Ecological characteristics and polyphasic taxonomic classification of stable pigment-types of the genus *Chroococcus* (*Cyanobacteria*)

Ekologická charakteristika a polyfázická taxonomická klasifikace trvalých pigmentových modifikací z rodu *Chroococcus* (*Cyanobacteria*)

Ľubomír K o v á č i k[1], Jitka J e z b e r o v á[2], Jaroslava K o m á r k o v á[2,3], Jiří K o p e c k ý[4] & Jiří K o m á r e k [3,5]

[1]*Comenius University in Bratislava, Faculty of Natural Sciences, Department of Botany, Révová 39, SK-811 02 Bratislava, Slovakia, e-mail: kovacik@fns.uniba.sk;* [2]*Biological Centre, Academy of Sciences of the Czech Republic, Institute of Hydrobiology, Na Sádkách 7, CZ-370 05 České Budějovice, Czech Republic, e-mail: j.jezberova@email.cz, jarkakom@seznam.cz;* [3]*Institute of Botany Academy of Sciences of the Czech Republic, Dukelská 135, CZ-379 82 Třeboň, Czech Republic,e-mail: komarek@butbn.cas.cz;* [4]*Institute of Microbiology, Academy of Sciences of the Czech Republic, Opatovický mlýn, CZ-379 81 Třeboň, Czech Republic, e-mail: kopecky@alga.cz;* [5]*University of South Bohemia, Faculty of Biological Science, Branišovská 31, CZ-370 05 České Budějovice, Czech Republic*

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Two differently coloured strains of the genus *Chroococcus* were isolated from a cyanobacterial assemblage collected from the stony littoral of a backwater of the Danube River in southern Slovakia. When grown after isolation, both subcultures were similar morphologically and their growth parameters did not differ substantially, but their pigment content (PC: PE and carotenoid ratios), details in their morphology during their life cycles and slime production were different. Identical and different characters of both morphotypes remained stable during cultivation on both agarized and liquid media, even when the cultivation parameters were changed. Both of the subcultures were studied using electron microscopy and almost their complete 16S rRNA genes were sequenced, which showed that in terms of their genetic relationship there was a 96.4% sequence similarity and certain taxonomic interspecific differences between both subcultures were confirmed. The various chromatic modifications recorded in cyanobacteria and their ecological consequences are discussed. The results yielded further data on the changes that occur during the cyanobacterial differentiation processes and their genetic stabilization.

K e y w o r d s: chromatic adaptation, *Chroococcus,* cyanobacteria, ecology, phylogeny, pigment content, pigment mutants, Slovakia, taxonomy, ultrastructure

Introduction

Diversification strategies of cyanobacteria and criteria for characterizing the stabilized evolution steps are still poorly known. Horizontal exchange of genes, mutation pressure, and biochemical and genotypic adaptations play an important role in the formation of various phylogenetic modifications in natural habitats and numerous variations can occur in these processes. Using the polyphasic approach to classify cyanobacterial diversity it is possible to select more or less satisfactory common criteria for the identification of basic cyanobacterial phylogenetic (taxonomic) units associated with the clusters obtained using

16S rRNA gene sequencing (cf. Stackebrandt & Goebel 1995) and other molecular, biochemical, ecophysiological and morphological markers (autapomorphic characters). The basic taxonomic units, derived and delimited by this approach, correspond more or less to the traditional cyanobacterial category "genus" and this is the case for all the generic entities revised using modern methods. However, resolving the diversity within these generic clusters supported by molecular analyses is much more complicated, and establishing even approximate criteria for the delimitation of subgeneric categories (species) is difficult. Diversification within cyanobacterial genera is variable and its evaluation in various genera will require different criteria.

Both traditional and modern taxonomic criteria indicate that the genus *Chroococcus* is heterogeneous. Although considered to be one of the most common and basic unicellular types it is studied experimentally only sporadically. It does not belong to the simple cyanobacteria and its structure and life cycle is rather complicated (Castenholz 2001, Hoffmann et al. 2005, Komárek 2006). There are only a few strains that have been studied completely and whose phylogenetic position is known. Typical *Chroococcus* belongs to the line of more complicated unicellular cyanobacteria in which division of cells occurs in more than one plane in subsequent generations (Golubić 1967) and there is an irregular thylakoidal system (Potts et al. 1983, Hoffmann et al. 2005). Recently, Komárková et al. (2010) defined, on the basis of 16S rRNA gene sequences, the typical *Chroococcus* cluster, which contains only the benthic (metaphytic, terrestrial) large morphospecies with the type species *C. rufescens.* This study excluded morphotypes from this genus that have different phylogenetic positions and morphological and ecological characters (the planktic genus *Limnococcus,* several small types belonging to the genera *Synechocystis* or *Eucapsis*, etc.). However, the intrageneric diversity in the revised genus *Chroococcus* needs further study.

Two morphologically similar strains of typical *Chroococcus* were isolated from one mat in which there were differently pigmented cells of one morphological type (Fig. 1). Differences in the pigmentation of phenotypically similar populations are an interesting taxonomic and ecological problem in cyanobacteria and are usually explained in terms of chromatic adaptation. However, there exist also stable, differently coloured types, the taxonomic classification of which still needs to be resolved. The isolated strains do not differ substantially in their size, morphology of the cells and ecophysiological conditions, but do differ in their PE/PC pigment ratio (which remained stable in cultures) and structure of colonies. These genetic, ecophysiological and morphological differences were studied in detail and used to resolve the taxonomy of respective *Chroococcus*-taxa. Some aspects of the diversification and speciation processes within cyanobacteria at the subgeneric level are also demonstrated. The aim of this study was to determine the type and stability of the pigments in the cells, the taxonomic status of both strains and discuss their ecological position.

Material and methods

Locality, isolation and cultivation of strains

The material for this study was collected as an epilithic biofilm from gravel shore of a remnant of an oxbow lake Veľký Les, on the left-bank of a river arm system of the Danube river, located between the dike and channel of the Gabčíkovo Hydroelectric Power Plant in

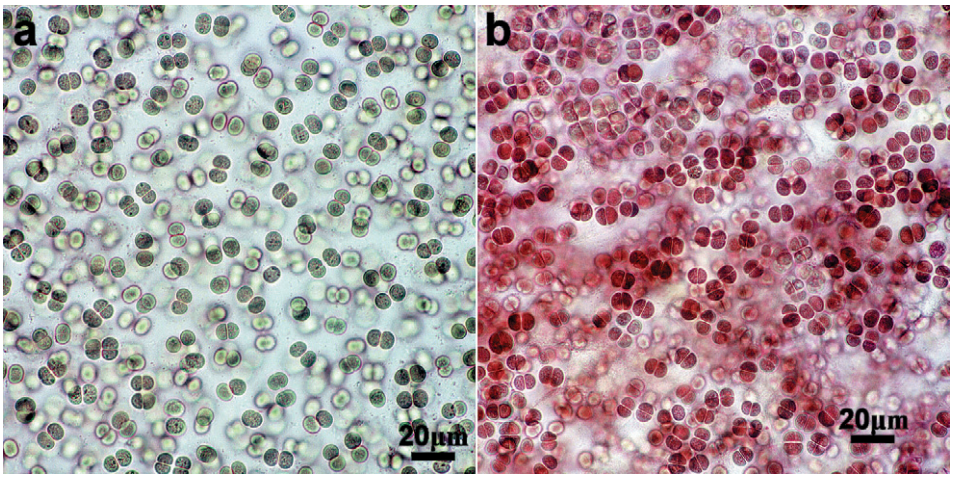


Fig. 1. – The *Chroococcus* strains CCALA 701 (a) and CCALA 702 (b) soon after isolation from a natural cyanobacterial community.

South Slovakia (rkm 1819 of the Danube, 47°51'33" N, 17°32'46"E). The material scraped from the surface of stones in the summer of 1982 was aseptically spread over the surface of Petri dishes containing 2% agarized BG-11 medium (Rippka et al. 1979) from which the two clonal colonies, distinguished by their colour, were isolated as uni-algal strains. Mediums BG-11 and WH (Guillard & Lorenzen 1972) (rather than "rich" BG-11 medium) were used for further cultivation. The WH medium contains a 7× lower concentration of PO_4 than BG-11, and contains a mixture of important vitamins that corresponds better to natural conditions.

Based on visual morphology and colour, the strains were identified as *Chroococcus* species, designated as KOVACIK 1982/11a ("the green strain" CCALA 701) and KOVACIK 1982/11b ("the red strain" CCALA 702) and deposited in the Algal Culture Collection of the Czech Academy of Sciences at Třeboň (www.butbn.cas.cz/ccala/index.php). Both strains are maintained either in liquid or agarized BG-11 medium at 15 °C under a 12:12 light-dark cycle with a photon flux density of 23 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by daylight fluorescent lamps and were monitored at regular intervals from the date of isolation.

Morphology of the cells (morphological limits) of field collected material, in unialgal cultures and in strains cultivated in cross-gradients of temperature and irradiation was examined. Morphology was examined using an Olympus BX51 light microscope equipped with Nomarski optics. Microphotographs were taken with an ARTCAM 300MI 3 Mpxl CMOS USB 2.0 Camera equipped with Quick PHOTO MICRO 2.1 software and mounted in figures using Adobe Photoshop 5.0. The limit values of cell length and width were recorded in all the experiments and at different stages during the life cycle, in order to evaluate the diameter of irregular cells, which divide in three or more planes in succeeding generations.

Growth parameters were recorded at a range of temperatures (9–34 °C) and irradiances (9–368 $\mu mol \cdot m^{-2} \cdot s^{-1}$) on agar plates in Petri dishes (Fig. 2) using the method of Albertano et al. (1993) and Albertano & Kováčik (1996). Initial inoculation was made from a homogeneous suspension of *Chroococcus* cells. Growth was evaluated by measuring dry weight (mg/Petri dish) after 14 days of continual cultivation under each set of conditions.

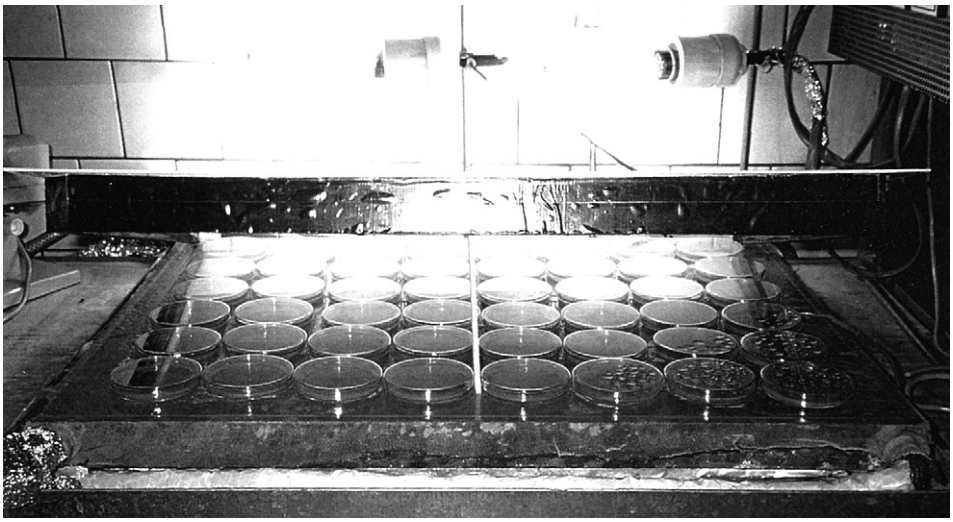


Fig. 2. – Cultivation apparatus for studying growth in an agarized medium at cross gradients of temperatures [°C] (horizontal axis) and irradiance intensities [$\mu mol \cdot m^{-2} \cdot s^{-1}$]. For more details see text.

Electron microscopy

For transmission electron microscopy, the cells were fixed overnight at 4 °C in 2.5% glutaraldehyde in a 0.2M phosphate buffer, pH 7.0. After washing in the same buffer, the cells were post-fixed with 2% osmium tetroxide for 4 hours at 4 °C. After dehydration in an acetone series, the cells were embedded in Spurr's resin (Spurr 1969). Thin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and investigated using a transmission electron microscope, Jeol JEM 1010 at 80KV.

Ecophysiology, pigment content

The pigments of the strains maintained in a stirred liquid culture illuminated by a metal-halide lamp TUNGSRAM HgMID (Daylight), 400 W, were analyzed. The cell suspension (5.0 or 10.0 ml) was centrifuged and the cell pellets disintegrated in 0.5 ml phosphate buffer (pH = 7.0) with 0.5 ml glass beads (diameter 0.3 mm) using a Vortex. Light microscope observations demonstrated that the procedure (5 minutes) disrupted the cells quantitatively. The spectra of disrupted cells re-suspended in phosphate buffer were recorded from 300 to 800 nm using the opal glass method described by Shibata (1958).

Chlorophyll *a* and total carotenoids were quantitatively extracted with aqueous acetone (90 % v/v) in dim light at room temperature. The samples were centrifuged and chlorophyll *a* and carotenoids present in the supernatants determined using the spectrophotometric method of Jeffrey & Humphrey (1975). Jeffrey & Humphrey's equations were used to estimate chlorophyll *a*. The concentrations of total carotenoids were calculated using the equation of Lichtenthaler & Wellburn (1983).

To estimate the phycobiliprotein contents, a slightly modified version of the procedure described by Bennett & Bogorad (1973) was used. Disrupted cells were re-suspended in phosphate buffer and aliquot volumes of suspension were centrifuged at +4 °C and

80.000 g for 1 hour. The absorption spectra of supernatants ("crude extracts") were recorded at light pass = 1.0 cm from 450 to 700 nm. The optical densities of crude extracts at 562, 615 and 652 nm and the following equations were used for calculating phycobiliprotein concentrations (PC = C-phycocyanin, PE = C-phycoerythrin, APC = allophycocyanin):

$$C_{PC} = \frac{A_{615} - 0.474 \cdot A_{652}}{5.34} \quad (mg \cdot l^{-1})$$

$$C_{APC} = \frac{A_{652} - 0.208 \cdot A_{615}}{5.09} \quad (mg \cdot l^{-1})$$

$$C_{PE} = \frac{A_{562} - 2.41 \cdot (C_{PC})_{562} - 0.849 \cdot (C_{APC})}{9.62} \quad (mg \cdot l^{-1})$$

Extraction of pigments and high performance liquid chromatography (HPLC) analyses were carried out according to Kopecký et al. (2000). Briefly, the algal cells were centrifuged and extracted twice at room temperature using 100% acetone. The extracts were clarified using 0.2 μm nylon filters (Micro-spin centrifuge filter, Alltech, Deerfield, IL, USA) before pigment analysis on a Beckman 114 series liquid chromatograph (Beckman, USA) with Waters 991 diode array detector (Waters, USA). Pigments were separated on a 5 μm particle Ultrasphere ODS-1 non-endcapped RP18 column (250 × 4.6 mm), with a flow rate of 2 ml·min^{-1} according to Gilmore & Yamamoto (1991). Chromatography started with a 10 min isocratic elution using pure solvent A (Acetonitrile : Methanol : H_2O : 0.1 M Tris[hydroxymethyl]aminomethane, pH 8.0 [72 : 28 : 6 : 3]), followed by a 4 min linear gradient from 100% solvent A to 100 % solvent B (Methanol : n-Hexane [9 : 2]). After another 8 min of isocratic elution, starting conditions were restored during a 2 min gradient followed by column equilibration for 4 min. HPLC grade solvents (Merck) were used for mobile phase preparation. Identification of individual carotenoids and chlorophyll *a* was confirmed by comparison with the spectral characteristics and retention behaviour of photosynthetic pigments in a reverse phase system.

Phylogenetic analyses

DNA was extracted from cells collected during the exponential growth phase using the classic phenol-chloroform method as described by Turicchia et al. (2005). Simultaneously with the DNA isolation, subsamples were photographed, preserved in formaldehyde (2% final concentration) and prepared for later morphological analyses. DNA amplification was performed using PCR (5min / 94 °C; 10 cycles of 45 s / 94 °C, 45s / 57 °C, 2min / 72 °C; 25 cycles of 45s / 94 °C, 45s / 54 °C; 2min / 72 °C; 7min / 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 Ltd). Alignment and phylogenetic calculations (sequence similarity, phylogenetic trees) were performed in MEGA4 (Tamura et al. 2007).

Phylogenetic trees were built using the Neighbour Joining method (Saitou & Nei 1987) and a bootstrap value of 1000. Nucleotide sequences were deposited at GenBank under the accession numbers GQ375046 (CCALA 701 – "green") and GQ375044 (CCALA 702 – "red"); (Komárková et al. 2010).

Taxonomic evaluation

The criteria of modern cyanobacterial taxonomy based on the molecular background and supplemented by complex of morphological and ecophysiological markers, were used for the taxonomic evaluation of the strains. Because the results indicated the strains were different species, they were compared with several type specimens obtained from herbaria, which were similar morphologically. The morphology was compared with descriptions in the literature, if available. Several type specimens from the National Herbarium Netherland in Leiden (L) and Naturhistorisches Museum in Wien, Austria (W) were examined.

Results

Morphology, phenotypic characters and variability

Both strains, CCALA 701 "green" and CCALA 702 "red", were similar in size when grown on agarized BG-11 medium and reared under the same temperature and light conditions. The colonies were formed of single, double or groups of cells embedded in a common hyaline mucilaginous matrix. Cells were at first subspherical or spherical, later slightly hemispherical, or in the form of a segment of a sphere and did not attain a spherical form before the next division (= typical diacritical feature of the genus *Chroococcus*; cf. Golubić 1967, Komárek & Anagnostidis 1998). The contents of the cells in fresh cultures were homogeneous, while in old cultures they were granular. In the "red strain" CCALA 702 there was a high frequency of typical "*Chroococcus*-packets" with colourless mucilaginous envelopes, which copy from outside the shape of cells, while in the "green strain" CCALA 701 the mucilaginous envelopes were rather diffluent and indistinct. Fresh cultures of young daughter cells had a blue-green colour in both strains. However, differences in colour between strains developed as the cultures matured. Cells of the "green strain" were dark blue-green to granulate dark green, while those of the "red strain" were brownish or reddish to dark red, but never deep red, independent of culture conditions. The cultivation of both two strains in batch culture resulted in different changes in various concentrations of BG-11 medium nutrients: the "green strain" produced mainly dead cells and cells with many anomalies, the "red strain" produced a large biomass of cells quickly when grown in the same nutrient medium and the cells were less deformed. In old cultures the morphology of cells did not change (Fig. 3) but the colour of the cells changed in the green strain CCALA 701 to dirty green and in the red strain CCALA 702 from reddish to olive-green to brownish.

Pigment composition

The differences in colour recorded in the two strains are reflected in the pigment analyses (Figs 4, 5, Table 1). Pigment composition (average values for all experiments) and concentration of photosynthetic pigments and total carotenoids under continual cultivation are

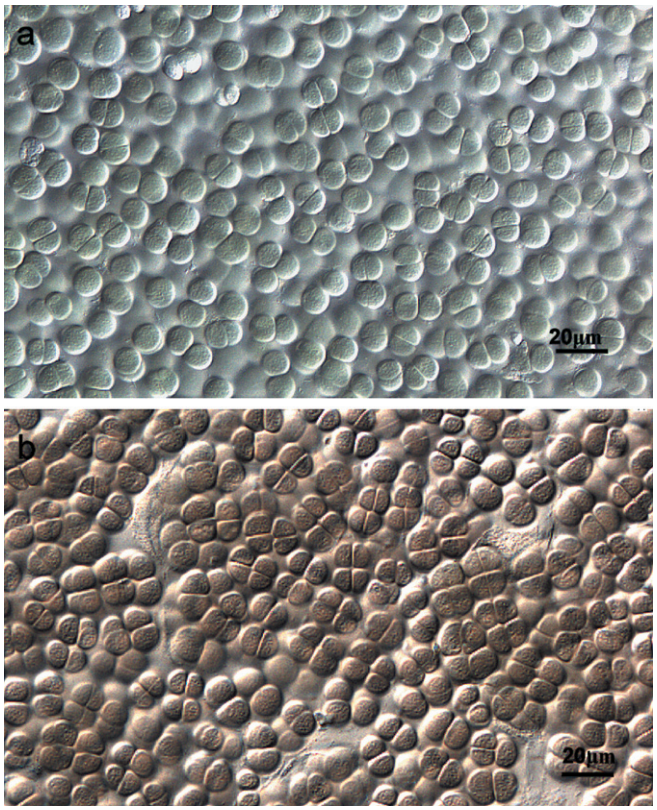


Fig. 3. – Cells of both strains, green CCALA 701 (a) and red CCALA 702 (b) from the batch culture after long-term cultivation under optimum conditions, which were determined using the results of the study using cross gradients of temperature and light intensity (cf. Table 3).

presented in Table 1. There was a 3-times higher ratio of chlorophyll *a* to total carotenoids and more than 4-times higher ratio of phycocyanin to phycoerythrin in the green strain CCALA 701 than in the red strain CCALA 702. The composition of carotenoids and their relation to chlorophyll *a* is documented in Figs 5, 6 and Table 2. The differences are particularly striking in the content of myxoxanthophyll, zeaxanthin and astaxanthin-ester. The pigment differences are stable in both strains and independent of rearing conditions and variable light intensities. Identical results were obtained for cultures kept in optimal and suboptimal conditions.

Table 1. – Average pigment composition and concentration ($mg \cdot l^{-1}$) of cell biomass produced under optimal culture conditions, which are derived from the results of the cultivation in cross gradients.

	strain CCALA 701 (green)	strain CCALA 702 (red)
Chlorophyll *a* (C_a)	17.70	7.72
Total carotenoids (TC)	1.99	2.56
C_a/TC	9.00	3.05
C-phycocyanin (C_{PC})	0.058	0.013
C-phycoerythrin (C_{PE})	0.033	0.249
C_{PC}/C_{PE}	1.81	0.441

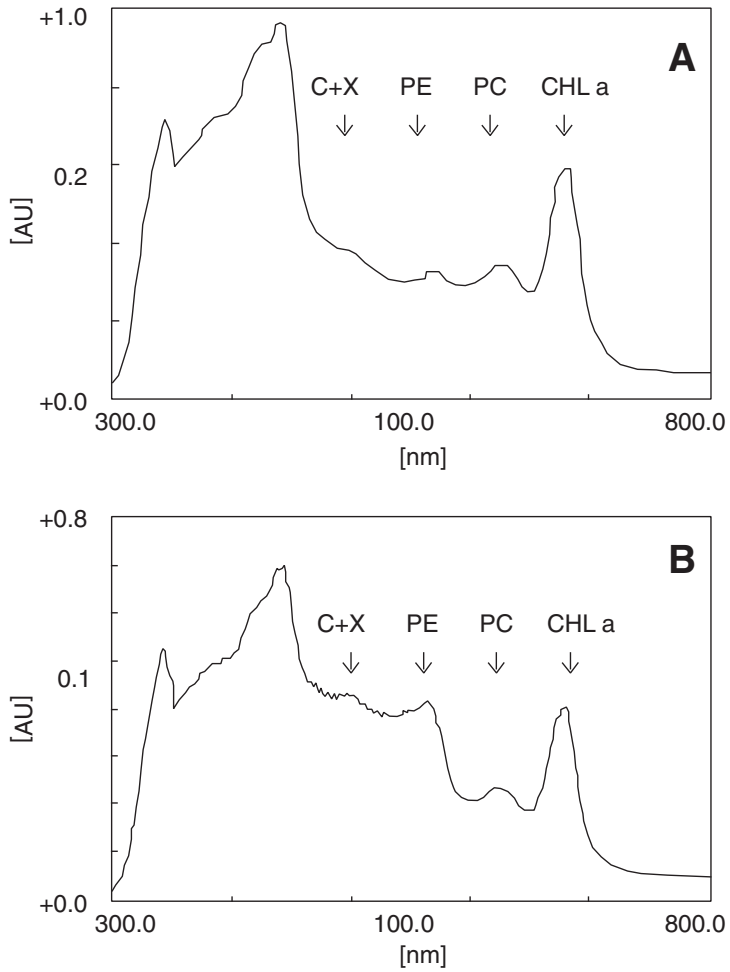


Fig. 4. – Absorption spectra of suspensions of disintegrated cells of the green strain CCALA 701 (A) and red strain CCALA 702 (B), recorded using a Shimadzu 160A spectrophotometer and the opal glass method of Shibata (1958); C+X = total carotenoids, PE = phycoerythrin, PC = phycocyanin, ChLa = chlorophyll *a*. Spectra were measured at the exponential phase of growth under optimal conditions.

Table 2. – Composition of carotenoids and chlorophyll *a* in both the *Chroococcus* strains studied, which were recorded from the exponential phase of their growth in liquid culture kept under optimum culture conditions.

	Compound	*Chroococcus* CCALA 701 (green)		Chroococcus CCALA 702 (red)	
		Peak area	%	Peak area	%
1	Myxoxanthophyll	0.001753	1.861942	0.002517	8.558023
2	Zeaxanthin	0.004263	4.527929	0.002291	7.789603
3	Chlorophyll *a*	0.065331	69.39107	0.009756	33.17126
4	Astaxanthin-ester	0	0	0.007031	23.90602
5	β-carotene	0.020565	21.84304	0.007305	24.83765
6	Cis-β-carotene	0.002237	2.376021	0.000511	1.737445

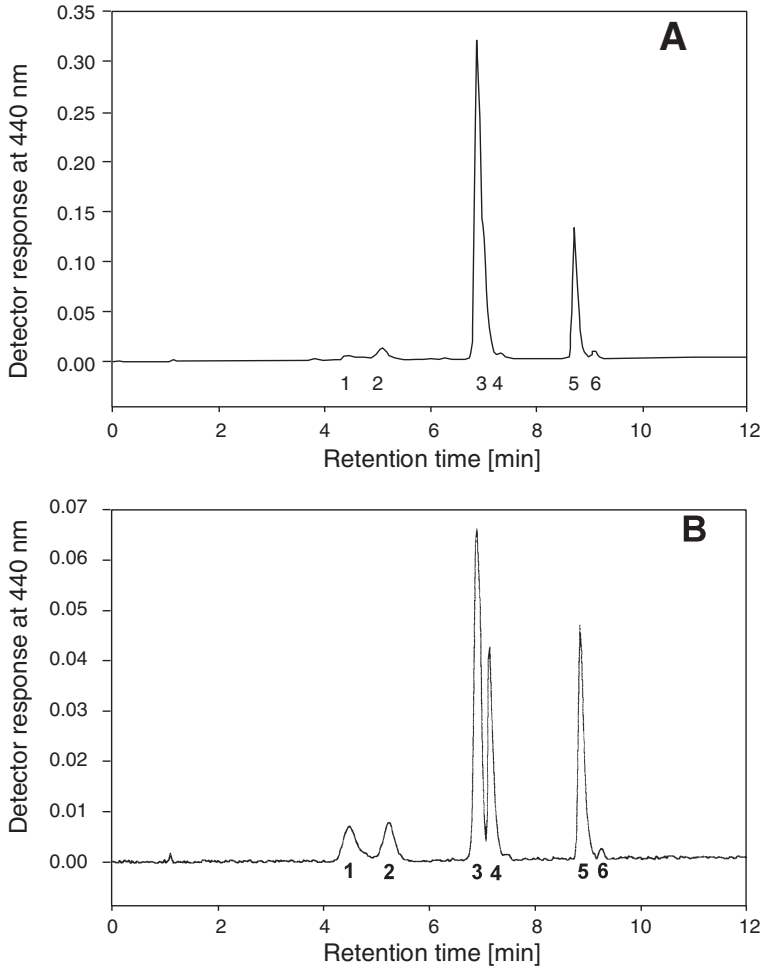


Fig. 5. – The composition of total carotenoids and chlorophyll *a* in both the CCALA 701 (green, A) and CCALA 702 (red, B) *Chroococcus* strains recorded at the exponential phase of the growth of the culture; 1 = myxoxanthophyll, 2 = zeaxanthin, 3 = chlorophyll *a*, 4 = astaxanthin-ester, 5 = ß-carotene, 6 = cis-ß-carotene.

Ultrastructure

The ultra-structure of both strains (CCALA 701 and CCALA 702) is more or less identical. It corresponds to the commonly known structure of typical *Chroococcus* (cf. Potts et al. 1983), which is probably more or less uniform in the whole genus (Figs 7, 8). Fasciculated thylakoids are irregularly spread throughout the cells and among the thylakoids are numerous phycobilisomes and ribosomes. Polyphosphate and cyanophycin granules are small, numerous, common in all preparations and situated irregularly throughout the cell. The enveloping mucilaginous layers form a complicated sheath, which is variable, but different in both strains. There are distinct clusters of nucleoplasmas among the thylakoids.

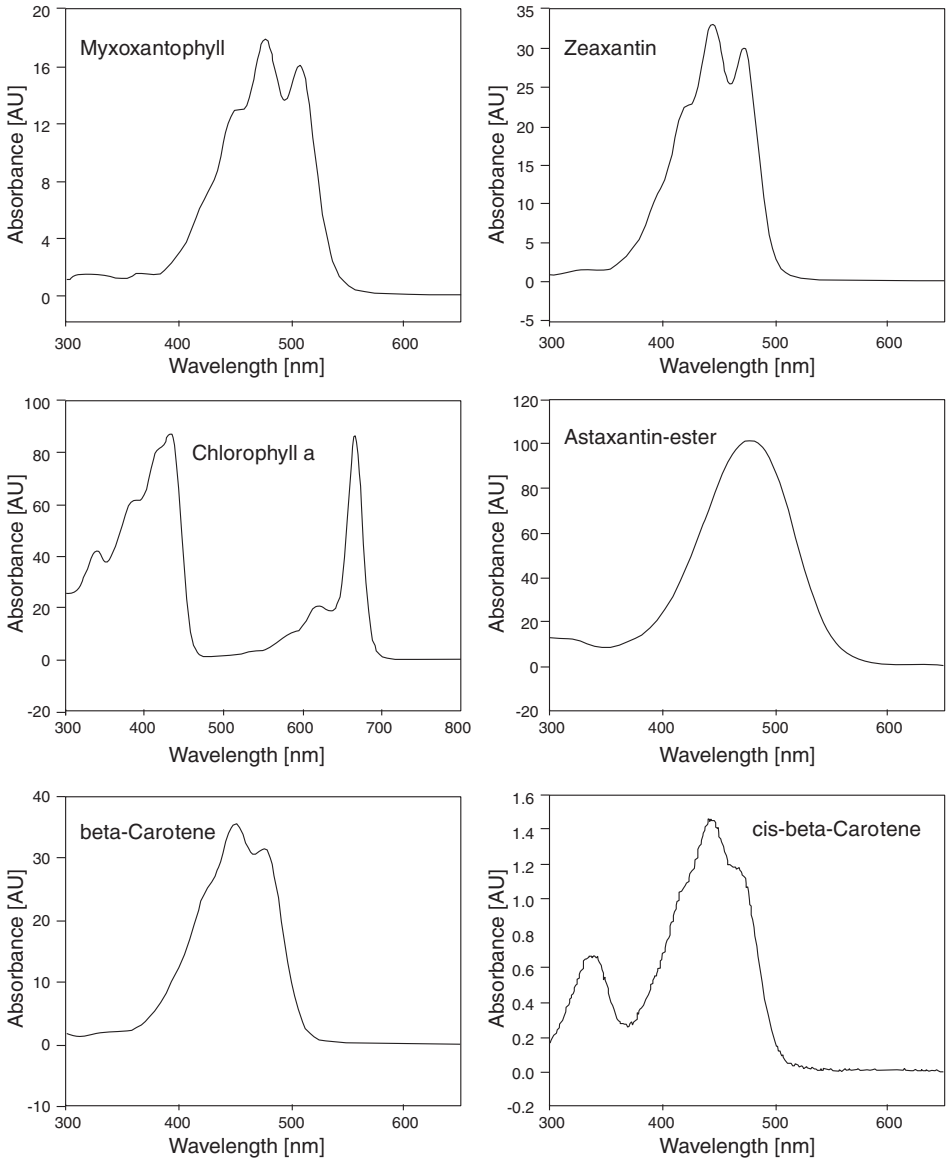


Fig. 6. – The composition (spectral analyses) of chlorophyll *a* and carotenoids in the cultures of the *Chroococcus* strains.

Ecophysiology

Cultivation of both of the strains under cross gradients of temperature and light intensity showed that their dependence on light and temperature were slightly different (Table 3). Optimal growth of the green strain CCALA 701 (measured by DW values) was recorded at 26 ºC and a low light intensity of 4–8 $W \cdot m^{-2}$. Optimal growth of the red strain CCALA 702

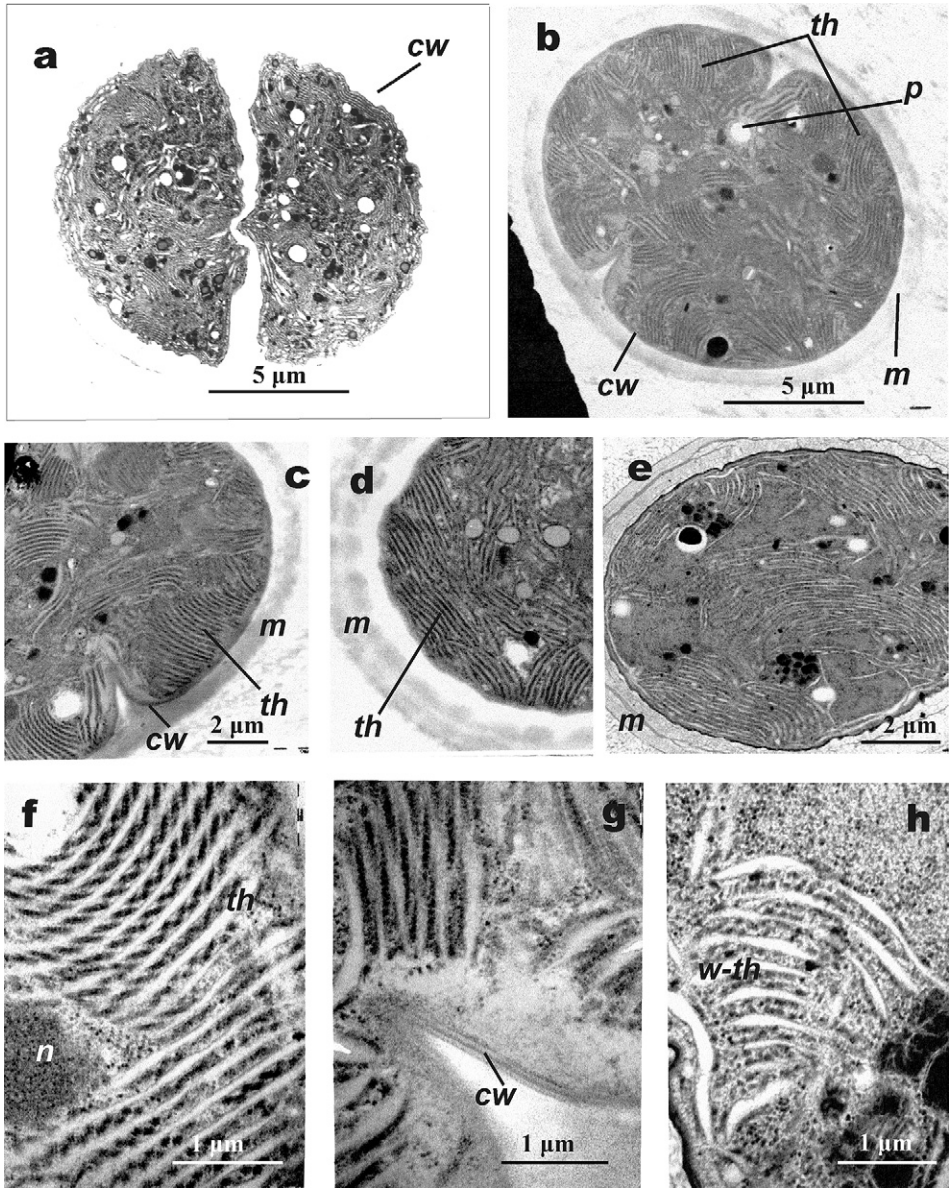


Fig. 7. – Ultrastructure of *Chroococcus* strain CCALA 701 (green). The structures within the cells are: the layered mucilaginous, mostly diffluent envelopes (m; b–e), thin cell wall (cw; a–c, g), fasciculated thylakoids (th; b–e), widened thylakoids (w–th; h), phycobilisomes (f–h), ribosomes (h), nucleoplasma (n;) and small, irregularly spaced cyanophycin granules (b–c), polyphosphate granules (p; b–e) and carboxysomes (d).

occurred under similar conditions but over a slightly wider range of temperatures (25–31 °C) and at a distinctly higher irradiance (9–23 $W \cdot m^{-2}$). Table 3 shows that the ecological plasticity of the red strain in relation to temperature and light intensity is wider.

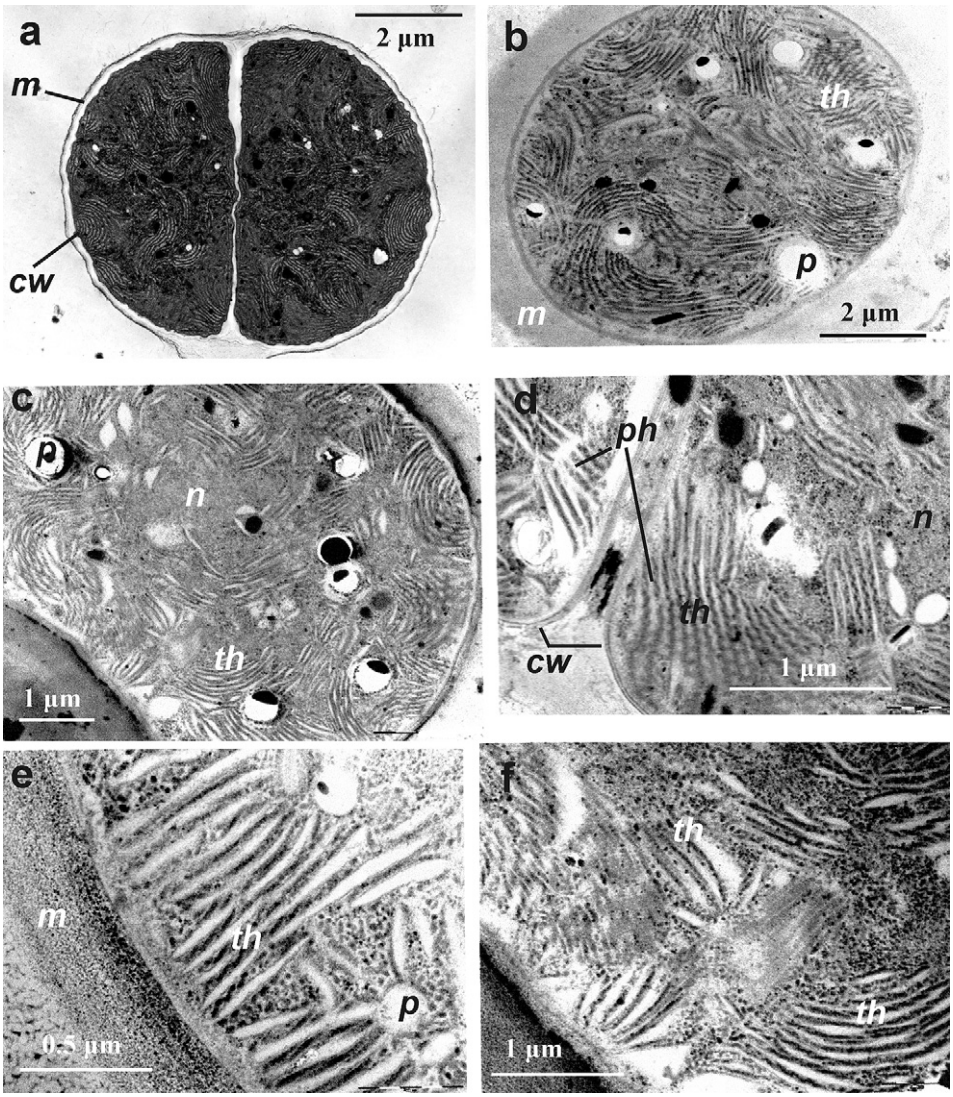


Fig. 8. – Ultrastructure of *Chroococcus* strain CCALA 702 (red). In thin sections the following can be seen: layered and limited mucilaginous envelopes (m; a–b, e), cell wall (cw; b, d), fasciculated thylakoids (th; b–f), widened thylakoids (w–th; e), phycobilisomes (d–e), ribosomes (e–f), nucleoplasma (n; c–d) and small irregularly placed cyanophycin granules (b–d), polyphosphate granules (p; b–e) and carboxysomes.

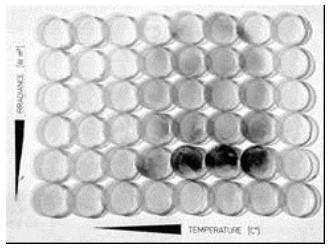
Phylogeny

The traditional genus *Chroococcus* (e.g. in the sense of Geitler 1932, Starmach 1966, Bourrelly 1970) is heterogeneous and both the strains studied belong to a morphologically distinct cluster with relatively large cells and more or less stratified sheaths. That is, in terms of morphology, they are similar to the type species of *Chroococcus*, *C. rufescens*. The generic identification of these strains within the revised genus *Chroococcus* sensu stricto is provided by Komárková et al. (2010). However, these strains are not genetically identical; the similarity between them based on the 16S rRNA gene sequence is 96.4%, which supports a different specific classification (Figs 9, 10).

Table 3. – Cross gradient cultures of *Chroococcus* spp. strain CCALA 701 (green) and CCALA 702 (red) indicating the optimal dependence on combined light intensity and temperature. The growth is visible as coloured Petri dishes. The dry weight (DW) values do not coincide exactly with the colour of the plates (= pigment content), because the pigment content (darkness of the colour in Petri dishes) is not directly related to biomass. Black wedges indicate decrease in irradiance and temperature.

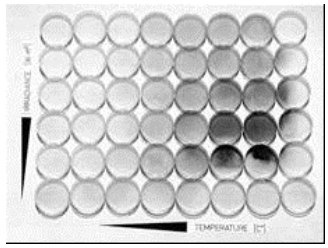
Strain CCALA 701 (green) DW [mg]

Irradiance	345	2	4	7	8	12	12	12	4
[μmol·m^{-2}·s^{-1}]	124	7	6	11	13	14	17	16	2
	69	7	9	10	17	19	18	18	2
	37	8	10	11	13	21	17	17	6
	18	9	10	13	11	23	17	16	5
	9	10	10	11	10	10	8	7	6
Temperature [°C]		10	15	18	23	26	30	33	37



Strain CCALA 702 (red) DW [mg]

Irradiance	368	3	8	11	16	23	20	18	6
[μmol·m^{-2}·s^{-1}]	106	7	8	12	18	26	27	24	15
	69	7	8	13	18	26	24	31	13
	41	8	8	10	15	24	24	24	10
	23	8	8	10	10	19	19	16	9
	9	6	9	7	7	9	7	6	5
Temperature [°C]		9	13	17	21	25	28	31	34



Taxonomy

The differently pigmented types ("green" CCALA 701 and "red" CCALA 702) are stable and of two different genotypes, but occurring in nature in one microhabitat. There is no evidence for chromatic adaptation, or the recent transfer of phycobilisome genes and subsequent rapid adaptation. They must therefore be designated as individual species within the genus *Chroococcus*. The preference is to designate these species using old available taxonomic names of species to which they morphologically and ecologically correspond (cf. Hansgirg 1892, Geitler 1932, Drouet & Daily 1956, Komárek & Anagnostidis 1998). Revised description of the specimens:

Chroococcus virescens Hantzsch in Rabenhorst 1862, Alg. Sachs. 133 & 134: 1332 (Fig. 11A), strain CCALA 701

Colonies few-celled or in small packets, usually up to 4(8)-celled. The masses are dirty green. Cells blue-green or greenish with homogeneous or a slightly granular content, 10.0–12.5 μm in diameter, or after division 6.2–10.0 (12.0) × 8.7–12.5 (17.5) μm. Mucilaginous envelopes are hyaline, colourless, diffluent and usually quite indistinct.

Reference strain: CCALA 701.

Type material from Germany, Dresden (deposited in L); isotypes have the nos. L0793662 and L0055202. The identification was controlled.

Ecology: In unpolluted pools and backwaters with aquatic vegetation or in shallow wetlands, metaphytic and periphytic on submerged plants and stones.

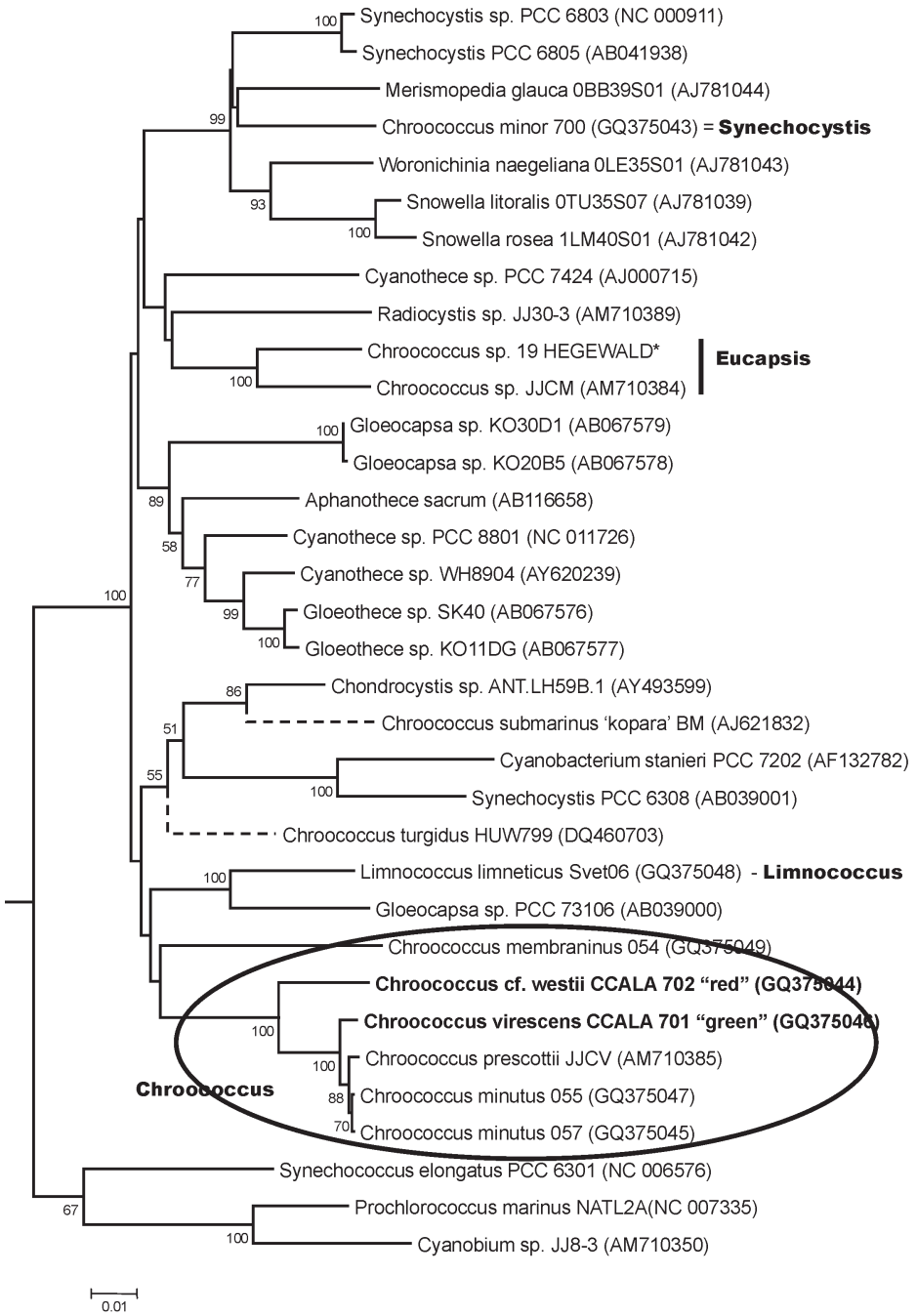


Fig. 9. – Phylogenetic tree with position of the genus *Chroococcus* and our strains CCALA 701 and CCALA 702, constructed with the Neighborjoining method (Saitou & Nei 1987). A bootstrap analysis involving 1000 resamplings was performed and bootstrap values higher than 50% are given in from of the concerned nodes. 34 sequences of chroococcal cyanobacteria deposited to GenBank were included. Dashed lines indicate positions of the *Chroococcus* strains from the GenBank that did not have sufficient sequence length.

Chroococcus cf. *westii* Boye-Petersen 1923, Bot. Icel. 2(2): 263 (Fig. 11B), strain CCALA 702:

Colonies in the form of three-dimensional, slightly irregular packets of cells, usually with up to 4 (–16)-cells, sometimes more or less in an agglomerate. The masses are dark violet-brownish to blackish. Cells reddish brown, when old brownish-yellowish, spherical, hemispherical or in form of a section of a sphere, sometimes slightly elongated (up to 2x longer than wide), 5.2–11.0 µm in diameter, (3.8) 7.5–9.5 (10.0) × 5.0–12.5 (15.0) µm. Sometimes produces smaller cells, ± 5.0 × 5.0–7.5 µm or 3–5 µm in diameter. Mucilaginous envelopes homogeneous, colourless, delimited at the margin, sometimes layered, 2–3 µm thick.

Reference strain: CCALA 702.

Type material is not available and the identification is based on descriptions in the literature. The specimens do not correspond exactly to the original diagnosis of material from Iceland by Boye-Petersen (1923), but agree with that of material collected more recently from populations in the northern parts of the European temperate zone. The ecology of other populations of *C. westii* and the material studied is similar. These strains do not correspond to any other *Chroococcus* species.

Ecology: In clear pools, small ponds with aquatic plants, and in the littoral zone of clear (mountain) and mesotrophic streams, usually periphytic on stones. The information on this species indicates it has a wider ecological range than *C. virescens*. This also accords with the experimental results on the ecological plasticity of this species.

Discussion

Modifications and strategies of chromatic differences

The occurrence of differently pigmented specimens (with different PC/PE ratios but similar morphology) was recorded as early as the beginning of the last century. Variously coloured populations occur in the cyanobacterial genera *Cyanobium, Synechocystis, Limnothrix, Phormidium, Lyngbya* and others. Such variously pigmented, morphologically very similar strains sometimes co-occur in nature in the same microhabitat and are stable in culture. The variation in colour in cyanobacteria is usually called "complementary chromatic adaptation", dependent on the environment, particularly the light conditions and considered as to be a more or less reversible. The first reviews of chromatic adaptation were published by Schindler (1913) and Boresch (1921), who summarized interesting data on this phenomenon at that time. In the 1970s and 80s, the strain UTEX 481 of *"Fremyella diplosiphon*" was commonly used for studying chromatic adaptation (Bennett & Bogorad 1973, Haury 1980, Rosinski et al. 1981, Bogorad et al. 1983, Cobley & Miranda 1983). The model strain was incorrectly identified (both the generic and specific names used to designate this strain are invalid according to nomenclatoric rules), but the data on phycocyanin and phycoerythrin syntheses and phycobilisomes function are commonly valid. Similar studies are reviewed by Bogorad (1975) and Tandeau de Marsac (1977). These later studies were focused particularly on chromatic adaptation in various ecological habitats, on the physiological conditions influencing the coloration of cells (Füglistaller et al. 1981, Tandeau de Marsac & Houmard 1983, 1993, Ohki & Fujita 1991, Latala & Misiewicz 2000, Pinevich et al. 2000) and on molecular mechanisms connected

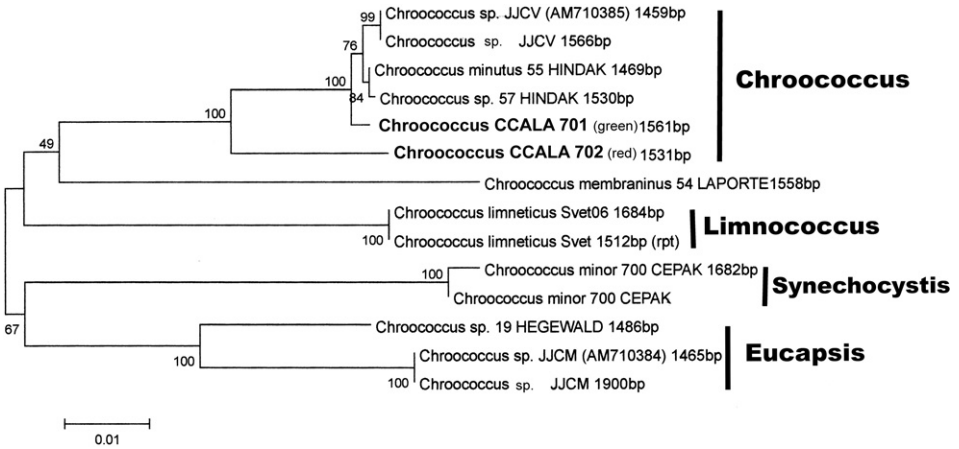


Fig. 10. – Phylogenetic analysis of the traditional genus *Chroococcus* and the position of the strains CCALA 701 and CCALA 702. The genetic (and specific) difference between the strains is clearly recognizable. The genus *Chroococcus* is separated from morphologically similar, but genetically distant generic clusters (*Limnococcus, Synechocystis* and *Eucapsis*), which were evaluated using the modern polyphasic approach.

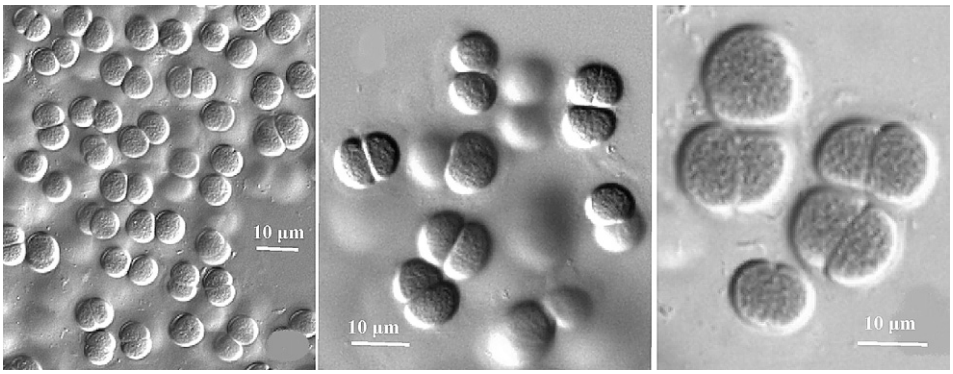
with chromatic adaptations (Bryant 1994, MacColl 1998, Kehoe & Grossman 1999, Everroad & Wood 2006).

However, many other questions, concerning evaluation of the diversity and ecology of variously pigmented and stabilized cyanobacterial populations, remain to be resolved. The problem of variously pigmented cyanobaterial populations is not uniform or simple. There occur various strategies of pigmentation in populations and strains (changes in ratios of photosynthetic pigments and carotenoids) and it is evident that all these features are in principle dependent on their ecology.

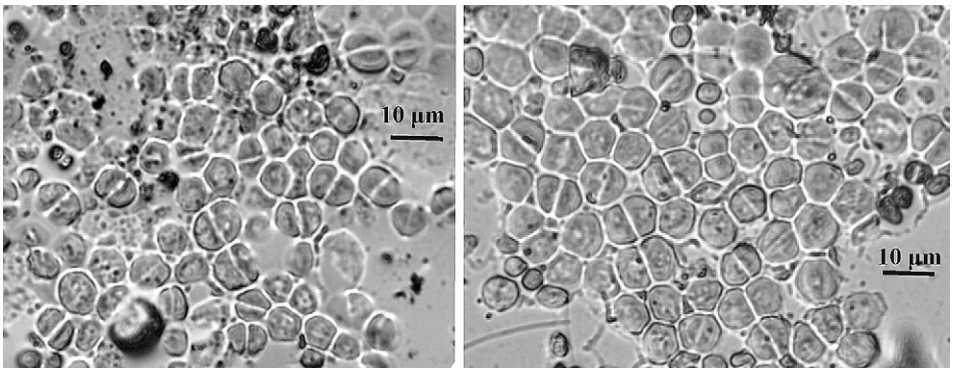
There has been great progress in studies on the genomic changes and lateral transfer of phycobilisome rod genes and gene clusters in populations in which there is a wide variety of cell pigmentation, e.g. in populations of the marine *Synechococcus/Cyanobium* (Ong & Glazer 1988, Palenik 2001, Six et al. 2007, Dufresne et al. 2008). In addition, there is a wide and variable mixture of numerous similar pigment types in natural population of planktic, freshwater "*Oscillatoria"* (= *Limnothrix*) *redekei* (Kohl & Nicklisch 1981).

Moreover, there are other adaptative changes in pigmentation and their stabilization in natural populations. Stable and differently coloured populations are known from nature, which differ sometimes only slightly morphologically but are clearly different in their ecological and other characteristics. The best known example is the complex *Planktothrix agardhii/rubescens,* in which both differently coloured types were considered as distinct and separate species already in the original description more than 100 years ago (cf. Geitler 1932). The differentiation and stabilization of these pigment types is interesting, because there are no distinct differences in the 16S rRNA gene sequences of the most common types (*P. agardhii* and *P. rubescens*) and it is hypothesized that they are conspecific (Humbert & Le Berre 2001). However, the stability of both types and the ecological and ecophysiological differences are so distinct that their separation in two taxa (species) is justifiable (e.g. Suda et al. 2002, Davis et al. 2003).

A *Chroococcus virescens* Hantzsch in Rabenhorst 1862 strain CCALA 701



type material (L 0793662)



B *Chroococcus* cf. *westii* Boye-Petersen 1923 strain CCALA 701

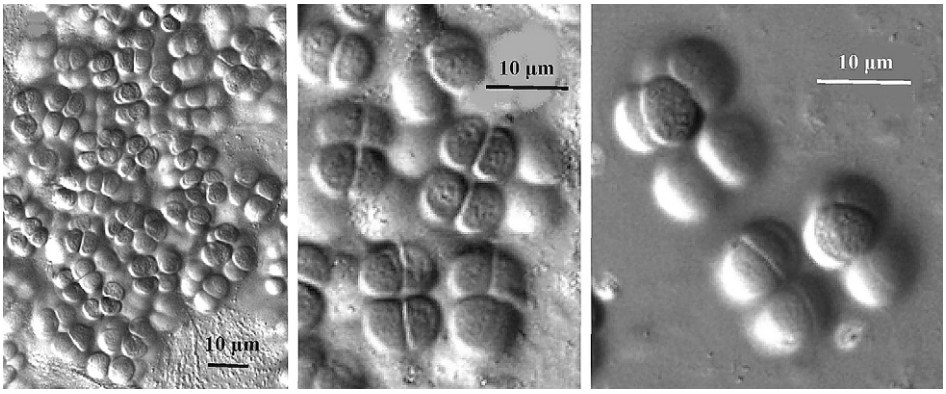


Fig. 11. – Strains CCALA 701 (*Chroococcus virescens*) and CCALA 702 (*Chroococcus* cf. *westii*) from the deposited strains kept in culture at CCALA. *Chroococcus virescens* is compared with the type material of Hantzch (wild culture), deposited in the herbarium of Leiden (L).

The different pigmented *Chroococcus*-strains studied are not chromatic adaptations. The dependence of both strains on light conditions differed in spite of their occurring in nature in the same habitat. This fact indicates the possibility of microzonation of both species within mats. This was not recorded during the collection of the original material, but similar zonation of differently coloured species in microcommunities is recorded for other habitats (see e.g. in Komárek & Komárek 2003). The molecular analyses confirmed that the two strains were genetically different.

Differently pigmented cyanobacteria include therefore a variety of modifications: (i) reversible chromatic adaptation (Kehoe & Gutu 2006), (ii) populations that are able to differentiate continually into variety of pigmented strains and rapidly adapt to a variety of light niches (Six et al. 2007), (iii) morphologically similar and ecologically distinct morpho-types with different but stable pigment compositions, in which the process of diversification and stabilization occurred a long time ago. The *Chroococcus* strains studied belong to such already stabilized types (similar to e.g. *Planktothrix rubescens/agardhii*). They belong to the typical *Chroococcus*-cluster (Komárková et al. 2010) and are genetically different species (Fig. 9).

Ultrastructure of Chroococcus

The ultrastructure of both strains is almost identical and is probably characteristic of the whole genus *Chroococcus* (cf. Potts et al. 1983). It is interesting that it was not easy to prepare *Chroococcus* cells (fixation) for ultrathin sectioning and the outline (periplast) of the cells in the sections was always wavy. This is commonly recorded for *Chroococcus* (cf. e.g. Figs 3a,b and 4a in Potts et al. 1983). Good sections were only obtained only after using a slightly different fixation procedure.

Taxonomic consequences

The modern classification of cyanobacteria is based on molecular methods, which elucidate and explain the phylogenetic relations. A revision of the systematic classification at the generic level has been carried out usually using 16S rRNA gene sequences (Castenholz 2001, Hoffmann et al. 2005, Komárek 2006). However, comparison of various genetic units from the GeneBank is sometimes difficult, because the similarity expressed in % is acceptable only as an indication and needs to be interpreted also with respect to a restricted number of sequenced bases. Moreover, according to the results of recent studies it is difficult to use common uniform criteria for the delimitation of subgeneric units in cyanobacteria (Johansen & Casamatta 2005, Komárek 2010).

Coccoid cyanobacteria are particularly heterogeneous and belong to various developmental lines, which differ in important phenotypic and ultrastructural markers. The large diversity of the genus *Chroococcus* (cf. Komárek & Anagnostidis 1998) was described in numerous morphological and ecological studies on strains and natural populations (Potts et al. 1983, Richert et al. 2006, Komárek & Komárková-Legnerová 2007 and others) and in the revisions based on molecular analyses. The strain "*C. turgidus* PCC 9340", designated by Castenholz (2001) as the reference strain for "*Chroococcus* cluster 2" (corresponding to the typical *Chroococcus* in the traditional sense) is probably close to *Chroococcus submarinus* strain BM (Richert et al. 2006). The planktic types ("*Chroococcus limneticus" = Limnococcus*) and "small" *Chroococcus* morphospecies, which are more closely related to *Aphanocapsa* or *Eucapsis,* differ from the typical morphotypes (*C. rufescens, C. turgidus*, etc.; Komárková et al. 2010), etc.

The heterogeneity of *Chroococcus* is evident also from the database of sequences, in which the strains designated as "*Chroococcus*" occur in different clusters in phylogenetic trees. In this genus (in the revised, restricted form) there are types in which the difference in colour (pigment ratio) is stable, and which correspond to stable morphotypes at the species level (*C. rufescens, C. turgidus, C. subnudus, C. virescens, C. westii* and others). This is an example of taxonomic classification of a number of differently pigmented types, among which the strains studied also belong. The molecular, morphological and ultrastructural analyses indicate that both of the strains studied are typical *Chroococcus*-taxa. The question if the studied strains belong into the category of "chromatic adaptations", or if they represent genetically stable types (species) of the genus *Chroococcus,* was therefore the particular question in our study.

The studied strains were isolated from one mat and therefore it is likely they have similar ecologies. Ecological differences are important arguments for deciding if two types can be separated at the specific level (e.g. *Planktothrix agardhii/rubescens*). However, these strains only differ slightly in their ecophysiological characters, but are clearly distinguishable phenotypically (stable pigment ratios) and have different positions in phylogenetic trees (96.4% genetic similarity). Their positions in the phylogenetic trees were particularly important in the assessment of their differences at the specific level. They cannot be considered as individuals that are differently chromatically adapted or a consequence of a recent lateral transfer of phycobilisome rod genes or gene clusters, as described, e.g. by Six et al. (2007) and Dufresne et al. (2008) for oceanic planktic populations of small-celled *Synechococcus/Cyanobium* spp. The differences between the strains studied are stable, and therefore important in evaluating them as separate taxa. These two more or less delimited types do not differ morphologically from previously described *C. virescens* and only slightly from *C. westii*. The preference is to use these taxonomic names, because they do not differ morphologically from the original descriptions and type exsiccates (Drouet & Daily 1956). It is a pity it was not possible to sequence the 170 and 120 year old dried out types. However, the creation of new names in this case would be superfluous and complicating a nomenclatoric review of the genus. Both of the species studied can evidently occur in one and the same habitat at the same locality, but may occupy different microniches.

There is an ongoing debate about the classification of stable, variously pigmented types in nature. Chromatic adaptations or populations with continually occurring differently pigmented strains (Kohl & Nicklisch 1981, Six et al. 2007, Dufresne et al. 2008) must be recognized, but it is difficult to evaluate them as taxonomic units. On the other hand, stable and isolated pigment mutants should be classified as different species, if they differ in ecology, as in the genus *Planktothrix* (*P. agardhii/rubescens*), or if the ecology is similar but they differ in other specific criteria (molecular sequencing), as is the case of the strains studied.

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Souhrn

Jedním z taxonomických problémů u cyanobakterií (sinic) je hodnocení populací s různým obsahem fykobilínů (poměr PC:PE). Dříve se takové případy vysvětlovaly pouze tzv. komplementární chromatickou adaptací, kde poměr fotosyntetických barviv je závislý na vnějších podmínkách, především na světelném režimu a teplotě. Moderní, zejména molekulární metody však prokázaly, že morfologicky podobné ale různě zbarvené populace mohou být způsobeny různými mechanismy a strategiemi, a mohou být různě stabilní s různou taxonomickou hodnotou. Vedle reverzibilní, „klasické" chromatické adaptace může v populacích dojít k vytvoření celé škály stálých barevných odchylek, které jsou v přírodních podmínkách vyvolány především horizontálním transferem příslušných genů (stabilizují se jen po izolaci do monokultur), nebo se mohou dlouhodobě ustálit některé pigmentové mutace ve stálých odlišných ekologických podmínkách. Takové stabilizované přírodní typy se vyskytují zpravidla i v odlišných ekologických situacích, ale to neplatí absolutně. Pokud se mají podobné případy řešit, je nutno provést podrobné ekofyziologické, cytomorfologické i fylogenetické analýzy.

V našem případě šlo o vyřešení dvou důležitých, fenotypově podobných, ale pigmentově rozdílných typů z rodu *Chroococcus*, izolovaných ze stejného biotopu (mrtvé rameno Dunaje u Gabčíkova, Slovensko). Z našich výsledků vyplynulo, že se jedná o geneticky i fenotypově rozdílné typy, které je nutno považovat za odlišné druhy. Kontrolou typových materiálů z Leidenu (L) a Vídně (W) vyplynulo, že lze dokonce tyto druhy ztotožnit již s dříve popsanými, ale dosud taxonomicky ne zcela jasnými druhy (*Chroococcus virescens, C. westii*). V našem případě se jedná o výskyt ve stejném biotopu, což je u podobných případů určitá zvláštnost. V našem článku je vyřešena taxonomická pozice obou studovaných druhů a jsou zde též poprvé stručně porovnány různé případy pigmentových modifikací u sinic a diskutován způsob jejich taxonomického hodnocení.

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