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PHYTOPLANKTON

Variability of *Chroococcus* (Cyanobacteria) morphospecies with regard to phylogenetic relationships

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Abstract Recent advances in the taxonomy of cyanobacteria due to the utilization of molecular methods (such as 16S rRNA analysis) require a comparison of genetically identified items with their morphological expression. Morphological variability of seven monoclonal populations of the planktonic coccoid genus *Chroococcus* Nägeli, isolated mostly from reservoirs, fishponds, and littoral substrata from localities in the Czech and Slovak Republics, were studied experimentally under various combinations of nutrient concentration, light intensity, temperature, and water movement. Two cultivation media (WH-WC and BG11) commonly used in algal collections were applied in a liquid state. Of these, the WH medium was found to be more convenient for planktonic forms. Impacts of combined temperature and light gradients, concentration of P–PO_4, and a stable versus shaken medium were found to stress different morphological modifications as a consequence of varied growth intensity and media convenience. Cell width was chosen as the parameter for testing changes in morphology; formation of mucilage and packets of cells were also taken into account. According to 16S rRNA gene analysis, the sequences of 10 strains (including seven studied in the experiments), which were assigned to the genus *Chroococcus* in the Culture Collection CCALA Třeboň (www.cas.ccala.cz) formed four distinct phylogenetic groups. While two of them showed no affiliation to the genus *Chroococcus*, two other groups proved the polyphyletic character of the genus. Apart from the group of typical species of the genus *Chroococcus*, a group of planktonic species could be distinguished, i.e., *Chroococcus limneticus* Lemmermann 1898 (*Limnococcus*), and was established as a new genus after recombination.

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Introduction

Since the first scientific studies on blue-greens, they were perceived as algae and classified among botanical objects. Thus, taxonomic evaluation of populations of Cyanophyta (Schizophyta) was ruled by botanical nomenclature according to the Botanical Code (Greuter et al., 1988). However, as blue-greens are prokaryotes, they have been correctly subsumed to Bacteria and the Bacteriological Code (see e.g., Castenholz & Waterbury, 1989), which became relevant to the taxonomy and nomenclature of this group. Since the 1980s (Wayne et al., 1987), the 16S rRNA sequence and 95% similarity between relative sequences have been accepted as criteria for distinguishing genera of cyanobacteria. For cyanobacteria, the newly used Bacteriological Code stresses genomic information as a unique, reasonable, and objective approach in taxonomy.

Even if taxonomic research may, and should, concentrate now on solving genera problems, and there is still much to be done, the practice must work also with lower level taxa, i.e., with species, for which no explicit molecular criteria were found up to now. Such categorization is necessary for the study of morphological diversity and plasticity of populations in nature. Neither the Botanical nor Bacteriological Codes reflect cell structures, complex diversity in thallus organization, specialization of cells in heterocytous cyanobacteria or changes in morphology due to different environmental conditions, together with highly important information about the phylogenetic position of a taxon among related sequences. It seems that problems of cyanobacterial taxonomy call for a special Code that would respect both the Bacteriological and Botanical Codes as well as help to characterize lower than genus taxa, such as species.

A polyphasic approach can be an intermediate stage between the recent state and the establishment of a final specific Code for cyanobacteria. This means that molecular analysis (16S rRNA gene sequence and phylogenetic analysis) is combined with morphological characteristics, EM structures of cells, presence of extracellular products, and ecological demands of the population. These criteria can be used for characterization of lower level taxa, while requirements of the Bacteriological Code are respected. The Bacteriological Code requires assignment of so-called Reference strains (for a newly described taxon, it is a Type strain), which should represent characteristics of the identified strain (morphospecies) whose 16S rRNA gene sequence is placed into the GenBank. Both the Reference (Type) strain and the sequence should be available to everybody to be controlled and compared. Thus, knowledge of morphological plasticity and reaction of such a clone to changes of environmental parameters is important.

We tried to determine experimentally the ranges of variability of cell width together with formation of mucilage and colonies in seven strains of *Chroococcus* kept in cultures for different lengths of time. Monoclonal cultures enabled us to exclude morphological diversity of other genetically different clones, which can be present in natural assemblages, and provide isolation of DNA and sequencing of the product. Six 16S rRNA gene sequences of these strains, plus two more of strains available from the Culture Collection CCALA in Třeboň and two sequences of *Chroococcus* available already in the GenBank, were submitted to phylogenetic analysis.

Materials and methods

Two types of experiments in which environmental conditions were manipulated were conducted: cultivation on a cross gradient table of light and temperature in miniature batch cultures (CG experiments), and cultivation in shaken liquid media in Erlenmeyer flasks (Shaker experiments, Table 1).

Seven strains originally designed as *Chroococcus* are listed in Table 2 (lines 1–7). All strains had the same history starting at least half a year before the experiments. They were cultivated in our collection at the Institute of Hydrobiology, České Budějovice, in a liquid WH medium (Guillard & Lorenzen, 1972), at 22°C and radiation of 35 μmol m^{-2} s^{-1}. The strains coming from CCALA (Culture Collection of the Centre of Algology, www.butbn.cas.cz/ccala) were originally cultivated in 1.8% agar Z medium (Staub, 1961), at 20°C and 25 μmol m^{-2} s^{-1}, 16:8 h light–dark cycle since their isolation or a transfer from another culture collection. Strain 054 of CCALA

Chroococcus cf. *membraninus* was transferred to Třeboň from the Culture Collection of Algae and Protozoa at Argyl, Scotland.

Cross gradients (CG) of light and temperature

Experimental clones were cultivated in sets of 12-well microtitration plates filled with 2.5 ml of BG11 (Allen & Stanier, 1968; Stanier et al., 1971) and WH media (Guillard & Lorenzen, 1972), and situated at four places on a cross gradient table in duplicates (Lukavský, 1982; Kvíderová & Lukavský, 2001). Four combinations were used: high radiation, high temperature, HR+HT (120 μmol m^{-2} s^{-1}, 25°C); high radiation, low temperature, HR+LT (120 μmol m^{-2} s^{-1}, 8°C); low radiation, high temperature, LR+HT (10 μmol m^{-2} s^{-1}, 25°C); and low radiation, low temperature, LR+LT (10 μmol m^{-2} s^{-1}, 8°C). All the factors were chosen to correspond to natural conditions; however, maximum radiation intensities were modified according to the intensities used during long-term cultivation of clones in the culture collections, reaching values optimal for growth of cyanobacteria in cultures (Wyman & Fay, 1987).

Shaken media and different concentrations of phosphorus

The strains were cultivated in 100-ml Erlenmeyer flasks filled with 50 ml of media. Two modifications of P–PO_4 concentrations in both WH and BG11 media (Table 1), each in duplicate, were exposed to 35 μmol m^{-2} s^{-1} and 22°C. A shaking velocity of 120 min^{-1} (orbital shaker Labatron©) was chosen to simulate a turbulent water column. Concentrations of reactive phosphorus in media were different enough to test the reaction of cell and colony morphology to the highest and possibly limiting (stressing) conditions. BG11 is a rich medium and contains seven times higher concentration of P–PO_4 than WH medium. In both experiments, the strains were cultivated for 10 days and then analyzed. The cells were photographed in fresh state under a microscope (Olympus BX51 and digital camera DP 70, magnification 400×). Afterward, 100 items were randomly chosen for measuring of cell width using Olympus DG Soft image analysis. Width of the cells best characterizes the size of the morphological unit of *Chroococcus*, as the length of cells varies greatly in this genus. Results were evaluated using Analysis of Variance (ANOVA) in Statistica (Anonymous, 1996). The amount, pigmentation, and macroscopic appearance of biomass were estimated visually.

Table 1 Concentrations of reactive phosphorus in shaker experiments

	P–PO_4 (μg l^{-1})	pH
BG 11 full medium	6150	9
BG 11 with 1/50 P–PO_4	120	9
WH full medium	880	7.6
WH with 1/50 P–PO_4	17.6	7.6

Phylogenetic analysis

Six of the strains used for the morphological experiments were newly sequenced (see Table 2, GenBank accession numbers italicized). For new sequencing we added two more strains from the CCALA collection designed also as *Chroococcus*: *Chroococcus* sp. CCALA No. 019 isol. HEGEWALD 1952 originating from the Pasteur Collection in Paris and *Chroococcus minor* CCALA No. 700, isolated by CEPÁK 1993 from rice fields in Indonesia, Nias island, Telukdaham. Two strains, Nos. 1 and 8 in Table 2 determined later as *Chroococcus prescottii* Drouet et Daily, and *Eucapsis* Clements et Shantz sp. had already been sequenced at the Laboratory of K. Sivonen, University of Helsinki, Department of Applied Biochemistry and Microbiology, Finland and sent to GenBank before our experiments started. With these, altogether 10 sequences designated as *Chroococcus* were used for construction of phylogenetic tree (Table 2); all of them were checked under microscope and photographed.

The DNA was extracted from the exponentially growing cells using the classical phenol–chloroform method. DNA amplification was performed by PCR (5 min/94°C; 10 cycles of 45 s/94°C, 45 s/57°C, 2 min/72°C; 25 cycles of 45 s/94°C, 45 s/54°C, 2 min/72°C, 7 min/72°C) in combination with an unspecific prokaryotic forward primer 16S27F and cyanobacterial specific reverse primer 23S30R (Taton et al., 2003). An additional primer, WAW1486R/K8 (Flechtner et al., 2002), was used for sequencing. Sequences were checked and corrected manually using Chromas Lite (version 2.01; Technelysium Pty

Table 2 List of strains and sequences for construction of phylogenetic tree

	Original name	Isolated by	CCALA Třeboň	Locality	Final name	GenBank number
1	*Chroococcus* cf. *minor*	KOMÁRKOVÁ 2005/1	889	Trnávka Res., plankton, Czech Rep.	*Eucapsis* sp.	AM710384
	Chroococcus sp. JJCM					
2	*Chroococcus* sp. "red"	KOVÁČIK 1982/11b	702	Danube backwater, epilithon, S. Slovakia	*Chroococcus* cf. *westii* Boye-Petersen	*GQ375044*
3	*Chroococcus* sp. "green"	KOVÁČIK 1982/11a	701	Danube backwater, epilithon, S. Slovakia	*Chroococcus virescens* Hantzsch in Rabh.	*GQ375046*
4	*Chroococcus minutus*	HINDÁK 1965/1	055	Lake Ochrid, littoral, Macedonia	*Chroococcus minutus* (Kütz.) Nägeli	*GQ375047*
5	*Chroococcus* sp.	HINDÁK 1971/52	057	Bratislava, pool, plankton Slovakia	*Chroococcus minutus* (Kütz.) Nägeli	*GQ375045*
6	Chroococus limneticus	ZAPOMĚLOVÁ 2006/4	890	Fishpond Svět plankton, Czech Rep.	*Limnococcus limneticus* gen.nov.	*GQ375048*
7	*Chroococcus* cf. *membraninus*	LAPORTE 1965	054	Unknown thermal spring	*Gloeocapsa*? sub *Chrooc*. cf. *membraninus* (Menegh.) Nägeli	*GQ375049*
8	*Chroococcus* sp. JJCV	KOMÁRKOVÁ 2003	922	Klíčava Reservoir, plankton, Czech Rep.	*Chroococcus prescottii* Drouet et Daily	AM710385
9	*Chroococcus minor*	CEPÁK 1993	700	Rice fields in Indonesia	*Synechocystis* sp.	*GQ375043*
10	*Chroococcus* sp.	HEGEWALD 1952	019	Pasteur Collection, Paris, France	Not known	*Not submitted*

Strains 1–7 were used for experiments. Accession numbers of newly sequenced strains italicized

Ltd). Alignment and phylogenetic calculations (sequence similarity, phylogenetic trees) were performed in MEGA4 (Tamura et al., 2007). Phylogenetic trees were built with the Neighbor-Joining method (Saitou & Nei, 1987) using a bootstrap value of 500.

Results

Diversity in the morphology of seven studied *Chroococcus* morphospecies

All the data sets were submitted to statistical analyses. Tables 3 and 4 demonstrate the reliability of the results and their mutual relations.

1. *Chrococcus* cf. *minor* (= *Eucapsis* sp.) Figs. 1A1–3, 3a, b: Cultivation in BG11 in all positions of the CG table caused formation of large dense colonies composed of tight 4–8 or more celled packet groups without any distinct amount of mucilage, which is a typical feature of the genus *Eucapsis*. In the HL–HT treatment, the packets and cells had a brownish color (Fig. 3a) and formed brownish flakes on the bottom of the wells. The LL–LT combination in WH medium also caused packet-like forms. These seem to function as resting stages, formed under stress conditions, or they may start a transformation to a periphytic mode of life. In the other growth supporting combinations, the strain in WH medium formed colonies with single, double, or grouped cells in a loose common mucilage, a feature typical for planktonic *Chroococcus*. As our isolate came from phytoplankton of a reservoir, it had been determined as *C*. cf. *minor*. Minute cells varied in width, larger cells were found in batch cultivation in the BG11 medium (Fig. 1A1, 5.4 μm in average), while cells in the shaker cultures were smaller as a consequence of weaker growth in loosened mucilage and lower nutrient concentrations of the medium (Fig. 1A3, 2.4–2.1 μm in average).

There was a negative relationship between concentration of phosphorus and colony size and distance between the cells. Inoculated packets disintegrated

Table 3 Table of F and P values for ANOVA statistical evaluation of width data measured in the CG experiments (light and temperature influence in BG11 and WH media)

	Value	F	Error	P
Intercept	0.003108	28687.28	626	0.00
Medium	0.286070	223.18	626	0.00
Temperature	0.498689	89.90	626	0.00
Light	0.330317	181.31	626	0.00
Medium × Temperature	0.633256	51.79	626	0.00
Medium × Light	0.544877	74.70	626	0.00
Temperature × Light	0.648375	48.50	626	0.00
Medium × Temperature × Light	0.587205	62.87	626	0.00

Wilks' test for seven experimental strains. All the relationships were statistically significant

Table 4 Table of F and P values for ANOVA statistical evaluation of width data measured in the shaker experiments in full media (BG11 and WH) and those with reduced P concentration

	Value	F	Error	P
Intercept	0.002480	22414.10	390	0.000000
Full Medium	0.245709	171.03	390	0.000000
Medium with P 1/50	0.390536	86.95	390	0.000000
Full Medium × Medium with P 1/50	0.802784	13.69	390	0.000000

Wilks' test for seven experimental strains

and the intensity of doubling diminished (*Chroococcus minor* state, Fig. 1A3). Large cells were found in the shaken cultures with WH and P–PO_4 1/50 medium. However, the cells were spherical and evidently stopped dividing as the P-concentration became limiting.

2. *Chroococcus* sp. "red" 702 (*C.* cf. *westii* Boye-Petersen) Figs. 1B1–3, 3c, d: In the CG cultivation, this strain formed a brownish-red powder at the bottom of the batch cultures. The HL + HT and LL + HT treatments in BG11 medium fit best to this strain description, as this cultivation produced the highest biomass and cells grew quickly, even if they were only blue-green to brownish and never deep reddish in color. In WH medium, the strain formed brownish packets in the HL + HT treatment (Fig. 3c). In the LL + HT combination, daughter cells were blue-green in both media, while mother cells remained dark red. In both low temperature combinations (HL + LT and LL + LT), cells were brownish or reddish and formed packets in both media. In general, cells of this strain were rather large (8–18 μm in width). The large width in Fig. 1B2 corresponded to the slower development of mother cells and formation of packets.

The environment in the shaken cultivation corresponded best to the demands of this strain, especially in the case of full BG11 medium. The cells were dark reddish-violet, of a "chroococcus" form and produced the highest biomass (Fig. 3d). A lower concentration of phosphorus induced the formation of smaller cells (Fig. 1B3). Packets occurred in the WH, P–PO_4 1/50 medium. Cells were reddish to violet in all of the phosphorus concentrations in the shaken experiments.

3. *Chroococcus* sp. "green" 701 (*C. virescens* Hantzsch in Rabh.) Figs. 1C1–3, 3e, f: Batch cultivation in high nutrient concentrations (BG11 medium) led to cell mortality as well as to the formation of anomalies and polyhedric forms in all combinations of light and temperature (Fig. 3e). The strain showed a better state in WH medium in all treatment combinations. Cells were dividing, regular in shape with a smooth or granular content. The strain behaved as an oligotrophic, lower temperature preferring population, as the best development was found in the WH medium in the HL–LT combination. The strain preferred lower concentrations of nutrients, lower temperature and lower pH. Owing to anomalies in the BG11 medium, the cell width reached high values in comparison with

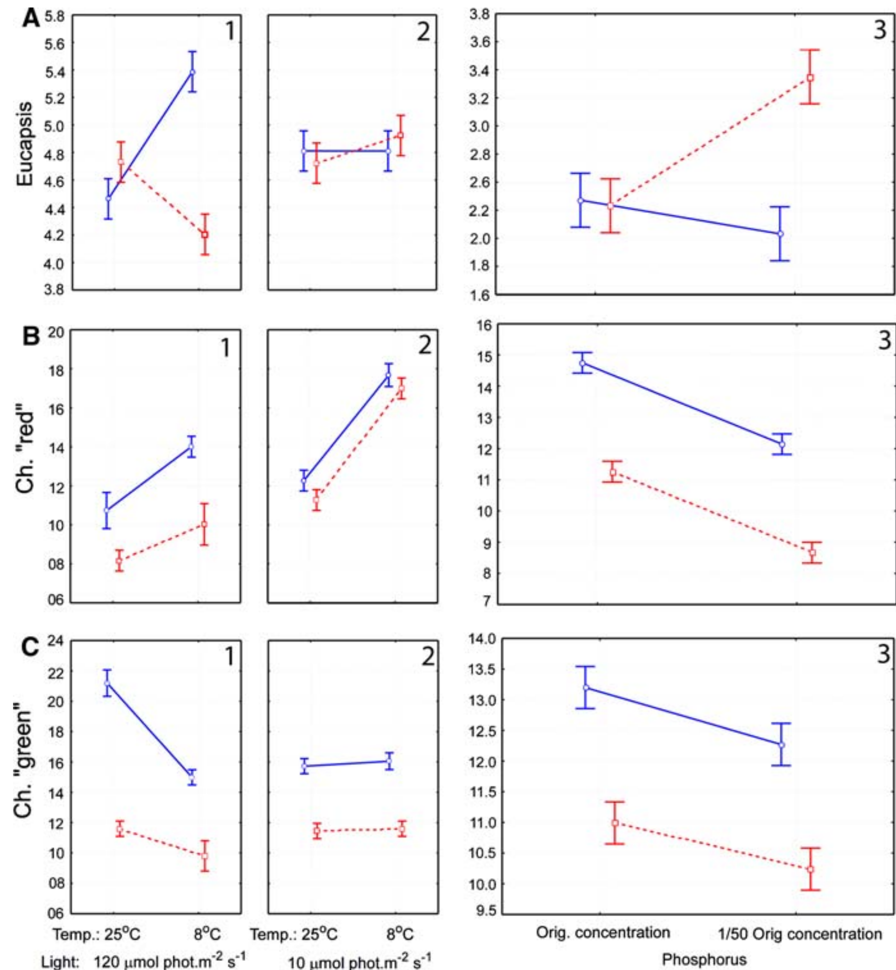


Fig. 1 ANOVA analysis of data—width of cells on *y*-axis. *Full line, empty circles*—BG11 medium. *Dashed line, squares*—WH medium. Central average, *vertical lines*—±std.

A1, 2—*Chroococcus* cf. *minor* (*Eucapsis*), CG experiment. 3—Shaker experiment.
B1, 2—*Chroococcus* sp. “red” (*C.* cf. *westii*), CG experiment. 3—Shaker experiment.
C1, 2—*Chroococcus* sp. “green” (*C. virescens*), CG experiment. 3—Shaker experiment

the WH medium (Figs. 1C1, 3e; 15–22 and 10–12 μm on average, respectively).

Cell shape was better in different concentrations of P when shaken, although the largest and slightly deformed cells were also produced in full BG11 medium. The best developed “chroococcal” cells were found in WH medium with 1/50 concentration of P–PO_4 (Fig. 3f).

4. *Chroococcus minutus* 055 Figs. 2A1–3, 3g, h.
5. *Chroococcus* sp. 057 (*Chroococcus minutus* (Kütz.) Nägeli): These two strains were similar in cell shape and in their reaction to various experimental conditions; they evidently represented the same species. Therefore, we combined the two sets of data and only one graph was constructed for both strains. CG cultivation in the LL–HT and LL–LT combinations in BG11 medium was the most successful (Fig. 2A1, 2). The highest biomass and most regular form of cells were reached under these conditions. Cells were embedded by their own sheath of amorphous mucilage (Fig. 3g). Under suboptimal conditions, the sheaths stacked together and formed blue-green flakes (Fig. 3h). Contrary to the other studied strains, the LL combinations were preferred.

Cell width depended on concentration of P–PO_4 when shaken. The differences between the BG11 and WH media were distinct, and the responses were not the same (Fig. 2A3). Low concentration of P evoked production of a thick layer of amorphous mucilage (Fig. 3h). No formation of packets, changes in cell color, or abnormities were registered in any experimental modifications. The width of cells for all experiments corresponded roughly to the size range of natural populations (7.5–10.5 μm on average).

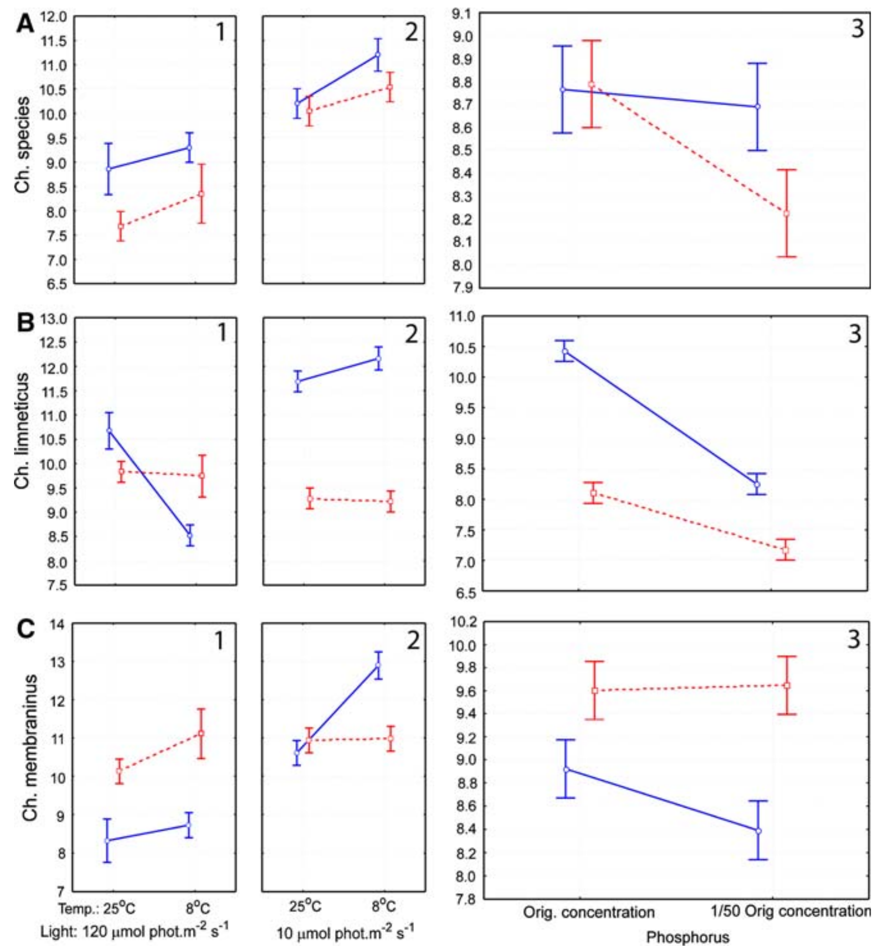


Fig. 2 ANOVA analysis of data—width of cells on *y*-axis. *Full line, empty circles*—BG11 medium. *Dashed line, squares*—WH medium. Central marks—average, *vertical lines*—±std.

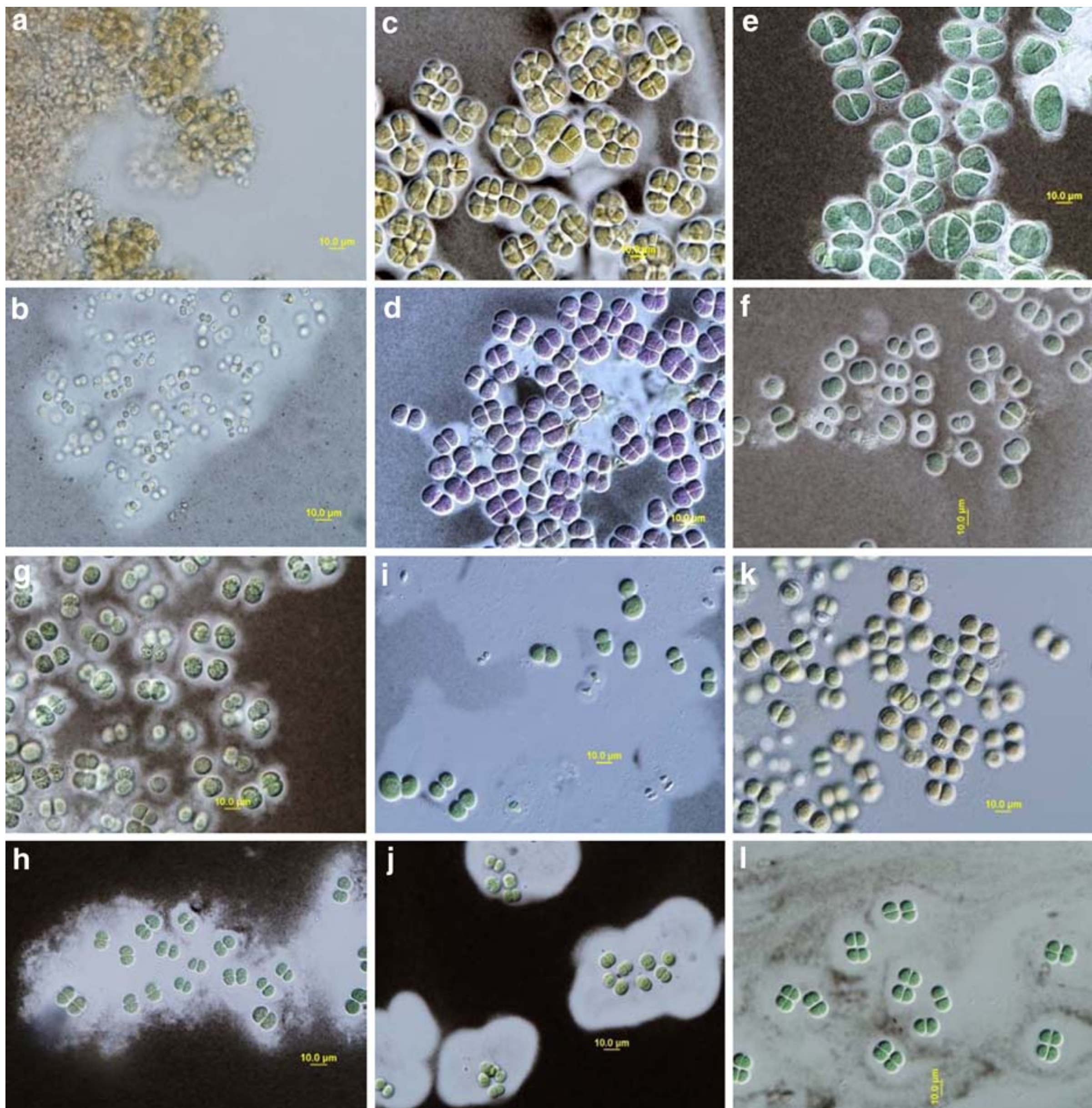
A1, 2—*Chroococcus minutus*, CG experiment. 3—Shaker experiment. **B**1, *Chroococcus limneticus* (*Limnococcus l.*), CG experiment. 3—Shaker experiment. **C**1, 2—*Chroococcus* cf. *membraninus*, CG experiment. 3—Shaker experiment

6. *Chroococcus limneticus* Lemmermann (Figs. 2B1–3, 3i, j): Experiments on the CG table showed the sensitivity of this strain to BG11 medium and batch culture cultivation in high radiation and temperature. The cells that lost vitality were dying and colonies were destroyed. Under HL + LT in BG11, the cells only grew and reached a large size, produced a lot of mucilage, but did not divide (Figs. 2B2, 3i). More sound development was found in WH medium in the HL + LT and LL + LT combinations.

The shaken cultures conformed better to the environmental demands of the plankton especially in WH medium (Fig. 3j). The strain grew well and the cells and colonies fit better to the description. Cell size decreased with P–PO_4 concentration (7.5–8.0 μm in average, compared with 10.5 μm in higher P–PO_4 concentrations), but did not stop dividing (Fig. 2B3). The amount of mucilage in colonies increased. Cells were always larger in BG11 than WH medium except for HL + LT, Fig. 2B1 (8.5–12.5 and 7.2–10.2 μm on average, respectively).

The strain behaved like a typical planktonic organism, preferring lower temperature, lower supply of nutrients, and shaken cultivation. The width of cells was rather uniform, around 7–11 μm, which corresponded well to the description of the species (Komárek & Anagnostidis, 1998).

7. *Chroococcus* cf. *membraninus* (Menegh.) Nägeli 054 Figs. 2C1–3, 3k, l: CG combinations demonstrated the large diversity in the morphology of populations. The largest biomass was found in BG11 in the LL + HT combination (Fig. 2C2), while slightly smaller biomass developed in the same



combination in WH medium. The HL + HT in BG11 combination produced large packets of smaller brownish cells (Fig. 2C1). The same combination in WH medium resulted in the best-shaped, regular cells also in brownish color (Fig. 3k). Lower intensity of light intensified the green color of the cells. Low temperature in both media stressed cell division, but without enough light the cells were minute and remained in small green packets.

The shaken cultures provided a set of very similar regular cells of intense blue-green color. Cell width remained almost the same in WH medium (Fig. 2C3), while low P–PO_4 concentration in BG11 resulted in smaller width of the cells (Fig. 2C3, 8.4 µm on average, compared with 8.9 µm in higher P–PO_4 concentrations). All of the cultures, except for WH with P1/50, were intensely blue-green without much difference in growth intensity. A mucilaginous envelope was produced in lower concentrations of P–PO_4. The mucilaginous layer was the thickest in WH medium with limiting concentration of P and the four-celled colonies were distant from each other

◀ **Fig. 3** Largest morphological differences registered during the experiments by photographs. **a** *Chroococcus* cf. *minor* (*Eucapsis*). CG experiment, BG11 medium, HL + HT combination. Packet-form colonies, brownish color. **b** *Chroococcus* cf. *minor* (*Eucapsis*) in shaker experiment, WH medium. Colonies in loose mucilage, planktonic form, blue-green color. **c** *Chroococcus* sp. "red" (*C.* cf. *westii*). CG experiment, WH medium, HL + HT combination. Packet form colonies, brownish color. **d** *Chroococcus* sp. "red" (*C.* cf. *westii*) in shaker experiment—in BG11 medium with 1/50 P–PO_4 concentration. Large colonies forming flakes, cells in typical form, intensively violet. **e** *Chroococcus* sp. "green" (*C. virescens*). CG experiment, BG11 medium, LL + HT combination. Polyhedric form of cells, large size, flake form of colonies. **f** *Chroococcus* sp. "green" (*C. virescens*). Shaker experiments, WH medium with 1/50 P–PO_4, concentration. Solitary cells embedded by narrow mucilage sheath, small but of typical shape. **g** *Chroococcus minutus* (055 and 057). CG experiment, full BG11 medium, LL + LT combination. Cells large and forming small colonies with a narrow layer of mucilage around the cells. **h** *Chroococcus minutus* (055 and 057). Shaker experiment, WH medium with 1/50 P–PO_4 concentration. Cells spread in small few-celled colonies in cloudy mass of mucilage. Cells small as the P concentration is limiting. **i** *Chroococcus limneticus* (*Limnococcus*). CG experiment, BG11 medium, HL + HT combination. Cells large but some in monstrous form, they do not divide, mucilage layer vast. **j** *Chroococcus limneticus* (*Limnococcus*). Shaker experiments, full WH medium. Colonies and cells small, but in typical planktonic form. **k** *Chroococcus* cf. *membraninus*. CG experiment, WH medium, HL + HT combination. Cells typical "chroococcoid" in form, but not forming packets. **l** *Chroococcus* cf. *membraninus*. Shaker experiment, BG11 medium with 1/50 P–PO_4 concentration. Cells in typical form, groups embedded by simple, wide mucilaginous envelope. Cells blue-green in color. WH medium, HL + HT combination. Cells of typical size and form, brownish color with a firm, narrow layer of mucilage

(Fig. 3l). Except for the slowly growing LL + LT combination in the CG experiment, the cells in all other cases were smaller in BG11 medium (8.4–10.5 μm on average, compared with 10.1–11 μm in WH), which did not correspond to the reactions of the other "chroococcus" strains studied.

Phylogenetic relationships

Ten strains were included in the phylogenetic analyses of 16S rRNA sequences, from which eight strains were newly sequenced within the framework of this study (Table 2). The neighbor-joining tree based on 16S rRNA sequences (1451 bp) is presented in Fig. 4, while the matrices of sequence similarities are demonstrated in Tables 5 and 6.

The clustering in Table 5 shows four groups of "*Chroococcus*" morphospecies situated between the *Microcystis* cluster and a cluster of heterocytous cyanobacteria. The detailed center of the tree (Figs. 4, 5; Table 6) demonstrates the affinity inside individual groups. The first two ones, group (Cluster, Cl) 1, *Eucapsis* (Fig. 5Cl1a–c) and Cluster 2 (Fig. 5Cl2a–c) are considerably different from Clusters 3 and 4.

There are two morphotypes in Cluster 1: strain *Chroococcus* sp. 019 HEGEWALD 1952 (Fig. 5Cl1a) and our strain *Chroococcus* sp. JJCM (AM710384) (Fig. 5Cl1b, c). The first strain contains small (4–4.5 × 2.5 μm), short ellipsoid cells in an irregular mucilaginous layer. The affiliation of the strain is not clear and needs more study. The sequence will not be submitted to GenBank for the present. The second strain of the first Cluster was isolated by KOMÁRKOVÁ in 2005 from plankton and originally determined as *Chroococcus* cf. *minor*. However, it forms packet-like colonies in very high nutrient concentrations and also under less-than-optimal conditions. According to phylogenetic position, this packet-form way of life is probably typical for the species and in no case could be classified within the genus *Chroococcus*. It should be classified among the species of the genus *Eucapsis*. Its sequence is already included in the GenBank as *Chroococcus* sp. JJCM (AM710384), but its name will be corrected to *Eucapsis* sp.

Cluster 2 includes the strain *Chroococcus minor* CEPÁK. The strain was determined as *Chroococcus* because of four-celled groups of spherical, very small cells in mucilaginous envelopes (Fig. 5Cl2a–c). However, its sequence position excludes the strain from the genus *Chroococcus*. The cluster contains sequences of well-determined morphospecies of *Snowella* and *Woronichinia* (Rajaniemi-Wacklin et al., 2005) relative to our strain. The sequence will be correctly named *Synechocystis* sp. CEPÁK prior to submission to GenBank.

Cluster 3 gathers all of our "large" *Chroococcus* morphospecies (Fig. 5Cl3a–f). The bootstrap values in Fig. 4 show a very close relation and it is evident that all of these strains belong to one genus, i.e., genus *Chroococcus* Nägeli in the sense of the Botanical Code.

The inconsistent Cluster 4 contains sequences of two isolates of *Chroococcus*: *C. limneticus* (Fig. 5-Cl4a, b) and *C.* cf. *membraninus* (Fig. 5Cl4c). The second one is related more to sequence 2SD05bisS08 of *Gloeocapsa* sp., a strain isolated from periphyton

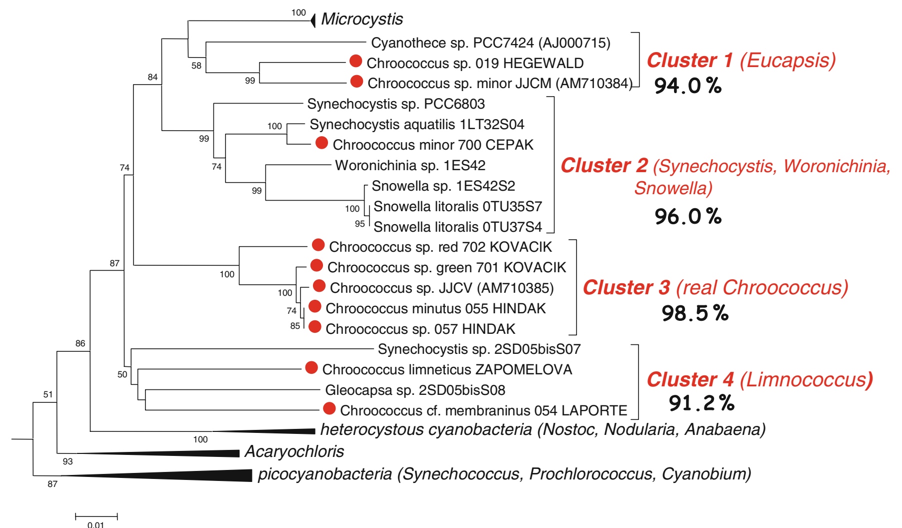


Fig. 4 Neighbor-joining (Jukes-Canton model, complete deletion) tree based on almost complete 16S rRNA gene sequence (1377 bp) showing the clustering of studied *Chroococcus* strains (group 1–4). Percentage of similarity inside of the Cluster is shown at each group

Table 5 Matrix showing similarities (%) between the clustered groups based on the 16S rRNA gene (1451 bp)

	Root	MIC	G1	G4	G2	HTC	G3	ACAR	PICO
Root—*E. coli*									
Microcystis	77.2								
Cluster 1	77.4	93.2							
Cluster 4	77.4	90.4	91.0						
Cluster 2	76.8	92.6	91.2	90.8					
Herocytous cyano	77.5	89.8	88.9	89.5	90.0				
Cluster 3	78.0	92.0	91.6	91.2	91.0	89.5			
Acaryochloris	78.2	90.2	89.5	90.0	89.6	90.0	91.5		
Picocyanobacteria	78.0	88.4	87.0	88.0	88.0	88.5	88.0	90.4	

All the positions containing alignment gaps and missing data were only eliminated in pairwise sequence comparison. All the groups (clusters) are distant from each other

in Svålbard by S. Ventura (S. Ventura, pers. com.). While the first isolate is a typical and frequent member of phytoplanktonic assemblages and was freshly isolated from plankton, the other strain came originally from the Pasteur Collection in Paris and had been isolated and determined by Laporte in 1965 from an unknown hot spring. Both have very different plasticity and reaction to changes of environmental parameters (see above), and more such types must be studied in the future. The strain *C.* cf. *membraninus* neither corresponds fully to the original description (*C. membraninus* (Meneghini) Naegeli 1849) nor could be designated to the genus *Chroococcus*. The species should form flat, leathery, violet to green to blackish colonies and the cells should be ± spherical, blue green, with an individual envelope, but smaller, 3–8 µm. In some combinations in experiment, reddish or brownish cells also appeared (Figs. 3k, 5Cl4c). However, our cultivation did not correspond to periphyton demands, and thus, the strain should be further studied to determine its species and generic designation. As concerns its generic affiliation, we decided for the name *Gloeocapsa* with a cf. abbreviation and to submit the sequence to the Genbank for future usage. All the information about the strains, sequences, and changes of names are listed in Table 2.

Using phylogenetic analysis, we showed that *Chroococcus limneticus* does not belong to the real *Chroococcus* genus and should be designated and described as a new genus. The difference is evident not only from the phylogenetic position of its 16S

Table 6 Matrix showing similarities (%) among the sequences of individual morphospecies based on the 16S rRNA gene (1451 bp, see Fig. 4)

		1	2	3	4	5	6	7	8	9	10
1	#*Chroo*_sp._019 HEGEWALD (genus not known)										
2	#*Chroo*_sp._JJCM (*Eucapsis*)	*95.4*									
3	#*Chroo_minor*_700 (*Synechocystis*)	90.5	90.9								
4	#*Chroo*_sp._red_702 (*Chroococccus* cf. *westii*)	92.2	91.4	90.5							
5	#*Chroo*_sp._green_701 (*Chroococccus virescens*)	91.8	91.5	90.5	*96.4*						
6	#*Chroo*_sp._JJCV- (*Chroococccus prescottii*)	91.7	91.2	90.5	*96.4*	*99.2*					
7	#*Chroo_minutus*_055 (*Chroococccus minutus*)	91.9	91.4	90.5	*96.5*	*99.4*	*99.7*				
8	#*Chroo*_sp._057 (*Chroococccus minutus*)	91.9	91.4	90.7	*96.4*	*99.4*	*99.7*	*99.9*			
9	#*Chroo_limnet*_ZAPOMSvet06 (*Limnococcus limneticus*)	91.2	91.1	90.7	91.6	92.0	91.8	91.8	91.8		
10	#*Chroo_membraninus*_54 (cf. *Gloeocapsa*)	91.1	89.4	89.2	90.8	91.8	91.7	91.9	91.9	91.6	

All the positions containing alignment gaps and missing data were only eliminated in the pairwise sequence comparison. Numbers higher than 95% are italicized

rRNA gene sequence, but also from its distinct morphology and ecology. The sequence is to be named "*Limnococcus*".

Taxonomic and nomenclatoric solution

The name of the taxon *Limnococcus* is transferred from the status of subgenus *Limnococcus* (Komárek & Anagnostidis 1999) to the rank of genus.

***Limnococcus* (Komárek & Anagnostidis) Komárková et al. genus novus**

Basionym: subg. *Limnococcus* Komárek et Anagnostidis 1999, Süsswasserflora von Mitteleuropa 19/1, p. 281).

Description: Cells irregularly deposited in wide, homogenous, amorphous, colorless, and diffluent mucilage, distant one from another in microscopic colonies usually of a small number of cells (2–40). Cells spherical, after division subspherical, up to 22 μm in diameter, usually with gray-green, blue-green, or olive-green content. Cell division by binary fission, in 2–3 planes in successive generations. The cells reach the original spherical shape before the next division. Reproduction proceeds by disintegration of colonies or liberation of cells from colonies.

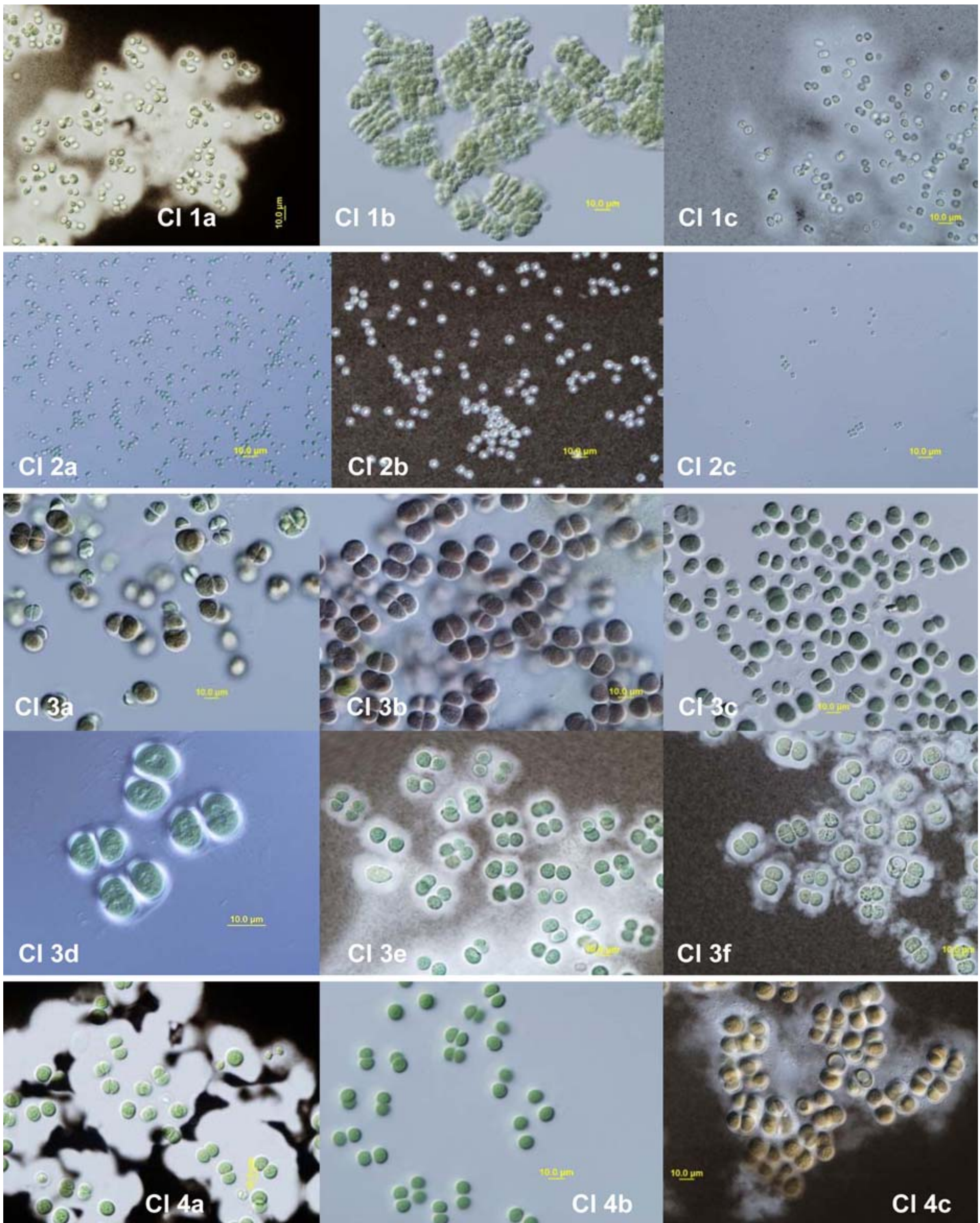
Latin description: *Genus cyanocaryoticum, cum cellulis in coloniis parvis consociates, non filamentosum. Coloniae mucilaginosae, paecipue microscopicae, natantes, lobatae vel abbitu irregulari, (1)2-ad 40 cellullares, cum cellulis praecipae distantis, irregualriter ordinatis, vel 2-4-8 indistincte aggregatae. Mucus coloniae tenuis, sine colore, homogeneus, interdum ad marginnem diffluens. Cellulae sphaericae, post divisionem hemisphaericae, aeruginosae, griseo-virides, olivaceo-virides vel lutescentes, ad 22 μm in diametro. Divisio cellularum in planos duas vel tres in generationes subsequentibus, cellulae filiales in forma sphaerica ante divisionem sequentem crescent*.

Typus generis: Limnococcus limneticus (*Lemmermann*) *comb. nova* (basionym: Chroococcus limneticus *Lemmermann*, *Bot. Zentralbl. 76: 153, 1898.*

Type strain: *Limnococcus limneticus* ZAPOMĚLOVÁ 2006/4, CCALA No. 890.

The incident sequence of the type species *Limnococcus limneticus* comb. nov. was sent to GenBank and was given the accession number GQ375048. The Type strain is cultivated in CCALA culture collection of the Botanical Institute AS CR at Třeboň, Czech Republic (www.cas.ccala.cz) and the Cyano-collection of the Institute of Hydrobiology AS CR, České Budějovice, Czech Republic. Preserved sample is maintained in Moravian Museum in Brno (BRNMU), Czech Republic.

Reference strains for other relevant sequences are kept at the same collections. Their nucleotide sequences were deposited in the GenBank (Table 2).


Cl 1a
Cl 1b
Cl 1c
Cl 2a
Cl 2b
Cl 2c
Cl 3a
Cl 3b
Cl 3c
Cl 3d
10.0 µm
Cl 3e
Cl 3f
Cl 4a
Cl 4b
Cl 4c

◀ **Fig. 5** Images of sequenced strains built in the phylogenetic tree (Fig. 4). Individual clusters are divided by wider spaces. **Cl1a** Strain *Chroococcus* sp. 019 HEGEWALD 1952, No. 019 = unknown species. **Cl1b**, **c** Strain *Chroococcus* sp. JJCM (AM710384)/*C.* cf. *minor*, KOMÁRKOVÁ 2005/1 = *Eucapsis* sp. **Cl2a**, **b**, **c** Strain *Chroococcus minor* 700 CEPÁK 1993 = *Synechocystis* sp. **Cl3a**, **b** Strain *Chroococcus* sp. "red" 702 KOVÁČIK 1982/11b = *C.* cf. *westii*. **Cl3c** Strain *Chroococcus* sp. "green" 701 KOVÁČIK 1982/11a = *C. virescens*. **Cl3d** Strain *Chroococcus* sp. JJCV KOMÁRKOVÁ 2003 = *C. prescottii.* **Cl3e** Strain *Chroococcus minutus* 055 HINDÁK 1965 = *C. minutus.* **Cl3f** Strain *Chroococcus* sp. 057 HINDÁK 1971 = *C. minutus.* **Cl4a**, **b** Strain *Chroococcus limneticus* Svet06 ZAPOMĚLOVÁ 2006/4 = *Limnococcus limneticus.* **Cl4c** Strain *Chroococcus* cf. *membraninus* 054 LAPORTE 1965 = cf. *Gloeocapsa*

Discussion

Morphological studies of cultivated cyanobacterial strains became frequent during the last few years, but already in 1969 the morphology of *Synechococcus* (*Anacystis nidulans*) strains were studied under different light and temperature conditions (Komárek, 1969). Further studies (Weisse, 1993; Palinska et al., 1996; Jezberová and Komárková, 2007) confirmed the dependence of cell shape and growth parameters on nutrient supply, and light and temperature intensity. Formation of so-called involution forms in HL + HT and high nutrient concentrations, and an unequal division of cells, distinguished two genera of picoplanktic cyanobacteria, i.e., *Synechococcus* and *Cyanobium*, in agreement with their phylogenetic positions. Morphological diversity among the strains of *Microcystis*, studied using CG cultivation, was not confirmed by genetic analysis (Komárková et al., 2005). Morphology of heterocytous cyanobacteria was intensively studied using a polyphasic approach (Li et al., 2000; Gugger et al., 2002; Zapomělová et al., 2008a, b, 2009) also under combinations of light and temperature and different supply of nutrients. Such studies concerning species of the genus *Chroococcus* have not been performed yet. The only study (Richert et al., 2006) concerning a 16S rRNA sequence and a polyphasic approach describes the occurrence of *Chroococcus* (*C. submarinus*) in microbial mats on the Tuamotu Archipelago, French Polynesia. Unfortunately, the sequence was not available in GenBank.

Many authors suppose a weak stability of morphological and physiological features resulting from long-term stable cultivation conditions (Anand, 1988; Tandeau de Marsac & Houmard, 1993). Rudi et al. (1998) even suspected changes in the genome due to the impact of mutagenic factors on the population present in a natural environment. These factors can be even more active during cultivation. However, morphological characteristics will probably remain the main tool for taxonomic identification of species of blue greens in practice. Thus, it is still questionable whether the study of variability of a strain under artificial conditions can help in identification of the taxon. The variability range studied in 61 populations from natural samples and eight strains of *Anabaena* species provided a positive response (Zapomělová et al., 2009, in press). Such data can be compared with data measured in natural populations, which develop in a lake, pool, or a fishpond.

Our morphological study of *Chroococcus* strains, in which also DNA isolation and 16S rRNA gene sequencing were performed, provided a first approach to widen the knowledge about Reference and Type material concerning several *Chroococcus* and one *Eucapsis* morphospecies and their phylogenetical classification. Our results showed a wide variability of measured cell size, which depended on variables influencing the growth of the studied strains. In the case where sizes and shapes overlapped in most combinations of experimental conditions, genetically neighboring strains showed the same responses (*Chroococcus minutus* 055/sp. cf. *minutus* 057). Also, morphology of individual groups of the studied strains corresponded with the phylogenetical position of the strains in the tree. The small cells of the minute *Chroococcus* "species" from Cluster 1 do not belong to the genus, and probably, many minute planktonic *Chroococcus* species will have to be reclassified with other genera after 16S rRNA sequence control.

Cluster 4 (Fig. 5Cl4a, b) is represented by the evidently planktonic form of *Chroococcus limneticus*. Its distant position from the true *Chroococcus* phylogenetic cluster was guessed already by Geitler (1932, p. 222) and the name *Limnococcus* for the subgenus of phytoplanktonic *Chroococcus* species was submitted already by Komárek & Anagnostidis (1998). Even if the process should be supported by a few more sequences, the installation of the genus *Limnococcus* is evident. However, inside of Cluster 3 there also exist planktonic *Chroococcus* species, such as *C. minutus* (Pollingher, 1991, Lake Kinneret) and

C. prescottii, isolated from phytoplankton of Římov reservoir in the Czech Republic.

Conclusions

Four clusters of 10 strains designed originally as species of the genus *Chroococcus* were distinguished in the Neighbor-Joining phylogeny tree based on 16S rRNA gene sequences. Cell morphology and the formation of colonies studied in experiments verified the existence of these four clusters. Clusters 1 and 2 did not correspond to the *Chroococcus* phylogenetic position. They included small cell-sized species with different structure of colonies. Cluster 3 included typical morphospecies of *Chroococcus: C. virescens*, *C.* cf. *westii*, *C. prescottii*, and two strains of *C. minutus*. The last Cluster 4 verified a distant generic position of the genus *Limnococcus*, which could not be classified to the genus *Chroococcus* and was established as an independent genus. Generic position of the strain *Chroococcus* cf. *membraninus* No. 054 from the CCALA collection remained unsolved. The strain and its sequence were provisionally named *Gloeocapsa* with a question mark.

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