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A detailed morphological, phylogenetic and ecophysiological analysis of four benthic *Anabaena* (Nostocales, Cyanobacteria) strains confirms deep heterogeneity within the genus

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Abstract: Four benthic *Anabaena* strains isolated from different localities in the Czech Republic were examined from the morphological, phylogenetic, and physiological points of view. The results of combined analysis showed distinct morphological dissimilarity between the studied strains, which were further found to belong to different phylogenetic groups based on the 16S rRNA gene phylogeny. To assess the temperature and light (irradiance) optima of the strains, we exposed them to various combinations of these two parameters. The experiment revealed unexpectedly high temperature and light optima for some of the strains, while others showed optima that were similar to those of previously studied planktic species of related heterocytous genera. Our study is the first of its kind to be applied to benthic *Anabaena* strains. Our results indicate that the benthic *Anabaena* genus is much more complex than previously thought and provide novel insights into the biology and ecology of benthic *Anabaena* species. With the collection of more data, we expect the genus *Anabaena* will be split into several new monophyletic taxa, each covering distinct morpho– and ecotypes.

Key words: *Anabaena*, crossed gradients, ecology, growth optima, morphology, phylogeny, polyphyly

Introduction

Cyanobacteria are photosynthetic prokaryotes that are considered to be among the first organisms of the early Earth (Brock 1973; Schopf 1996). They are observed worldwide, in all kinds of environments (Oren 2000; Stal 2000; Castenholz 2001). In aquatic habitats, they represent one of the major groups of organisms in both planktic and benthic communities, and are capable of inhabiting wide temperature and irradiance ranges (Castenholz 1969, 1973; Ward & Castenholz 2000). One explanation of their broad distribution is the small amount of energy they require to maintain cell function and structure (Van Liere & Mur 1979; Gons 1997).

Methodological improvements and new approaches applied in cyanobacterial studies have recently revealed many new taxa. Numerous traditional genera have been subdivided, usually on the basis of results of molecular analyses (Komárek et al. 2014). After the introduction of new characterization methods based on a polyphasic approach (Johansen & Casamatta 2005; Komárek 2010; Osorio–Santos et al. 2014), the traditional genus *Anabaen*a established by Bory in 1822 (Geitler 1932) has recently been divided (Komárek & Zapomělová 2007, 2008; Wacklin et al. 2009; Zapomělová et al. 2009, 2012). A large group of planktic taxa with aerotopes (gas vesicles) was reclassified into the new genera *Dolichospermum*, *Sphaerospermopsis*, and *Chrysosporum*, primarily based on the results of 16S rRNA sequencing (Iteman et al. 2002; Gugger et al. 2002; Rajaniemi et al. 2005a,b; Hoffmann et al. 2005; Willame et al. 2006). Several morphospecies, characterized by the possession of a subsymmetrical filament structure and a special type of large akinete, and forming metaphytic mats in tropical regions, have been included in the newly described genus *Macrospermum* (Komárek 2008). After these revisions, the generic name *Anabaena* was preserved to include the remaining species without gas vesicles (benthic, periphytic, soil), and the

genus is now considered to be more or less consistent. However, up until now, only a few studies have dealt with non–planktic *Anabaena*, whose classification is based solely on morphology (KOMÁREK 2005, 2008; SKÁCELOVÁ & ZAPOMĚLOVÁ 2010; MAREŠ 2010) or on phylogeny (HALINEN et al. 2008). The main weak point with this approach is the inadequate morphological characterization of the majority of non–planktic *Anabaena* strains for which 16S rRNA gene sequences are available. Thus, we have hardly any idea how big the molecular diversity of this cyanobacterial group is and how the morphological diversity of this group observed in nature is related to their evolutionary diversity. Considering this gap in current knowledge, a detailed investigation of benthic *Anabaena* spp. combining morphological and phylogenetic approaches is highly desirable.

Anabaena species are a common component of many types of benthic, periphytic and metaphytic mats worldwide (KOMÁREK 2013), including newly emerging habitats. Therefore, understanding their ecological demands is of exceptional importance. Knowledge about the growth preferences of benthic cyanobacteria in shallow waters is particularly interesting since their living conditions can change dramatically, even during a single day. Unlike planktic cyanobacteria, they face daily extreme fluctuations in environmental factors such as temperature, salinity, and grazing pressure (STAL 2000). The ability to live across a wide range of temperatures is a corollary of their autecological features, i.e., metabolic rate, cell composition, and population differences in growth and temperature optima (DE NICOLA 1996). Several studies, such as those of STULP & STAM (1985) and ZAPOMĚLOVÁ et al. (2008b), have dealt systematically with the growth demands of nostocacean cyanobacteria, but up to now there have been

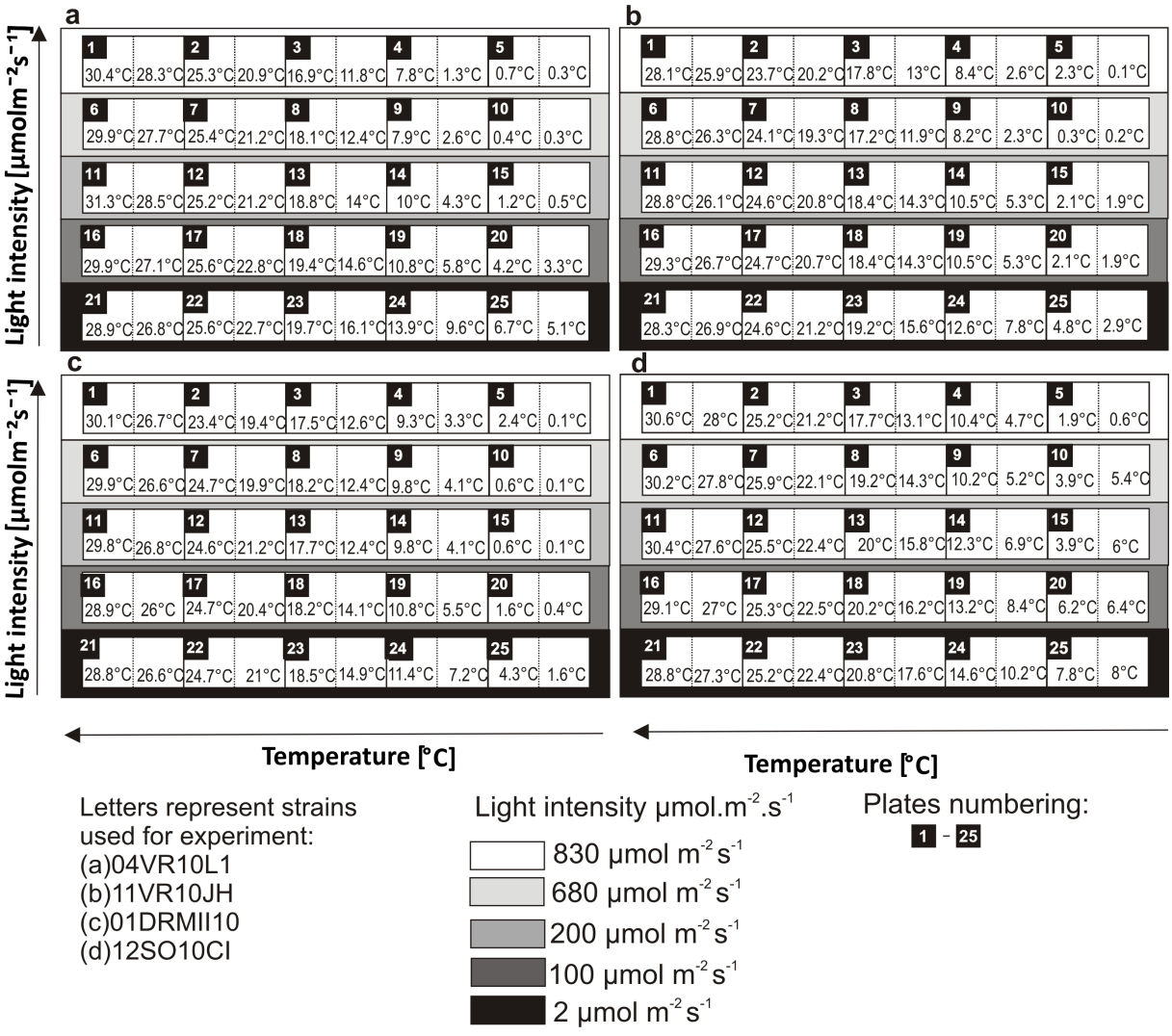

Fig. 1. Design of the crossed–gradient experiment used for the determination of the light and temperature growth optima of the studied *Anabaena* strains. Experimental setups for the four strains studied: (a) 04VR10L1; (b) 11VR10L1; (c) 01DRMII10; (d) 12SO10CI. Gradients of light intensity and temperature are indicated with arrows. The gradient of light intensity is also indicated by shading.

no studies assessing the optimal growth conditions of benthic *Anabaena*. Growth responses to environmental factors are also important because they are fundamental for water management targeting. From this point of view, a full understanding of the ecology of benthic cyanobacteria and their environmental preferences is valuable.

The aim of this study was to examine the morphological traits of four benthic *Anabaena* strains and assess their phylogenetic relationships to each other and to other nostocacean cyanobacteria based on 16S rRNA gene sequences. Another objective was to experimentally evaluate their temperature and light preferences to enable a prediction of their ecological niches and a comparison of these preferences with those of species belonging to related planktic taxa. It is hoped that, by comparing the outputs of these three approaches, correlations/discrepancies in morphological, phylogenetic, and ecophysiological similarities of the studied strains will be uncovered, thereby yielding a more complex picture of the little studied benthic *Anabaena* and providing a starting point for further research.

Materials and Methods

Cyanobacterial Strains. Samples used for this study were collected in 2010 from localities with a water conductivity of cca 600–6000 µS.cm^{-1} and a pH in the range 5.9–7.8 (Table 1), which are probably caused by the high concentration of dissolved minerals released from coal mines in the vicinity, with the exception of SO–CI, which is a natural mineral spring locality. Localities were situated in the northern part of the Czech Republic (Sokolov area). VR–JH is a small lake in a former coal mining pit near Vřesová; VR–L1 is a rainwater puddle in a mine disposal site (on a soil substrate left over after coal mining) near Vřesová; and DR–MII contains drainage water from a coal mine and a SO–CI – Císařský mineral spring in the Soos protected area near Františkovy Lázně. Single trichomes were isolated from the environmental samples using a glass capillary pipette (Zapomělová et al. 2007) and the resulting strains have been maintained in the culture collection of the Biology Centre of AS CR, Institute of Hydrobiology, in WC medium (Guillard & Lorenzen 1972) at 21°C with a light intensity of 50 µmol.m^{-2}.s^{-1} (16:8 L:D cycle). The strains were used for all analyses (examination of morphological traits, phylogenetic analysis, and growth demands) within 1 year after isolation to avoid possible changes induced by long–term cultivation, especially those that result in a loss of important features (Lehtimäki et al. 2000; Gugger et al. 2002b).

Morphological Study. The morphology of the strains was examined using an Olympus BX 51 light microscope equipped with an Olympus DP 70 digital camera. Microphotographs of at least 30 trichomes per strain were taken at a magnification of 400×. Morphometric characterization of the studied *Anabaena* strains was done based on microphotographs using the image analysis software Olympus DP Soft. Lengths and widths of all cell types were measured. Five vegetative cells per trichome were measured in 30 trichomes, 30 heterocytes, and 30 akinetes (if present) in each strain. The positions of akinetes relative to those of heterocytes were determined. Shapes of terminal cells and the length:width ratios of vegetative cells, heterocytes, and akinetes were assessed as additional characters. The identification of strains was done according to classical morphology (Komárek 2013).

Molecular and Phylogenetic Study. The biomasses of the strains were harvested in the exponential phase of growth by repeated centrifugation. Samples were washed by mixing with physiological solution (NaCl solution, concentration 1 g.l^{-1}) to remove mucilaginous substances. The centrifuged biomass samples were stored at – 20 °C until DNA extraction. DNA was extracted using an UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA). The 16S rRNA gene and the adjacent rRNA ITS region was amplified using primers 16S27F (5'–AGAGTTTGATCCTGGCTCAG–3') and 23S30R (5'–CTTCGCCTCTGTGTGCCTAGGT–3') (Taton et al. 2003). Amplification was carried out as follows: an initial denaturation step 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; followed by a final elongation step of 7 min at 72 °C. Primers K6 (5´–GACGGGCCGGTGTGTACA–3´), which is

Table 1. Summary of analyzed strains, including year of isolation, isolation locality, and the parameters of the localities

Strain code	Locality	Environment	Conductivity (µS.m^{-1})	pH	GPS coordinates	Year of isolation
04VR10L1	Vřesová (VR-L1)	black crust	1230	7.1	50°15'51.97"N, 12°43'4.972"E	2010
11VR10JH	Vřesová (VR-JH)	pond	590	7.8	50°15'51.94"N, 12°43'4.973"E	2010
01DRMII10	Sokolov area (DR-MII)	drain	6050	6.7	50°16'18.079"N, 12°45'23.399"E	2010
12SO10CI	Soos Nature Reserve – Císařský spring (SO-CI)	spring	5200	5.9	50°08'51.903"N, 12°24'11.871"E	2010

the reverse complement of Primer 14 described by Wilmotte et al. (1993), K7 (5′–AAGGAGGTGATCCA GCCACA–3′) (FLECHTNER et al. 2002), and 27F (5'AGAGTTTGATCCTGGCTCAG–3') (TATON et al., 2003) were used for PCR product sequencing at the Laboratory of Genomics, Biology Centre of CAS, České Budějovice, Czech Republic. Raw data from the DNA sequencer were analyzed and assembled into final nucleotide sequences using the SeqMan 5.06 (BURLAND 1999) computer program and edited manually to remove unclear bases and the variable ITS region. The DNA sequences from four studied strains were deposited in the NCBI GenBank database under accession numbers KJ679568–571. Additional sequences for phylogenetic analyses were selected from the GenBank online database (http://www.ncbi.nlm.nih.gov). All GenBank sequences included in this study are listed in Supplementary Materials. Sequences were aligned using MAFFT v. 7 (KATOH & STANDLEY 2013) and eventual adjustments of the resulting alignments (deletion of ambiguous sites) were carried out in BioEdit v. 7.0.9.0 (HALL 1999). The alignment was analyzed by Bayesian Inference (BI), Maximum Likelihood (ML), and Neighbor–Joining (NJ) methods. BI trees were constructed using MrBayes 3.2.3 (RONQUIST et al. 2012) and the best ML tree was obtained using RaxML v. 8 (STAMATAKIS 2014); both phylogenies were computed using the CIPRES supercomputing facility (MILLER et al. 2012). NJ tree calculations were conducted in Seaview 4.5.3 (GOUY et al. 2010). The GTR+I+G evolutionary model of substitution used during the ML analysis was obtained for the best fit to the data using jModelTest–2.1.4 (GUINDON & GASCUEL 2003; DARRIBA et al. 2012,). Bayesian analyses were performed using two independent runs, each with four Markov chains that were run for 1 375 000 generations with the default likelihood model (without weighing of bases or base changes) until the average standard deviation of split frequencies was lower than 0.01. For NJ and ML analyses, 1000 bootstrap pseudoreplications with default parameters were performed to evaluate the relative support of branches. The topology of the final phylogenetic tree was derived from that of the BI tree. Trees were rooted using 16S rRNA gene sequences of outgroup non–heterocytous cyanobacteria *Gloeobacter violaceus* PCC 7421, *Trichodesmium erythraeum* IMS 101, S*ynechococcus* sp.1tu21s05, and *Cyanobium* sp. JJ10–3. Trees were edited using FigTree v. 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

Crossed–Gradient Experiment. Crossed gradients (KVÍDEROVÁ & LUKAVSKÝ 2001) were used to determine the growth preferences of the four *Anabaena* strains. The experimental table enabled us to establish different combinations of light intensity and temperature. The strains were exposed in sterile culture plates (9×12 cm, 6 wells, 16 ml each) to 50 different combinations (Fig. 1). The temperature gradient was set directly on the table and particular levels of light intensities were achieved by shading. Two replicates of strains were done for each combination of light and temperature. Ranges of temperature and light were modified for each strain according to the results of a pilot experiment (data not shown). The temperature ranged from 0.0 to 31.3 °C, and the range of light intensity was 2–830 $\mu mol.m^{-2}.s^{-1}$. The duration of each experiment was about 2 weeks. The experiments were terminated when the fastest–growing culture (corresponding to the optimal experimental condition) reached the late exponential phase of growth. The late exponential phase was indicated by a slightly yellowish colour of the growing biomass. After the termination of experiments, chlorophyll *a*

Table 2. Morphological characteristics and features of the studied *Anabaena* strains. Dimensions are given as mean values (minimum–maximum). Abbreviations and symbols: L: W, length to width ratio; **A**, akinete; ●, heterocyte; ■, vegetative cells.

	Vegetative cells, n=150					Heterocytes, n=30			Akinetes, n=30			
Strain	**Length (µm)**	**Width (µm)**	**L:W ratio**	**Shape of terminal cells**	**Shape of veg. cells**	**Length (µm)**	**Width (µm)**	**L:W ratio**	**Length (µm)**	**Width (µm)**	**L:W ratio**	**Position**
04VR10L1	4.2 (2.5–7.5)	4.1 (3–5.3)	1 (0.5–1.8)	Pointed arrow like	Isodiametric, cylindrical	6.7 (4.4–9)	5.6 (4.5–5.7)	1.2 (0.7–1.7)	11 (9.6–12.9)	7.6 (5.3–8.9)	1.5 (1.1–2.4)	A●A
11VR10JH	4.7 (3.4–6.2)	6.4 (5.4–7.3)	0.7 (0.5–1)	Spherical rounded	Shorter than wide, barrel shaped	6.4 (57.8)	6.6 (5.1–7.8)	1 (0.9–1.1)	less than 30 occurred	less than 30 occurred	less than 30 occurred	A●A
01DRMII10	6.1 (4.1–8.7)	6 (5–7.1)	1 (0.7–1.5)	Cylindrical rounded	Isodiametric, barrel shaped	9.2 (7–12.5)	7.3 (5.6 –8.2)	1.3 (1.1–1.5)	17 (8.9–23.8)	8.8 (7.1–10.8)	2 (1–2.6)	A■■■●
12SO10CI	4.3 (2.7–6.2)	3.5 (2.7–4.6)	1.2 (0.8–1.9)	Cylindrical rounded	Isodiametric, cylindrical	6.6 (4.8–8.4)	4.3 (3.8–5.4)	1.5 (1.2–1.9)	16.8 (12.7–22.5)	6.2 (5.5–7.3)	2.7 (2–3.6)	A●A

Table 3. Summary of temperature and light preferences of the four studied strains. The temperatures and light intensities providing the highest chlorophyll *a* concentration are printed in bold font. The highest chlorophyll *a* concentration is also indicated in bold.

Strain codes	04VR10L1		11VR10JH		01DRMII10		12SO10CI	
Light intensity ($\mu mol.m^{-2}.s^{-1}$)	Temperature (°C)	Highest chl–*a* concentration ($\mu g.l^{-1}$)	Temperature (°C)	Highest chl–*a* concentration ($\mu g.l^{-1}$)	Temperature (°C)	Highest chl–*a* concentration ($\mu g.l^{-1}$)	Temperature (°C)	Highest chl–*a* concentration ($\mu g.l^{-1}$)
830	30.4	5786.97	**28.1**	**3404.35**	**23.4**	**4498.91**	25.2	1513.68
680	25.4	**7200.33**	28.8	2141.29	19.9	3647.11	27.8	1814.63
200	28.5	4540.68	**24.6**	**3400.78**	17.7	3968.41	**25.5**	**2664.65**
100	19.4	1446.92	26.7	671.16	18.2	1196.31	22.5	860.73
2	5.1	417.69	12.6	228.84	14.9	504.08	7.8	328.80

concentrations were determined spectrophotometrically after acetone extraction (Lorenzen 1967) and compared between the individual positions of the crossed gradients. The light and temperature growth preferences of the strains were obtained from a Scatter Chart using R (v.3.1.1; R Development Core Team, 2014).

Results

Morphology

Morphological characters and dimensions of the studied *Anabaena* strains are shown in Table 2. Important morphological features (vegetative cells, terminal cells, heterocytes and akinetes) are further visible in Figure 2. The vegetative cell dimensions and their proportions differed between the different strains, which enabled the recognition of two main morphological types. Strains 04VR10L1, 01DRMII10 and 12SO10CI represent a group with more or less isodiametric vegetative cells, while another morphotype with shorter than wide vegetative cells with a barrel–shape is represented by strain 11VR10JH. Moreover, akinetes were found adjacent to heterocytes in all strains except for 01DRMII10, in which they were situated distant from heterocytes, usually at a distance of 3–10 vegetative cells. Terminal cells in strain 04VR10L1 were pointed, unlike in the other strains where the terminal cells were rounded. The morphological comparisons demonstrated that the strains belong to different morphological groups. Two morphospecies were identified according to currently–used species–defining morphological criteria: strain 12SO10CI was identified as *Anabaena oscillarioides*, and strain 01DRMII10 as *Trichormus variabilis* (syn. *Anabaena variabilis*). Morphological identification of these two strains was in agreement with their phylogenetic position (see below). The remaining two strains (04VR10L1 and 11VR10JH) were impossible to identify because of the insufficient number of observed akinetes, which are crucial for species determination within the *Anabaena*/*Trichormus* group (Rajaniemi et al. 2005).

Phylogenetic Relationships

Partial 16S rRNA gene sequences (1361 bp) of the four studied *Anabaena* strains were compared with a representative set of sequences available from GenBank. BI, ML and NJ phylogenetic algorithms produced similar topologies; thus only the BI tree is presented here. The bootstrap supports obtained using the ML and NJ methods are given in this tree (Fig. 3). Sequences of non–planktic *Anabaena* were recovered as highly polyphyletic in our phylogeny, forming at least eight separate clades. Each of the four *Anabaena* strains analyzed in this study appeared in one of these different clusters, which was supported by relatively high bootstrap values (Fig. 3). Strains 04VR10L1 and 11VR10JH formed two isolated lineages that were remotely related

Fig. 2. Microphotographs of the four studied *Anabaena* strains. Strain codes: (a) 04VR10L1; (b) 11VR10JH; (c) 01DRMII10; (d) 12SO10CI. Scale bar 20 µm.

to two different clades of benthic *Anabaena* spp. Strain 12SO10CI grouped tightly in an *Anabaena oscillarioides/Anabaena* sp. cluster, in agreement with its morphological identification (*A. oscillarioides*). Similarly, strain 01DRMII10, identified as *Trichormus variabilis* based on its morphology, clustered together with *Anabaena* sp. and two *Trichormus variabilis* strains with high bootstrap support.

Growth Preferences

Temperature and light growth optima were taken to be those that yielded the highest chlorophyll–*a* concentration. While the temperature and light intensity optima that produced the highest chlorophyll–*a* concentration were evident for three of the strains, strain 11VR10JH gave ambiguous results. The highest chlorophyll *a* concentrations for this strain occurred at 28.1 °C and 830 µmol.m^{-2}.s^{-1}, both of which are unexpectedly high. The second highest concentration, differing in only ~ 4 µg.l^{-1}, occurred at 24.6 °C and 200 µmol.m^{-2}.s^{-1}. The temperature preferences of the other strains were roughly similar, while the light preferences of strains 04VR10L1 and 01DRMII10 were remarkably high and differed from each other (Table 3, Fig. 4). The optimal light preference of strain 12SO10CI (200 µmol m^{-2} s^{-1}) was lower than those of the other strains. The results showed that the chlorophyll–*a* concentration was low in all the strains at low irradiance (2 µmol.m^{-2}.s^{-1}) and across the entire temperature range. At temperatures below 10–15 °C, the chlorophyll–*a* concentration was low in all the strains irrespective of light intensity. In general, the chlorophyll–*a* concentrations of the strains differed. Some of the strains, for example 04VR10L1, accumulated the highest chlorophyll–*a* concentration (over 7000 µg.l^{-1}) at 630 µmol.m^{-2}.s^{-1} and 25.4 °C, while strain 12SO10CI accumulated the lowest concentration (6.78 µg.l^{-1}) at 830 µmol.m^{-2}.s^{-1} and 4.7 °C. Average values of chlorophyll–*a* concentration also differed between the strains (04VR10L1, 1497.29 µg.l^{-1}; 11VR10JH, 628.36 µg.l^{-1}; 01DRMII10, 1378.42 µg.l^{-1}; and 12SO10CI, 635.33 µg.l^{-1}).

Discussion

The phylogenetic tree inferred from 16S rRNA gene sequences revealed clustering of the four *Anabaena* strains into separate, relatively little related clades that broadly correlated with their morphologies. Members

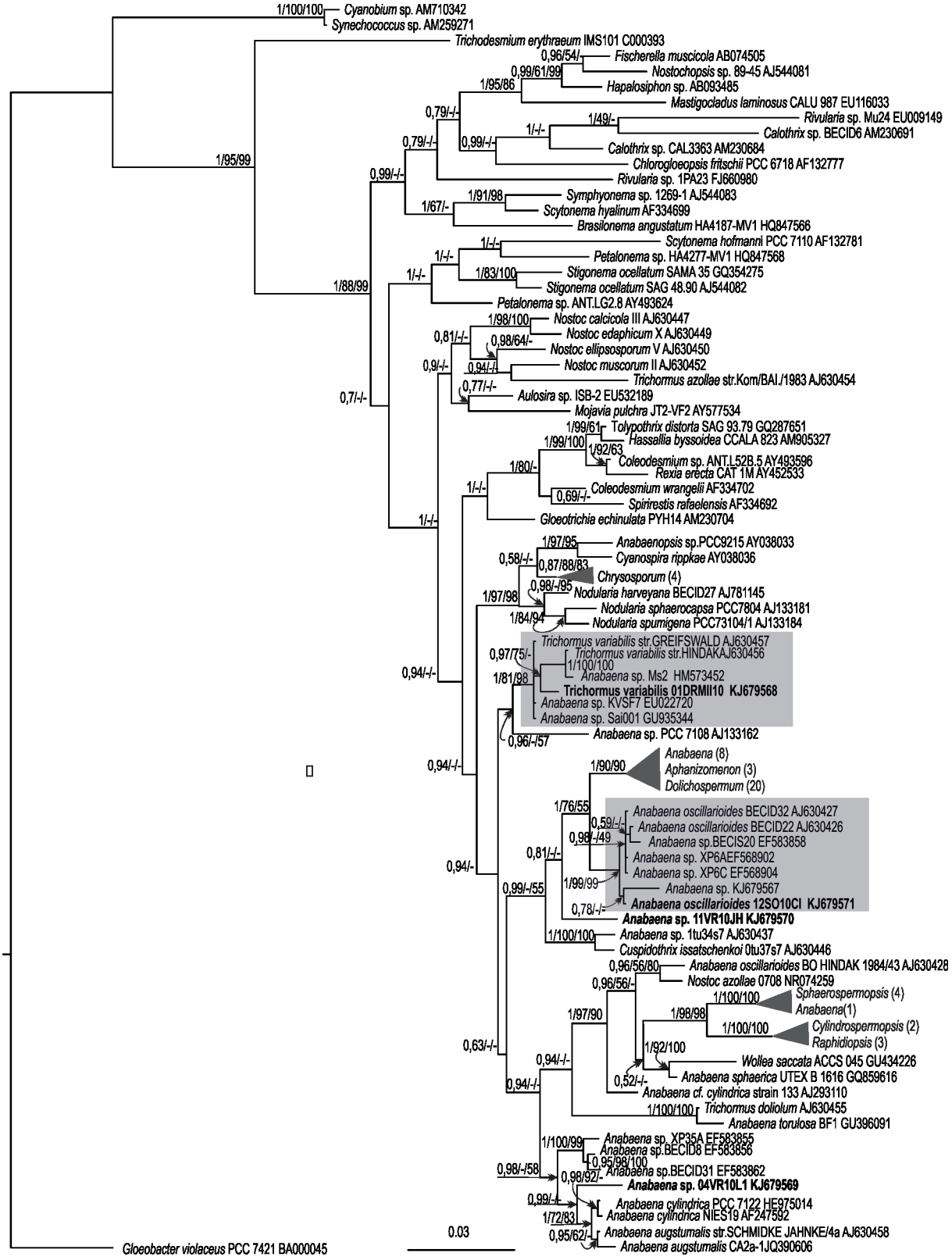

Fig. 3. Bayesian inference (BI) tree based on 16S rRNA data (1361 bp) showing the clustering of *Anabaena* morphospecies without gas vesicles from the Czech Republic (sequences obtained in this work are in bold, clusters containing identified strains are highlighted). Numbers near the nodes indicate branch support values over 50% for BI, Maximum Likelihood (ML), and Neighbor–Joining (NJ) analyses in the following order: BI/ML/NJ. Sequences are labeled with taxon name, strain code, and GenBank accession number. *Synechococcus* sp. 1tu21s05, *Cyanobium* sp. JJ10–3, *Gloeobacter violaceus* PCC 7421, and *Trichodesmium erythraeum* IMS 101 are outgroup taxa.

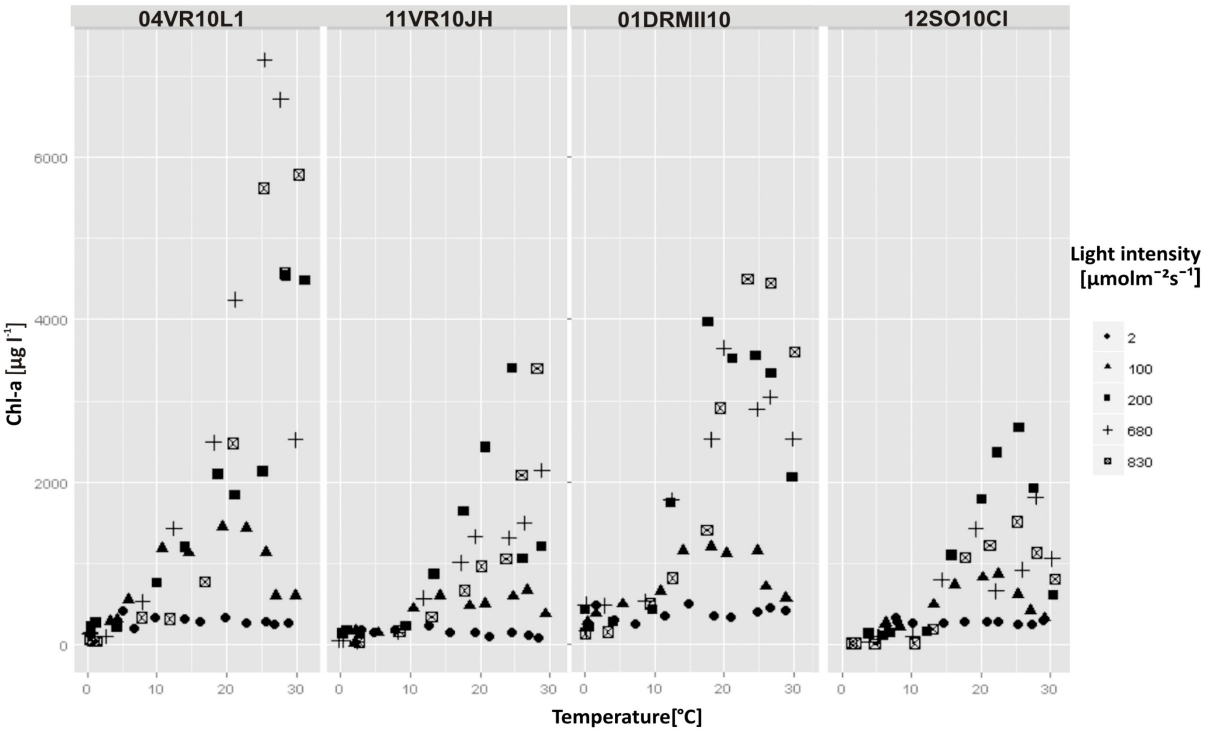

Fig. 4. Scatter Charts describing chlorophyll–*a* concentrations [µg.l^{-1}] of the strains as a function of temperature and light. Identical inocula of each strain were exposed to various combinations of temperature and light intensity. The biomass was harvested in the exponential phase of growth corresponding to that of the fastest–growing culture, and chlorophyll–*a* concentrations were determined.

of non–planktic *Anabaena* were resolved as highly polyphyletic, with sometimes a more distant relationship between themselves than with other species of other Nostocacean genera (for example, *Trichormus*, *Dolichospermum*, and *Aphanizomenon*). These findings are in accordance with those of HALINEN et al. (2008), who also identified several relatively unrelated phylogenetic clusters of benthic *Anabaena* spp. *Trichormus,* belonged for a long time to the genus *Anabaena* and only recently was separated from this genus on the basis of its apoheterocytic akinete development (RAJANIEMI et al. 2005). Some of the *Anabaena* strains (01DRMII10 and related *Anabaena* sp.) in our phylogeny formed a tight cluster together with members of the genus *Trichormus* (Fig. 3), namely, with a couple of strains identified as its type species *T. variabilis* (former *Anabaena variabilis*). Based on the high bootstrap support, and the fact that the morphology of 01DRMII10 matched that of *T. variabilis*, an affiliation of these strains with true *Trichormus* should be considered. However, there is no clear morphological description available for the other *Anabena* sp. in this cluster, and further polyphasic examinations will be required before any decision can be made. Strains of other species assigned to *Trichormus* (*T. azollae* and *T. doliolum*) clustered in completely different positions in the tree, once again emphasizing the polyphyly of the genus (RAJANIEMI et al. 2005; HROUZEK et al. 2013), which is in urgent need of a comprehensive taxonomic revision.

Strain 12SO10CI was identified as *Anabaena oscillarioides* based on its morphology and source habitat. This conclusion was further supported by its position in a cluster of several strains designated as *A. oscillariorides* and *Anabaena* sp. Because *A. oscillarioides* is the type species of *Anabaena*, this lineage may be a candidate for the definition of "true" *Anabaena*. Nevertheless, as demonstrated earlier (RAJANIEMI et al. 2005; KOZHEVNIKOV & KOZHEVNIKOVA 2011), putative *A. oscillarioides* can be found at least in one more, relatively distant lineage (see also Fig. 3, strain BO HINDAK 1984/43). There are currently not as many sequences available for this genus as there are for planktic taxa, and, moreover, the strains are usually poorly defined. One of the reasons for this lies in the morphological plasticity of *Anabaena* and their frequent lack of morphological features that are critical for identification during the different parts of its life cycle or when it is grown under culture conditions. This was also the case for two strains in our study that could be identified solely as *Anabaena* sp. because they lacked akinetes.

The results of this study further support the distinction of planktic *Dolichospermum, Sphaerospermopsis, and Chrysosporum* (ZAPOMĚLOVÁ et al. 2009, 2011, 2012) from benthic *Anabaena* strains. On the other hand, HALINEN et al. (2008) showed that some non–gas–vacuolate *Anabaena* spp. are intermixed with planktic *Dolichospermum* strains. The occurrence of aerotopes was demonstrated to be an unstable feature

of species in some related cyanobacterial taxa, especially after long–term cultivation (Komárek et al. 1993; Laamanen et al. 2001). Thus, detailed molecular and morphological studies involving a larger set of populations are still required to test this hypothesis.

Alongside phylogeny, the morphology of the studied *Anabaena* strains confirmed the considerable morphological and genotypic polymorphy within benthic *Anabaena*. However, the morphology and phylogeny of the studied strains were generally congruent, which is in contrast to studies where the observed morphological variability is sometimes not reflected in the genetic relationships based on 16S rRNA sequences (Lyra et al., 2001; Iteman et al. 2002; Gugger & Hoffmann 2004; Řeháková et al. 2014). On the other hand, it was difficult to find a correlation between the growth response of the studied strains to various light intensities and temperatures and their morphological or phylogenetic variability. Strain 01DRMII10 was clearly distinct from other strains based on its morphology and phylogeny, whereas it was not different from other strains with respect to its growth optima. Strain 12SO-10CI had growth preferences that were the most different from those of the other studied strains, while the results of strain 11VR10JH were ambiguous, similar to both 01DRMII10 and 04VR10L1. In contrast to our results, Miller & Castenholz (2000) showed that strains of *Synechococcus* isolated from Oregon hot springs that grouped into different phylogenetic lineages have different temperature growth optima. Apparently, ecophysiological niche differentiation in cyanobacteria can rapidly evolve in response to the selective pressure of the environment (Coleman et al. 2006). Hence, the correlation of growth optima with morphology or phylogenetic position can be expected only in extremely tightly related populations, which was not the case for our strains.

Since benthic or periphytic species often have to face severe fluctuations in temperature and light intensity (Stal 2000), we expected their growth optima or tolerances to be lower or higher than those of their planktic counterparts. Indeed, some of the studied strains showed remarkably high temperature and light preferences. Interestingly, strains showing preference for a higher light intensity (01DRMII10 and 04VR10L1) were isolated from localities with shallow water, with high exposure to sun irradiance. Strains preferring higher light intensities had also higher chlorophyll *a* concentrations (an approximation of biomass), which is in agreement with the greater photosynthetic yield expected from an increased energy input. We did not observe any link between the conductivity or the water pH of the localities and morphology or phylogenetic clustering.

Our strains survived over the whole experimental temperature range (0.0–31.3 °C), and at light intensities 2–830 $\mu mol.m^{-2}.s^{-1}$. Most of the studied strains (04VR10L1, 11VR10JH, and 01DRMII10) showed similar light intensity optima, which were relatively close to those of previously studied planktic species of *Dolichospermum* (Zapomělová et al. 2008a). The *Dolichospermum* strains exhibited an ability to grow over a wide temperature range (10–28 °C), but some of them did not survive extremely low or high light intensities (20 $\mu mol.m^{-2}. s^{-1}$, 750 $\mu mol.m^{-2}.s^{-1}$) (Zapomělová et al. 2008a). Although cyanobacteria generally require little energy to maintain cell function and structure (Gons 1997; Van Liere & Mur 1979), the low light intensities employed were apparently insufficient to provide the minimal amount of energy necessary for the growth of *Dolichospermum* strains studied by Zapomělová et al. (2008a). Their death at high light intensities could be explained by oxidative stress and photoinhibition (Van Liere & Mur 1980; Herman & D'Ari 1998; Muller 2000). In general, planktic strains seem to be adapted to a more homogenous and stable environment without the need for mechanisms to protect against abrupt changes in environmental conditions, whereas the benthic strains investigated in this study presumably possess protective mechanisms that allow them to grow over a wide range of temperatures and light intensities. Such mechanisms possibly involve increased carotenoid production to protect cells from photoinhibition (Paerl et al. 1983) or efficient cyclic electron flow (Marathe 2012). Considering that the four strains used in this study were collected from different sampling sites, their morphological differences, clustering in different phylogenetic lineages, and variable growth preferences are not very surprising. Previously, Rapala & Sivonen (1998) referred to strain–specific differences in growth rates of various planktic *Anabaen*a strains (recently reclassified to *Dolichospermum*) exposed to different temperature and light conditions. Similarly, Zapomělová et al. (2008b) described the markedly different and non–overlapping temperature and light optima of different strains in the species complex of *Dolichospermum circinale* / *D. crassum*.

Our study contributes to the current knowledge on non–gasvaculate *Anabaena* mostly by combining the morphological, phylogenetic and eco–physiological approaches in attempt at obtaining a congruent picture of the studied strains. Although the study itself is based solely on four strains, it summarizes and validates the methodological approaches, and provides the starting point for further studies within this intriguing group of cyanobacteria. The main weak point of previous phylogenetic studies of non–planktonic *Anabaena*–like cyanobacteria was the missing analysis of morphology. Furthermore, growth preferences of benthic *Anabaena* species have not been experimentally assessed before. Some of the strains included in the current study exhibited much higher light intensity preferences compared to their planktonic counterparts, raising further questions related to their biology and ecology.

Our study has once again stressed the importance of combining different approaches to reach reliable conclusions about individual cyanobacterial species and populations, which in turn should result in improvements in the taxonomy of cyanobacteria and better resolution of their life history. The phylogenetic heterogeneity (polyphyly) of the studied strains indicates that benthic and periphytic *Anabaena* will certainly have to be divided into several genera. Further investigation is necessary to reveal the whole range of the diversity of this cyanobacterial group and to create a reliable taxonomic classification. Taken together with the results of previous studies, our results show that the growth demands of related planktic and benthic cyanobacteria differ substantially from each other. This can be explained by their different life strategies, resulting from the different properties of their habitats. Planktic species usually live in relatively stable environments, whereas benthic species inhabit environments with extremely fluctuating conditions (light intensity, temperature, water and nutrient availability, grazing pressure etc.). Moreover, benthic or periphytic mats can occur during the whole year, whereas those of planktic species of cyanobacteria predominantly occur during the warmer season. The results of this study indicate that benthic *Anabaena* species can tolerate wider fluctuations in temperature and light intensity than their planktic counterparts. Further investigation of the physiological and biochemical aspects of these growth preferences is desirable.

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Supplementary material

the following supplementary material is available for this article:

Table S1.Summary of GenBank sequences used in this study.

This material is available as part of the online article (http://fottea.czechphycology.cz/contents)

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