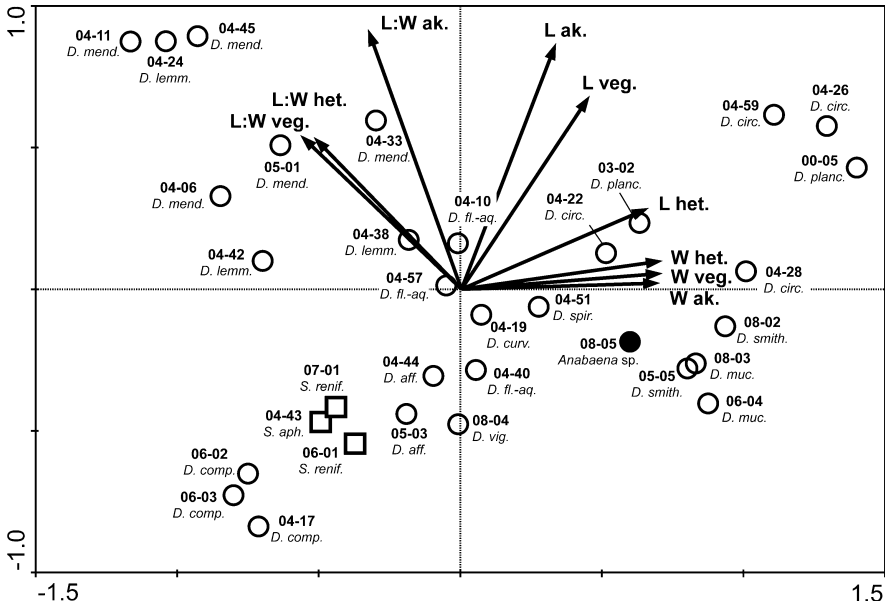

FIG. 1. Principal component analysis (PCA) diagram based on the morphological characteristics of the studied cyanobacterial populations from the field conditions. Each strain is symbolized by a plain circle (*Dolichospermum*), full circle (*Anabaena* sp.), or square (*Sphaerospermopsis*). The most variable morphological characteristics are shown by the arrows (veg., vegetative cells; het., heterocytes; ak., akinetes; L, length; W, width; L:W, length:width ratio). The first and the second canonical axes explain together 74.9% of the total variance. *D. aff., Dolichospermum affine; D. circ., D. circinale; D. curv., D. curvum; D. dan, D. danicum; D. fl.-aq., D. flos-aquae; D. lemm., D. lemmermannii; D. mend., D. mendotae; D. muc., D. mucosum; D. planc., D. planctonicum; D. smith., D. smithii; D. spir., D. spiroides; D. vig., D. viguieri; S. aph., Sphaerospermopsis aphanizomenoides; S. renif., S. reniformis.* The strain codes are explained in Table 1.

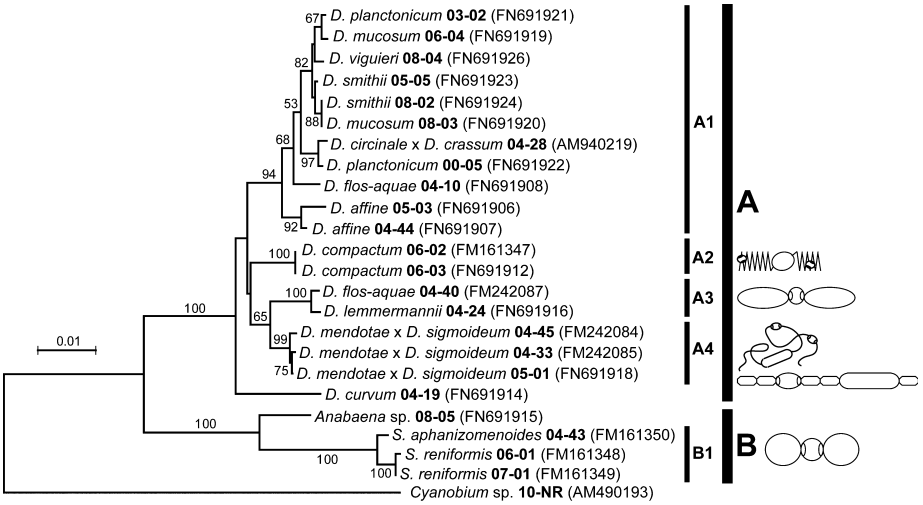

FIG. 2. Neighbor-joining tree based on 16S rRNA gene sequences (1,419 bp) showing the clustering of cyanobacterial strains from which fatty acid profiles were analyzed. Numbers near nodes indicate bootstrap values >50%. Drawings demonstrate morphological features that are typical and common for the strains of single genetic clusters. *D., Dolichospermum, S., Sphaerospermopsis.*

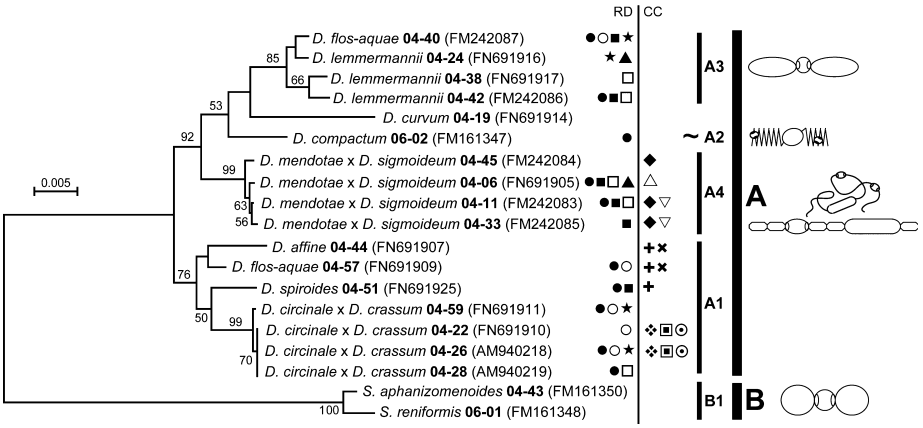

FIG. 3. Neighbor-joining tree based on 16S rRNA gene sequences (1,392 bp) showing the clustering of cyanobacterial strains from which secondary metabolites were analyzed. Symbols represent the most frequent randomly distributed compounds (RD) and compounds exhibiting correlation with 16S rRNA phylogeny (CC): ● (*m/z* 685.2, RT 11.6 min), ○(*m/z* 715.3, RT 21.6 min), ■ (*m/z* 701.3, RT 11.2 min), □ (*m/z* 256.3, RT 21.7 min), ★ (*m/z* 569.4, RT 20.4 min), ▲ anabaenopeptin B (*m/z* 837.4, RT 13.1 min), + (*m/z* 727.4, RT 12.6 min), × (*m/z* 534.4, RT 12.2 min), ❖ (*m/z* 714.4, RT 7.2 min), ▣ (*m/z* 756.3, RT 9.6 min), ⊙ (*m/z* 731.3, RT 19.4 min), ♦ unknown variant of anabaenopeptin (*m/z* 842.4, RT 20.1 min), △ anabaenopeptin A (*m/z* 844.4, RT 17.1 min), ▽ anabaenopeptin D (*m/z* 828.5, RT 19.6 min). Numbers near nodes indicate bootstrap values >50%. Drawings demonstrate morphological features that are typical and common for the strains of single genetic clusters.

demonstrate that for the reliable identification of morphospecies other than *Sphaerospermopsis* and *D. compactum,* additional morphological criteria (i.e., akinete shape, arrangement of heterocytes and akinetes, filament aggregation, occurrence and pattern of trichome coiling) have to be used alongside standard morphometric characters.

Molecular characteristics and phylogenetic analyses. The sequences of the 16S rRNA gene were determined from clonal cultures of *Dolichospermum* spp. and *Sphaerospermopsis* spp. Two NJ trees were constructed, one for the strains where fatty acids were analyzed (Fig. 2) and another for the strains where HPLC-MS analysis of secondary metabolites was performed (Fig. 3). Both of the trees displayed a consistent clustering of the strains that was in good agreement with their morphospecies affiliations.

The genera *Dolichospermum* and *Sphaerospermopsis* were located in two highly supported and distinct clades, A and B (Figs. 2 and 3), which is consistent with the establishment of the genus *Sphaerospermopsis* (Zapomělová et al. 2009, 2010a).

Clade A comprised four distinct subgroups, A1–A4, with significant bootstrap support, with the morphologically distinct strain *D. curvum* 04-19 as an outlier. Strain 04-19 displayed distinctive trichome coiling and clumps of trichomes in a distinct

mucilaginous envelope. It was identified as *D. curvum,* because its morphology resembled morphological features of the strain *Anabaena curva* Ana Ao presented by Li et al. (2000). In particular, the trichome coiling of our strain 04-19 and the accumulation of its filaments in thick mucilage was very similar to strain Ana Ao; however, the shape of akinetes, which was also identical in both strains, did not agree with the original description of *A. curva* (Hill 1976). According to the original description, the akinetes of *A. curva* should be markedly curved and elongated, while both our strain 04-19 and the strain Ana Ao by Li et al. (2000) exhibited kidney-shaped akinetes.

Subgroup A1 showed the highest genotypic and morphological diversity. It comprised a wide range of morphospecies, *D. planctonicum, D. mucosum, D. viguieri, D. smithii, D. flos-aquae, D. affine,* and strains from the morphological complex of *D. circinale* and *D. crassum.* Common morphological features of all these strains were the relatively small length:width ratios of all cell types (vegetative cells, heterocytes, akinetes) and their relatively large dimensions (Fig. 1). The grouping of these morphospecies is consistent with phylogenetic analyses of 16S rRNA gene sequences of Finnish isolates (Rajaniemi et al. 2005a).

Subgroup A2 comprised strains of *D. compactum,* which agreed exactly with the findings by Rajaniemi et al. (2005a,b). These strains were clearly characterized by tight regular coiling of their trichomes and widely ovoid akinetes with low length:width ratios, distant from heterocytes.

For the strains of subgroup A3, formation of ovoid akinetes next to heterocytes was typical. This akinete position was stable in all strains of *D. lemmermannii* and was only rarely disrupted in the strain *D. flos-aquae* 04-40. Though *D. lemmermannii* is known to be a highly diversified taxon morphologically (Komárková 1988, Zapomělová et al. 2007), the arrangement of akinetes is invariable and was shown to be a good indicator of its separation from *D. mendotae* and *D. sigmoideum,* morphospecies that occur in a different molecular cluster (Zapomělová et al. 2010b; Figs. 2 and 3, subgroup A4).

Subgroup A4 contained strains from the morphological complex of *D. mendotae* and *D. sigmoideum.* Typical morphological characteristics of these strains are the shapes of vegetative cells and akinetes (high length:width ratios; Fig. 1) and the position of akinetes distant from heterocytes.

Clade B contained a highly supported subgroup B1 (*Sphaerospermopsis* strains with nearly spherical akinetes adjacent to heterocytes as the autapomorphic feature) and an outlying strain *Anabaena* sp., 08-05, preliminarily identified as *D.* cf. *danicum* according to its morphology. The phylogenetic affiliation based on the 16S rRNA gene sequence indicated that the strain 08-05 should not be classified as a morphospecies of either *Dolichospermum* or *Sphaerospermopsis,* from which it is separated with 100% bootstrap support. This strain probably represents a specific group of *Anabaena*-like cyanobacteria that have yet to be described. It is questionable whether our strain 08-05 actually represented *D. danicum.* The width of its filaments, their mucilaginous envelope, and the dimensions and shapes of the akinetes corresponded with the original description of *D. danicum* (Komárková-Legnerová and Eloranta 1992). However, the trichomes of our strain 08-05 were distinctly narrowed toward the ends; the terminal vegetative cells were elongated, especially in the original natural population (Fig. S2, C); and the shape of vegetative cells in general was also not typical for the true *D. danicum.* The16S rRNA gene sequences of any true *D. danicum* strains are not available for comparison with strain 08-05.

Fatty acid profiles. Our cultured strains produced large amounts of linoleic and linolenic acids, whose content in some strains exceeded 50% of total fatty acids (Table 2). To our knowledge, such a high content has not been previously described for the genus *Dolichospermum* and closely related cyanobacteria (cf. Li and Watanabe 2001, 2004, Gugger et al. 2002b). This difference is probably explained by environmental conditions at the site of collection (see Temina et al. 2007). Worthy of note is the content of two positional isomers of hexadecenoic acid, which were found in previous analyses (Li and Watanabe 2001, 2004, Gugger et al. 2002b). Overall, the fatty acid profiles of our cyanobacterial strains, with the exception of the features influenced by ecological and geographic conditions, do not differ from those described previously.

The complete linkage cluster analysis of fatty acid profiles identified four clusters of strains (Fig. 4), which displayed a low correlation with morphospecies affiliation of the strains or phylogenetic clustering based on the 16S rRNA gene.

Li and Watanabe (2001, 2004) indicated that planktonic *Anabaena* (currently *Dolichospermum*) strains can be divided into two groups differing in their fatty acid production: type 2A producing 16:2 and 16:3 fatty acids, and type 2B where no 16:2 and 16:3 fatty acids were found. Type 2B comprised morphospecies *D. planctonicum, D. danicum, D. affine, S. kisseleviana,* and strains *Anabaena eucompacta* and *A. oumiana.* The taxonomic status of the two latter morphospecies *(A. eucompacta, A. oumiana)* has not yet been confirmed, but they both form spherical akinetes adjacent to heterocytes, suggesting that they may belong to the genus *Sphaerospermopsis* and not *Dolichospermum* (Zapomělová et al. 2009). Gugger et al. (2002b) reported neither 16:2 nor 16:3 fatty acids in their *Anabaena* strains.

We only detected the production of 16:2 fatty acids, with 16:3 not detected in any of our *Dolichospermum* and *Sphaerospermopsis* strains in amounts >0.1% of total fatty acid methyl esters. Very low or no production of 16:2 fatty acids, corresponding to

TABLE 2. Fatty acid compositions (%) of different *Dolichospermum* and *Sphaerospermopsis* strains.

Taxa	Strain code	14:0	15:0	16:2	16:1a	16:1b	16:0	17:0a	17:0b	18:3	18:2	18:1	18:0	19:0
Anabaena sp.	08-05	0.8	0.0	2.9	4.2	2.8	31.9	0.1	0.3	41.9	9.5	0.8	4.7	0.1
D. affine	04-44	2.1	0.7	5.2	4.5	5.1	25.6	0.0	0.1	43.5	11.1	1.1	0.9	0.1
D. affine	05-03	5.8	0.0	5.8	2.9	3.9	17.5	0.1	0.1	34.1	26.4	0.8	2.6	0.0
D. circinale and *D. crassum*	04-28	2.2	1.4	4.1	8.0	4.8	22.4	0.0	0.1	41.8	13.3	0.1	1.8	0.0
D. compactum	06-02	0.7	0.1	7.4	8.3	3.8	20.2	0.0	0.0	38.2	20.1	0.5	0.7	0.0
D. compactum	06-03	0.8	0.0	7.6	7.6	4.6	8.2	0.1	0.1	51.3	18.7	0.2	0.8	0.0
D. curvum	04-19	2.2	0.3	3.0	1.3	3.5	26.9	0.0	0.1	51.6	7.1	1.6	2.3	0.1
D. flos-aquae	04-10	0.7	0.1	7.1	5.2	2.4	21.9	0.0	0.1	40.4	19.4	0.2	2.4	0.1
D. flos-aquae	04-40	6.1	0.3	3.2	0.2	5.0	29.4	0.0	0.0	47.9	4.9	1.2	1.6	0.2
D. lemmermannii	04-24	5.0	2.0	2.5	0.2	5.2	24.8	0.0	0.1	50.8	3.9	2.4	3.0	0.1
D. lemmermannii	04-42	2.2	0.3	3.4	2.1	3.0	24.2	0.0	0.1	55.1	7.4	0.6	1.5	0.1
D. mendotae and *D. sigmoideum*	04-11	0.7	0.5	6.6	3.6	4.9	21.7	0.0	0.1	46.4	14.2	0.4	0.8	0.1
D. mendotae and *D. sigmoideum*	04-33	0.9	1.6	4.0	3.7	4.9	25.7	0.0	0.0	48.1	9.6	0.8	0.6	0.1
D. mendotae and *D. sigmoideum*	04-45	1.0	0.3	6.2	4.0	4.6	8.6	0.0	0.2	56.8	16.5	0.6	1.1	0.1
D. mendotae and *D. sigmoideum*	05-01	0.7	0.1	7.1	5.2	4.5	15.0	0.0	0.1	50.3	16.1	0.1	0.8	0.0
D. mucosum	06-04	0.5	0.5	4.6	2.1	7.8	28.2	0.4	0.2	36.7	12.9	4.2	1.8	0.1
D. mucosum	08-03	0.7	0.0	5.5	2.7	2.9	31.4	0.1	0.1	36.9	17.2	0.6	1.8	0.1
D. planctonicum	00-05	1.7	0.2	5.3	5.4	3.9	25.7	0.1	0.1	42.5	12.6	0.9	1.5	0.1
D. planctonicum	03-02	1.0	0.2	0.1	0.7	21.0	30.2	0.7	1.3	27.5	6.8	4.6	5.5	0.4
D. smithii	05-05	1.3	0.1	0.0	0.3	12.0	53.6	0.2	0.7	7.0	1.8	3.7	18.9	0.4
D. smithii	08-02	0.8	0.0	5.7	3.6	3.2	28.5	0.1	0.1	38.9	17.2	0.2	1.6	0.1
D. viguieri	08-04	0.3	0.2	0.1	0.0	19.3	28.2	0.2	0.4	44.0	3.8	1.8	1.6	0.1
S. aphanizomenoides	04-43	1.5	2.5	0.2	0.2	23.9	37.5	0.0	0.2	19.2	1.9	9.8	3.0	0.1
S. reniformis	06-01	2.7	0.2	0.1	0.2	19.8	52.9	0.0	0.3	8.1	1.8	8.8	4.8	0.3
S. reniformis	07-01	1.5	0.8	0.1	0.3	15.6	27.4	0.0	0.1	41.2	6.1	5.2	1.6	0.1
Cyanobium sp.	10-NR	10.7	0.0	0.0	36.2	39.8	8.2	0.0	0.9	0.2	0.0	2.9	1.1	0.0

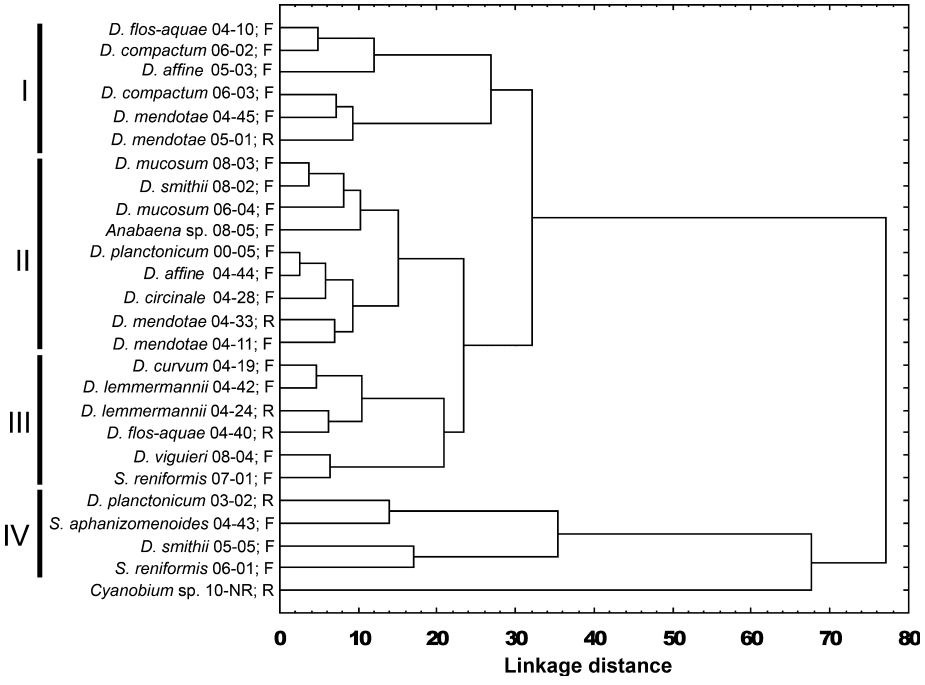

FIG. 4. Complete linkage cluster analysis dendrogram showing the similarities (Euclidean distances) of fatty acid profiles among planktonic *Anabaena* sp., *Dolichospermum* spp., and *Sphaerospermopsis* spp. strains of various morphospecies. The capital letters indicate the type of localities from which the strains were isolated: F, fishpond, R, reservoir.

type 2B, was confirmed in all of our *Sphaerospermopsis* strains (04-43, *S. aphanizomenoides*; 06-01, *S. reniformis;* 07-01, *S. reniformis*) and in one of the *D. planctonicum* strains (03-02), which is in good agreement with the results of Li and Watanabe (2001, 2004). Strains 05-05 (*D. smithii*) and 08-04 (*D. viguieri*) also belonged to type 2B, in contrast to the findings of Li and Watanabe (2001, 2004) who assigned these morphotypes to type 2A. Production of 18:1 fatty acids appeared to be characteristic of *Sphaerospermopsis*:

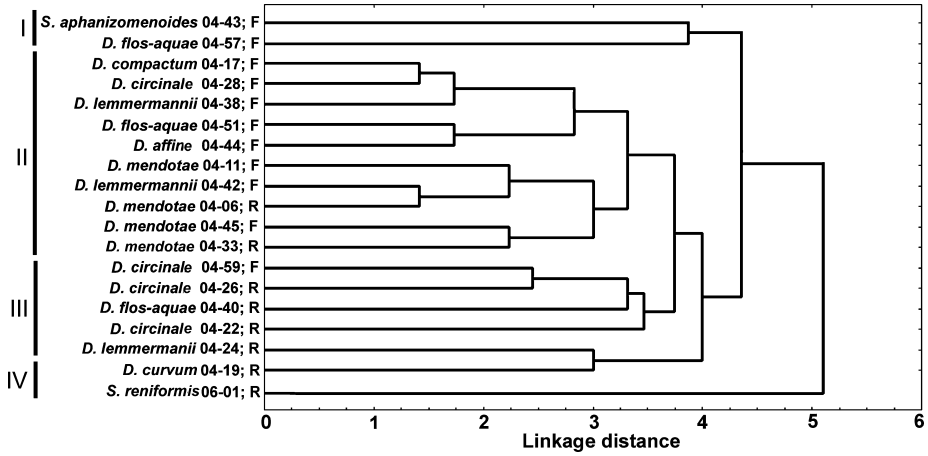

FIG. 5. Complete linkage cluster analysis dendrogram showing the similarities (Euclidean distances) of mass spectrometry spectra of secondary metabolites among planktonic *Dolichospermum* spp. and *Sphaerospermopsis* spp. strains of various morphospecies. The capital letters indicate the type of localities from which the strains were isolated: F, fishpond, R, reservoir.

all of the three *Sphaerospermopsis* strains studied displayed relatively high concentrations of 18:1 compared to the other strains analyzed (Table 2).

Secondary metabolite content. For the purposes of the present study, we selected an approach that considered each compound of a certain molecular mass (expressed as *m/z* value) and a certain retention time (RT) as one biochemical marker. The extraction and analytical method selected was designed to detect mainly cyanobacterial peptides, as they are the most abundant and diverse chemical structures in cyanobacteria. The main reasons for our approach were that an enormous diversity of cyanobacterial peptides exists (Welker and von Dohren 2006, Van Wagoner and Drummond 2007) and that most of their structural variants probably have not yet been described and remain unknown (Welker et al. 2006, Hrouzek et al. 2010). A similar approach was successfully applied to analysis of whole bacterial cell spectra by means of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS (Holland et al. 1996).

The extract analysis of 19 cyanobacterial strains revealed a total of 170 compounds, 26 of which were present in at least two strains (Table S1 in the supplementary material). The distribution of compounds that were common for two or more strains was compared to the 16S rRNA gene phylogeny in order to find possible correlations. The complete linkage cluster analysis of the metabolite profile identified four chemotype clusters (Fig. 5), which displayed a low correlation with morphospecies affiliation of the strains, phylogenetic clustering based on the 16S rRNA gene or with clustering based on fatty acid profiles. The strain *S. reniformis* 06-01 was clearly separate from all other strains in the complete linkage cluster analysis, which is in a good agreement with the results of phylogenetic analysis and was also reported previously (Zapomělová et al. 2009).

Metabolites were further categorized into three distribution groups: (1) metabolites that are randomly dispersed across the genotypic clusters based on 16S rRNA gene sequences, (2) metabolites that occur in several strains of a monophyletic genotypic cluster but not in all strains of the cluster, (3) metabolites that are present in the closest relatives and nowhere else in the phylogenetic tree and can be considered as autoapomorphies.

Most of the compounds (67%) fell into the first category, and their random distribution across the 16S rRNA gene phylogeny is demonstrated for the most frequently occurring compounds (Fig. 3). A detailed example of this random pattern is given using the compound of *m/z* 685.2 and RT 11.6 min (Fig. 3, full circles), which was spread both among all subgroups of *Dolichospermum* and also within these subgroups (Figs. S4 and S5 in the supplementary material).

Only 14% of the detected compounds fell into the second group and were produced by some of strains within a monophyletic cluster. Production of an unknown compound of *m/z* 842.4 and RT 20.1 min was recorded exclusively in three strains of the *D. mendotae* and *D. sigmoideum* complex (04-33, 04-11, 04-45), tightly clustering in subgroup A4; however, in the fourth member of this group (04-06) this compound was missing (Fig. S4). In all members of this cluster, the production of cyclic peptides anabaenopeptins was recorded based on the molecular weight, retention behavior, and characteristic fragmentation as described earlier in the literature (Erhard et al. 1999, Fujii et al. 2002). Strain 04-06 was found to contain anabaenopeptins A and B, while strains 04-33 and 04-11 synthesized anabaenopeptin D. Formation of a characteristic fragment 637.4 corresponding to the loss of arginine and a CO group in the side chain was observed in anabaenopeptin B. Identical fragmentation was described by Erhard and others using MALDI-TOF (Erhard et al. 1999).

Analogously, in anabaenopeptins A and D the presence of tyrosine and phehylalanine in their side chains was demonstrated by MS experiments (Fig. S6 in the supplementary material).

It is highly probable that the above-mentioned compound (*m/z* 842.4 and RT 20.1) produced by 04-33, 04-11, and 04-45 is an unknown variant of anabaenopeptin. This suggestion is supported by similarities of this compound with other anabaenopeptins in MS spectra. Thus, the strains of the phylogenetic cluster A4 can be characterized by anabaenopeptin production. Within the strains studied, production of anabaenopeptin B was also recorded in *D. lemmermannii* 04-24 located in cluster A3 (Figs. 3 and S5), and thus anabaenopeptin production cannot be used as an exclusive biochemical marker for cluster A4. However, its distribution definitely supports the grouping of cluster A4.

Tight clustering of *D. circinale* and *D. crassum* strains 04-22 and 04-26 was confirmed by the production of three compounds (*m/z* 714.4, RT 7.2 min; *m/z* 756.3, RT 9.6 min; *m/z* 731.3, RT 19.4 min) that were only detected in these two strains. These compounds were, however, missing in the third *D. circinale* and *D. crassum* strain (04-28) that exhibited 100% 16S rRNA gene sequence similarity with the previous two isolates.

Finally, five compounds (19% of the compounds detected in at least two strains) fell into the third category and were present in closely related strains (strictly monophyletic group) and nowhere else in the phylogenetic tree. Thus, they can be considered as autapomorphic characters with potential for use in taxonomy. In both strains of *S. reniformis*, a molecular ion of *m/z* 1235.7 and RT 19.8 min corresponding to puwainaphycin A was found (Zapomělová et al. 2009). Production of this compound was not documented for any *Dolichspermum* strains.

Strains *D. affine* 04-44 and *D. flos-aque* 04-57, which clustered within subgroup A1, were both found to produce two compounds (*m/z* 534.4, RT 12.2 min; *m/z* 727.4, RT 12.6 min). Also, their relation to *D. spiroides* 04-51 was confirmed by the production of a compound of *m/z* 727.4 in this strain. The production of anabaenopeptin D (*m/z* 828, RT 19.6 min) and unidentified compound of *m/z* 652.3 and RT 13.7 min by *D. mendotae* and *D. sigmoideum* strains 04-33 and 04-11 was in a good agreement with their tight clustering within the cluster A4.

General discussion, conclusions. The majority of the detected secondary metabolites and fatty acids seem randomly distributed, when compared with each other and with the 16S rRNA gene phylogeny of the strains studied. As was suggested by Thacker and Paul (2004), who also found a low consistency between the 16S rRNA gene phylogeny and chemical traits in the cyanobacterial genera *Lyngbya* and *Symploca*, divergence in chemosynthetic genes may not be reflected in 16S rRNA gene sequences as the 16S rRNA gene is a quite conserved molecular marker. Earlier studies demonstrated differences in toxin production among genetically similar strains and vice versa (Lyra et al. 2001, Baker et al. 2002, Gugger et al. 2002b). The random distribution of microcystin synthesis genes across the phylogenetic spectrum of cyanobacteria was suggested to be the result of the ancient origin of the synthetic pathway rather than the result of lateral gene transfer (Rantala et al. 2004). However, the evidence of lateral gene transfer of various synthetic genes has been recently reported not only from other prokaryotes (Jain et al. 1999, Schouls et al. 2003, Nesbø et al. 2006) but also from cyanobacteria (Green 2005, Leikoski et al. 2009, Bolhuis et al. 2010). Genes for metabolic pathways appear to be transferred more frequently than informational genes like the 16S rRNA gene (Jain et al. 1999), making metabolites problematic markers for determining phylogenetic relationships among cyanobacteria. Also, the synthetic pathways are probably rapidly evolving systems, since cyanobacterial strains of almost identical 16S rDNA can display markedly different metabolite profiles (Zapomělová et al. 2008). This is supported by our observation that 144 of the total 170 compounds were produced by only one of the studied strains. Little is known about the evolution of genes of fatty acid biosynthesis in prokaryotic autotrophs. Nevertheless, the effect of lateral gene transfer cannot be excluded, since some indications of lateral transfer of genes of fatty acid biosynthesis have been reported from other prokaryotes, for example, *Mycobacterium tuberculosis* (Kinsella et al. 2003).

Our results from *Dolichospermum* and *Sphaerospermopsis* strains confirmed that secondary metabolite and fatty acid profiles as a whole cannot directly and simply be interpreted in terms of evolution. On the other hand, we demonstrated that certain correlations between the production of particular compounds and the 16S rRNA gene phylogeny exist.

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Supplementary Material

The following supplementary material is available for this article:

Figure S1. Microphotographs of cyanobacterial strains studied. (A) *D. affine* 04-44, (B) *D. affine* 05-03, (C) *D. flos-aquae* 04-10, (D) *D. circinale* × *D. crassum* 04-22, (E) *D. circinale* × *D. crassum* 04-26, (F) *D. circinale* × *D. crassum* 04-28, (G) *D. flos-aquae* 04-40, (H) *D. flos-aquae* 04-57, (I) *D. circinale* × *D. crassum* 04-59, (J) *D. compactum* 04-17, (K) *D. compactum* 06-02. Scale bars represent 10 μm.

Figure S2. Microphotographs of cyanobacterial strains studied. (A) *D. compactum* 06-03, (B) *D. curvum* 04-19, (C) *Anabaena* sp. 08-05, (D) *D. lemmermannii* 04-24, (E) *D. lemmermannii* 04-38, (F) *D. lemmermannii* 04-42, (G) *D. sigmoideum* × *D. mendotae* 04-06, (H) *D. sigmoideum* × *D. mendotae* 04-11, (I) *D. sigmoideum* × *D. mendotae* 04-33, (J) *D. sigmoideum* × *D. mendotae* 04-45, (K) *D. sigmoideum* × *D. mendotae* 05-01. Scale bars represent 10 μm.

Figure S3. Microphotographs of cyanobacterial strains studied. (A) *D. mucosum* 06-04, (B) *D. mucosum* 08-03, (C) *D. planctonicum* 00-05, (D) *D. planctonicum* 03-02, (E) *D. smithii* 05-05, (F) *D. smithii* 08-02, (G) *D. spiroides* 04-51, (H) *D. viguieri* 08-04, (I) *S. aphanizomenoides* 04-43, (J) *S. reniformis* 06-01, (K) *S. reniformis* 07-01. Scale bars represent 20 μm (A) or 10 μm (B–K).

Figure S4. Secondary metabolite profiles of *Dolichospermum* strains of cluster A4 (base peak HPLC-MS chromatograms). Production of different anabaenopeptin conformers and probably unknown anabaenopeptin variants was found in all members of the cluster A4, which supports clustering of this group. Compounds that were detected in more than one strain of cluster A4 are in bold and marked by asterisks. *ph,* peak corresponding to phtalate contamination.

Figure S5. Secondary metabolite profiles of *Dolichospermum* strains of cluster A3 (base peak HPLC-MS chromatograms). None of observed compounds was found to be characteristic for this cluster. Particular compounds are characterized by their *m/z* values. Compounds that were detected in more than one strain of cluster A3 are in bold and marked by asterisks. Production of cyclic peptide anabaenopeptin B was recorded in *D. lemmermannii* 04-24. *ph,* peak corresponding to phtalate contamination.

Figure S6. Fragmentation spectra of anabaenopeptins detected in some of the studied strains. The molecular ion is marked by dashed line. The characteristic loss of aminoacid and CO group in the side chain can be clearly seen. (A) MS2 spectrum of anabaenopeptin B detected in the strain *D. sigmoideum* and *D. mendotae* 04-06. Formation of ion 637.4 corresponding to loss of Arg+CO in the side chain of the molecule is identical as described by Erhard et al. (1999). (B) MS2 spectrum of anabaenopeptin A detected also in *D. sigmoideum* and *D. mendotae* 04-06. (C) MS2 spectrum of anabaenopeptin D detected in *D. sigmoideum* and *D. mendotae* 04-33.

Table S1. Secondary metabolites detected by HPLC-MS analysis in extracts of the *Dolichospermum* and *Sphaerospermopsis* strains studied. *m/z* values and retention times (RT) are given for each compounds.

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