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Cronbergia gen. nov., a new cyanobacterial genus (Cyanophyta) with a special strategy of heterocyte formation

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Abstract – We describe a new genus, *Cronbergia,* based on a particular mode of heterocyte formation. This pattern consists in the division of one cell into two heterocytes followed by the rupture of the filament at the junction between the two heterocytes. The resulting trichomes have solitary heterocytes in the terminal position. A similar heterocyte development is known only in the genus *Anabaenopsis*, but the process of their formation is different. The strains/samples that correspond to this heterocyte formation and are included in the new genus are: (1) one strain (SAG B11.82) identified as *Anabaena siamensis*, but later assigned to the genus *Richelia* by Hindák (2003), (2) two natural populations from Slovak waters, (3) *Cylindrospermum planctonicum* from a Swedish fjord and (4) *Cylindrospermum* sp. strain PCC 7417. These four strains/samples are assigned to the new species *Cronbergia siamensis, C. paucicellularis, C. planctonica* and *Cronbergia* sp., respectively. The morphological differences between the four species are the cell length and numbers of cells, and the form and position of akinetes in filaments. The morphological differences between the new genus and *Cylindrospermum, Richelia, Anabaenopsis, Cylindrospermopsis, Nostoc* and *Anabaena* are also discussed. Available 16S rRNA sequences are compared to those of *Cronbergia siamensis* strain SAG B11.82, showing that the strains of the new genus have at least 3% 16S rRNA divergence with them.

akinetes / combined characters / *Cronbergia* / cyanobacteria / Cyanophyta / heterocyte formation / molecular methods / phylogeny / polyphasic approach / taxonomic classification

Résumé – ***Cronbergia*, nouveau genre de cyanobactéries (Cyanophytes) caractérisé par un mode spécial de formation des hétérocytes.** Un nouveau genre, *Cronbergia,* est décrit sur la base d'un mode particulier de formation des hétérocytes. Ce mode implique la division d'une cellule en deux hétérocytes suivie par la rupture du filament à la jonction entre les deux hétérocytes si bien que les trichomes qui en résultent ont des hétérocytes solitaires en position terminale. Un tel développement des hétérocytes n'est connu que du genre *Anabaenopsis* mais leur mode de formation est différent. Dans ce nouveau genre sont

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inclues les souches et échantillons qui correspondent à ce mode de formation. Il s'agit de 1) une souche identifiée d'abord comme *Anabaena siamensis* (SAG B11.82) et ensuite attribuée au genre *Richelia*, 2) deux populations naturelles de Slovaquie, 3) une population de Suède décrite comme *Cylindrospermum planctonicum* et 4) la souche PCC 7417 déterminée *Cylindrospermum* sp. Dans le genre *Cronbergia* ces souches et populations forment respectivement les espèces suivantes : *C. siamensis, C. paucicellularis, C. planctonica* et *Cronbergia* sp., différenciées par divers caractères morphologiques comme la longueur des filaments et le nombre des cellules ou la forme, les dimensions et la position des akinètes dans le filament. Sont aussi discutés divers autres caractères morphologiques permettant de différencier *Cronbergia* des autres genres de Nostocales comme *Cylindrospermum, Richelia, Anabaenopsis, Cylindrospermopsis, Nostoc* et *Anabaena*. D'autre part, les séquences 16S rRNA disponibles ont été comparées avec celle de la souche *Cronbergia siamensis* (SAG B11.82). Cette comparaison a mis en évidence que la souche du nouveau genre montre une divergence 16S rRNA d'au moins 3 % avec ces autres séquences.

Akinètes / approche polyphasique / caractères combinés / classification / *Cronbergia* / cyanobactéries / Cyanophyta / formation des hétérocytes / méthodes moléculaires / phylogénie

INTRODUCTION

The modern revision of cyanoprokaryotic (cyanobacterial) taxonomic classification is based on a combined polyphasic evaluation of taxa. The molecular approach, of which 16S rRNA gene sequencing is considered the most important standardized method, usually splits the traditional genera into more distinct entities. These newly defined genera should be characterized also by clear phenotype markers and we accept the principle that the definition of the autapomorphic character should be an integral and obligatory requirement of the revised new genera (Johansen & Casamatta, 2005; Rajaniemi *et al.,* 2005; Řeháková *et al.,* 2007; Komárek, 2010). Numerous traditional polyphyletic heterocytous genera have already been subdivided, usually on the basis of molecular results (*Scytonema, Anabaena, Aphanizomenon, Nostoc* and others; cf. Iteman *et al*., 2002; Rajaniemi *et al*., 2005; Fiore *et al*., 2007; Řeháková *et al*., 2007; Zapomělová *et al*., 2009, 2010; and others). Distinct deviations in phenotype that are specific for single phylogenetic clades can be used as good markers for the recognition of these types and consequently for their classification into special taxa.

Three similar morphotypes (two morphospecies) of heterocytous cyanobacteria have been recently described that do not correspond exactly to any known (revised or not revised) genus. Uncertainties on the taxonomic status of these cyanoprokaryotes are evidently reflected by the fact that they divided their (related) taxa into three quite different genera:

• Antarikanonda isolated in 1976 and later described (Antarikanonda, 1980, 1985) one strain from experimental paddy fields of Kasetsart University, Bangkok, Thailand, as *Anabaena siamensis*. This strain has short trichomes without sheaths (max. 50 μm long) and terminal, unipored heterocytes, similar to the genera *Cylindrospermum* or *Anabaenopsis*. Akinetes develop in short chains (less frequently also in longer rows), possibly apoheterocytically, intercalarly, usually separated by several cells from terminal heterocytes. Heterocytes develop intercalarly in pairs and later trichomes disintegrate between them.

• Hindák (2000) studied the strain of Antarikanonda and stressed the special and unique formation of two neighbouring intercalary heterocytes (from one mother cell), between which the trichomes later disintegrate and new short filaments with polar heterocytes arise.
• Hindák (2003) described a few other similar populations from the metaphyton of stagnant waters in SW Slovakia and classified all these types (including Antarikanonda's type) into the genus *Richelia* (as *R. siamensis*) according to morphological similarity. However, akinetes of his populations develop next to heterocytes or slightly distant from them, while akinetes of the type material (Antarikanonda, 1985) develop in the centre of trichomes. The populations from Slovakia should therefore be considered at least as a special species.
• Cronberg (2003) described a very similar cyanoprokaryotic type, with maximally 72 μm long trichomes, from the plankton of brackish waters in the Bay of Östhammarsfjärden in Sweden, as *Cylindrospermum planctonicum*. The basic morphology of solitary filaments is almost identical with Antarikanonda's and Hindák's populations with terminal heterocytes and particularly with the development of intercalary heterocytes in pairs, but with subterminal akinetes (paraheterocytic).

The main autapomorphic character of this whole group of cyanoprokaryotes is quite a special strategy for the formation of intercalary heterocytes (Fig. 1). In principle, the filaments are metameric, where pairs of new heterocytes regularly develop in the middle of a row of vegetative cells after symmetric division of one mother vegetative cell. The trichomes later disintegrate between the new proheterocytes. This process is similar to that of the genus *Anabaenopsis* (for which it is considered as quite unique and specific of this genus). However, the neighbouring proheterocytes and heterocytes are formed differently in *Anabaenopsis* and the new taxon discussed here; in *Anabaenopsis,* this process starts after asymmetrical division of two cells (Hindák, 2000; Komárek, 2005). The resulting morphology of short filaments with heterocytes in

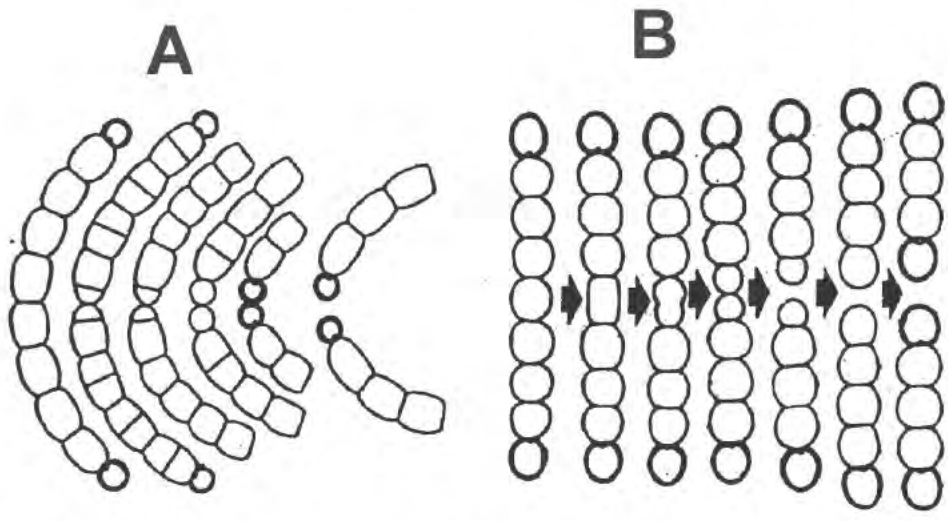

Fig. 1. Strategy of development of intercalary pairs of unipored heterocytes in the genus *Anabaenopsis* (A) and *Cronbergia* (B) (From Hindák, 2000).

terminal positions is similar in both genera, but the development of heterocytes is clearly different. The combined paraheterocytic and apoheterocytic akinete formation is the additional morphological characteristics of the above mentioned cyanoprokaryotic genera.

In the present study, we classify the three mentioned types from this cyanobacterial group in the new genus *Cronbergia.* We compare and partly revise their phenotype markers, and study the phylogenetic position of the type strain (*Anabaena* = *Cronbergia siamensis*) based on its 16S rRNA gene sequence, the strains of the other two species being unavailable.

MATERIAL AND METHODS

The original strain of *Anabaena siamensis* (strain Antarikanonda 1976 = SAG B11.82 = CCALA 756), isolated from a rice field near Bangkok, Thailand, was cultured originally in liquid medium BBM, at about 25°C and under continuous illumination with fluorescent tubes. Another important strain, *Cylindrospermum* sp. PCC 7417, was obtained from the PCC collection (headed by M. Gugger). The strains were studied and revised morphologically, and phylogenetically analysed. The morphology, development of trichomes and akinete formation of both strains were studied in BG 11 medium without nitrogen, in a liquid state and solidified by agar, under variable temperature and light conditions. Unfortunately, strains of other similar species were not available. We have derived their characteristics only from careful descriptions of recent authors (Hindák, 2000, 2003; Cronberg, 2003).

DNA extraction, PCR and sequencing

Biomass of the strain *Anabaena siamensis* SAG B11.82 (= strain Antarikanonda 1976) was harvested in the exponential growth phase by repeated centrifugation, during which the filaments were washed several times with a NaCl solution (1 $g.L^{-1}$), to remove mucilaginous substances. The biomass was stored at –20°C until the DNA extraction. DNA was extracted using the UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA). The 16S rRNA gene and ITS region were amplified with primers 16S27F and 23S30R (Taton *et al.,* 2003). Amplification was carried out as follows: one cycle of 5 min at 94°C; 10 cycles of 45 s at 94°C, 45 s at 57°C, and 2 min at 72°C; 25 cycles of 45 s at 94°C, 45 s at 54°C, and 2 min at 72°C; and a final elongation step of 7 min at 72°C. PCR product was used as a template for sequencing with primers 16S27F (Taton *et al.,* 2003), CYA781F(a) (Nübel *et al.,* 1997), and primer K6, which was a complement of Primer 14 by Wilmotte *et al.* (1993). The 16S rRNA gene sequence of strain SAG B11.82 was submitted at GenBank under the accession number GQ389643.

Phylogenetic analyses

A BLAST search was used to find the closest matches in Genbank. Strains that appeared in the BLAST search result were selected for the phylogenetic analyses so that they represented various nostocacean genera.

Sequences were aligned using the programme ClustalW in BioEdit Sequence Alignment Editor (Hall, 1999). The alignment was edited manually. Phylogenetic analyses were performed based on partial 16S rRNA gene sequences (1443 bp). Bayesian analysis was made using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). The general time reversible model (GTR), selected previously using MrMtgui 2.3 (Nylander, 2004) was used. All searches were conducted using four simultaneous Markov chains over five million generations and sampling every 100^{th} generation. The software tool Tracer version 1.3 (Rambault & Drummond, 2003) was used to examine the parameters and determine the number of trees needed to reach stationarity (burn-in) for each run. Bayesian posterior probability confidence value (bpp) was generated from trees found after this initial burn-in period. Neighbour Joining method (NJ) was performed in the program PAUP (http://paup.csit.fsu.edu/) with 1000 bootstrap replicates.

RESULTS

Morphology

The entire cluster of the studied strains and populations of *Cronbergia* has the following distinct morphological characters, which are identical for all studied populations, and which are typical only for this cluster (Fig. 2):

1. Heterocytes develop intercalarly always after symmetrical division of one vegetative cell into two daughter cells, which differentiate into proheterocytes. The process of development of paired intercalary heterocytes is started by the transformation of one intercalary vegetative cell into an elongated and often narrowed cell, which divides into two proheterocytes. They change into unipored heterocytes with a mirror-like orientation of their pores towards the vegetative cells. Shortly after formation of the intercalary heterocytes the trichome breaks at the junction between the new heterocytes, and short trichomes with only terminally localized heterocytes arise. This process is quite unique and does not occur in any other cyanobacterial genus (cf. *Anabaenopsis*, where the two adjacent heterocytes arise from two vegetative cells after their asymmetric division; Figs 1, 2).
2. The filaments grow solitarily or in loose colonies and are relatively short, with maximal length of 80(–120) μm.

In two morphotypes (Hindák's 2003 populations from Slovakia and Cronberg's *Cylindrospermum planctonicum*), the akinetes develop in principle paraheterocytically, just next to the terminal heterocytes, or less frequently separated from the heterocytes by a few (1-4) vegetative cells. The akinetes are elongated-oval, and they develop solitarily, or up to 4 (or more?) in a row, from one cell or possibly after fusion of two vegetative cells (cf. Fig. 6h). The akinetes develop on both ends of more or less symmetrical trichomes. In one morphospecies, *Anabaena* (= *Cronbergia*) *siamensis* (the type strain SAG B11.82) the widely oval akinetes of the similar shape arise in the middle of short trichomes, just between heterocytes, and later they form chains of several akinetes. The same type of akinete formation was observed also in cultures of the strain "*Cylindrospermum*" sp. PCC 7417, which is phylogenetically closely related to *Anabaena siamensis* and evidently does not belong to the genus *Cylindrospermum* (Fig. 8 e, g).

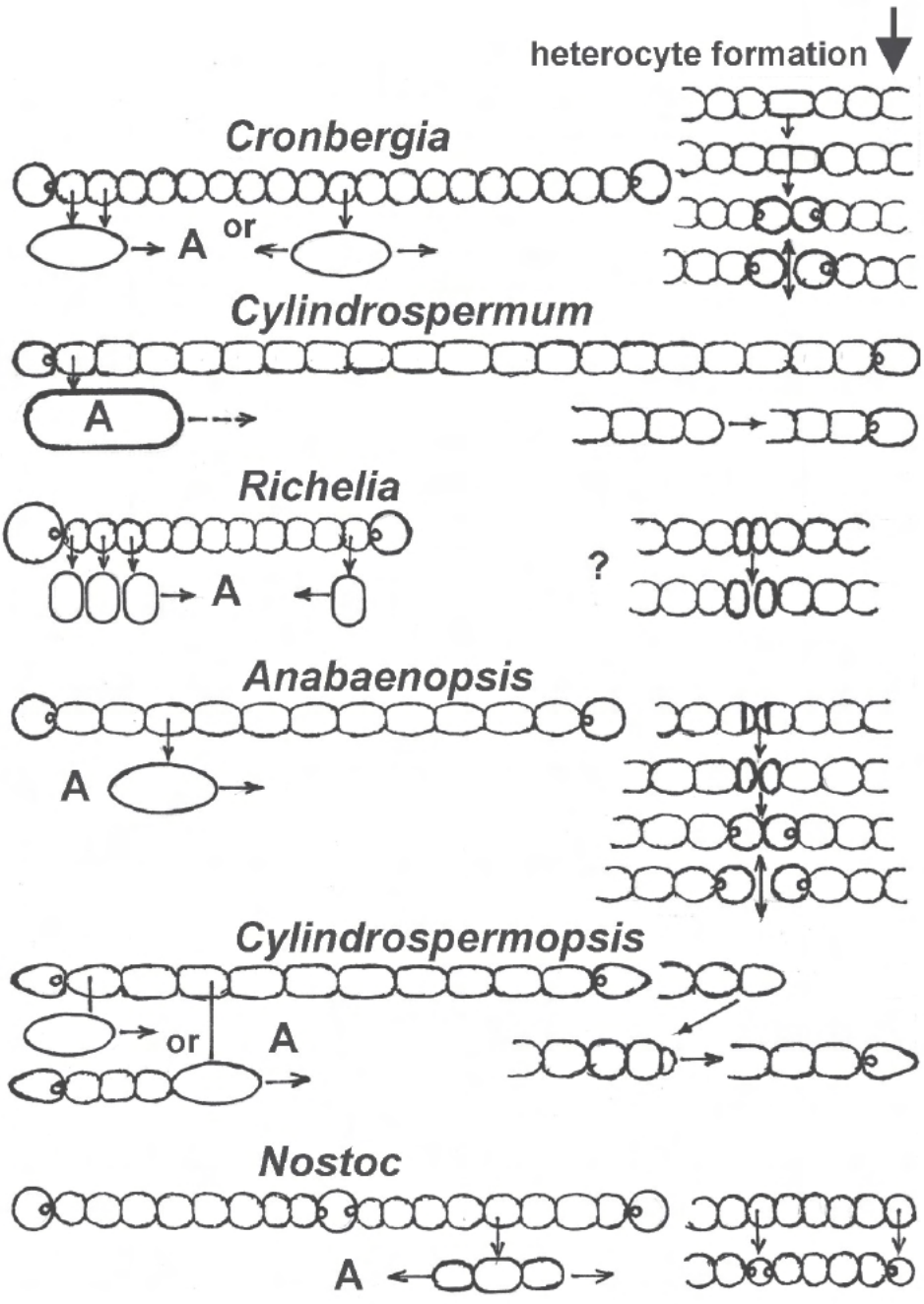

Fig. 2. Scheme of filaments of nostocacean genera with terminal heterocytes, with type of akinete formation (A) and various strategies of heterocyte development. The horizontal arrows in akinetes illustrate the direction of further possible akinete formation.

Phylogenetic position (Fig. 3, Tab. 1)

Based on 16S rRNA gene sequence, the type strain *Anabaena siamensis* SAG B11.82 represents a special subcluster closest to the genera *Nostoc* and *Cylindrospermum*. However, the strain "*Cylindrospermum*" sp. PCC 7417 with the highest similarity of 97.4% to SAG B11.82 (= *Anabaena siamensis,* Fig. 3) does not correspond morphologically to the genus *Cylindrospermum,* has several characteristic features of *Cronbergia* and must be classified to this genus. The percentage similarity of 16S rRNA gene sequence to other *Cylindrospermum* strains varies from 96.2 to 96.4%. Our "*Cronbergia*" cluster (type strain of *Anabaena siamenis* SAG B11.82) could be therefore joined to *Cylindrospermum* according to strictly bacteriological taxonomic criteria. However, the limit of 95% similarity of our "*Cronbergia*" cluster is also assessed in comparison to several

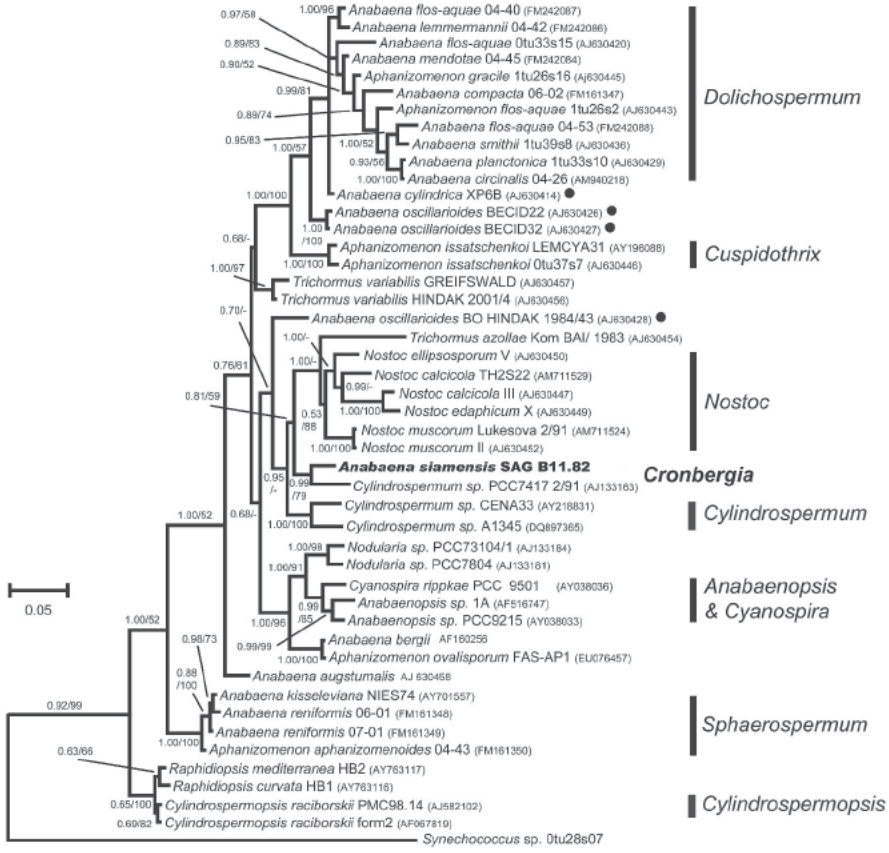

Fig. 3. Bayesian tree based on 16S rRNA gene sequences (1443 bp) showing the phylogenetic affiliation of the type strain of the genus *Cronbergia* ("*Anabaena siamensis*", strain SAG B11.82 = *Cronbergia siamensis*). Numbers near nodes indicate NJ bootstrap values over 50% Bayesian posterior probability confidence value. Names and codes of the strains in the phylogenetic tree correspond with those that are used in official culture collections. The generic names on the right side of the clusters reflect current taxonomic revisions of the traditional nostocacean genera. • = benthic *Anabaena* strains whose clustering is markedly different from "*Anabaena siamensis*", strain SAG B11.82 = *Cronbergia siamensis*.

Table 1. Genetic similarity between "*Anabaena siamensis*" (typical strain of the genus *Cronbergia*; Antarikanonda 1976 = SAG B11.82) and related strains of heterocytous (nostocalean) cyanobacteria. Matrix showing P-distances (%) is based on the 16S rRNA wgene (1443 bp). Only the positions containing alignment gaps were eliminated in pairwise sequence comparison.

		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*
1	**"*Cylindrospermum* sp." PCC7417 = *Cronbergia* like**															
2	*Cylindrospermum* sp. CENA33	95.4														
3	*Cylindrospermum* sp. A1345	95.6	97.4													
4	*Nostoc muscorum* AJ 630452	95.9	95.9	95.7												
5	*Nostoc calcicola* TH2S22	96.0	95.6	95.9	96.9											
6	*Trichormus azollae* AJ 630454	94.7	93.7	93.9	95.2	94.4										
7	*"Anabaena" bergii* AF160256	95.2	94.8	94.8	95.6	95.3	94.8									
8	*Anabaena oscillarioides* BECID22	94.1	94.2	93.7	94.3	93.7	94.0	94.6								
9	*Anabaena oscillarioides* BO HINDAK1984/43	95.9	95.5	95.4	94.6	95.0	94.5	95.2	95.7							
10	*Anabaena cylindrica* XP6B	94.5	94.5	94.0	94.3	93.8	94.2	94.9	98.1	95.9						
11	*Anabaena augstumalis* SCHMIDKE JAHNKE/4a	95.3	95.1	94.8	94.8	95.0	94.1	95.4	95.5	96.3	95.7					
12	*Anabaena* (= *Dolichospermum*) *planctonica* 1tu33s10	94.1	93.7	93.5	93.8	93.5	92.5	94.7	96.2	94.7	97.5	94.4				
13	*Anabaena* (= *Sphaerospermum*) *reniformis* 06-01	92.8	92.1	92.4	92.3	92.3	91.7	93.2	93.2	95.3	93.4	95.1	92.9			
14	*Anabaenopsis* sp. PCC9215	95.1	94.5	94.8	94.4	95.6	94.1	96.9	94.5	95.3	94.8	95.4	94.6	93.0		
15	*Cyanospira rippkae* PCC 9501	95.3	94.5	95.0	94.7	95.6	94.2	96.5	94.0	94.8	94.3	95.5	94.3	93.2	98.1	
16	***Anabaena siamensis* Antarikanonda 1976 (SAG B11.82)**	**97.4**	**96.4**	**96.2**	**96.1**	**96.6**	**94.3**	**95.4**	**94.7**	**96.6**	**95.0**	**95.7**	**94.5**	**93.4**	**95.0**	**95.2**

studied typical *Nostoc* strains (96.1-96.6%), benthic *Anabaena* (94.7-96.6%) and *Anabaenopsis* (95.0%). From all these genera *Cronbergia* differs particularly by the appearance (length) of trichomes and type of heterocyte formation. Because all these clusters differ one from another by distinct biological and phenotypic markers without transitions, their unification in one taxonomic unit (genus) is evidently incorrect. If we classify such different morphotypes as *Nostoc-Cylindrospermum-Anabaena-Anabaenopsis* as separate taxonomic genera, the separation of *Cronbergia* into a special genus, characterized also by clear autapomorphic characters (heterocyte and akinete development) is justifiable.

Taxonomy

Cronbergia genus novum

Nostocacean cyanoprokaryotic (cyanobacterial) taxonomic unit with isopolar, uniserial, unbranched filaments with heterocytes and akinetes. Trichomes solitary, without sheaths, free-floating, or in fine, mucilaginous thallus, more or less straight or slightly flexuous, short (up to 80-120 μm long), with terminal heterocytes on both isopolar ends, cylindrical, distinctly constricted at the cross-walls. Cells more or less spherical, barrel-shaped or slightly elongate or oval. Heterocytes terminal, spherical, ovoid or slightly oval, but developing intercalarly from one vegetative cell, which elongates, later symmetrically divides and from each daughter cell a heterocyte develops. Thus, the proheterocytes are originally situated in pairs in the intercalary position, but the trichomes quickly break at the junction between the young intercalary heterocytes. Akinetes arise next to the polar heterocytes, slightly distant from them by 1-2 vegetative cells, solitary or in short rows, or in the centre of short trichomes in chains; they are oval or oval-cylindrical.
Type species: *Cronbergia siamensis* (Antarikanonda) comb. nov.
Type (holotype): The strain SAG B11.82 (= CCALA 756) deposited as dry material (exsiccate), preserved in a metabolic active state (ICBN Art. 8.4) in BRNM (no HY-2333); typical and reference strain: Antarikanonda 1976 (= SAG B11.82 = CCALA 756) is deposited also in a lyophylised state (under the designation "CCALA 756") in the Phycological Department, Institute of Botany ASCR, Třeboň, Czech Republic.

Latin diagnosis: *Fila cyanoprocaryotica, isopolaria, uniseriata, simplicia, solitaria vel in aggregationibus liberis plus minusve irregulariter disposita, sine vaginis conspicuis, curta, recta, arcuata vel paulo flexuosa. Trichomata paucicellularia (ad 35-cellularia), usque ad 120 μm longa, cylindrica, cum heterocytis sporisque, ad dissepimenta constricta. Cellulae vegetativae sphaericae, doliiformes, ovales ad cylindricae. Heterocytae binatim in parte centrali trichomatis, per divisionem symmetricam unae cellulae vegetativae oriundae; trichomata postea inter heterocytis intercalaribus separantur; heterocytae sphaericae vel ovales, in trichomatibus adultis in positione terminali. Akineta solitaria vel in seriebus, ovalia vel cylindrica, apo- vel paraheterocytice evolventia.* – **Typus generis**: *Cronbergia siamensis* (Antarikanonda) comb. nova (basionym = *Anabaena siamensis* Antarikanonda, *Nova Hedwigia* 41: 345, 1985; syn.: *Richelia siamensis* (Antarikanonda) Hindák, Biologia Bratislava, 55(1): 3, 2000); exiccate no BRNM HY-2333.

List of species (Tab. 2)

Cronbergia siamensis (Antarikanonda) Komárek, Zapomělová *et* Hindák **comb. nov.**

Basionym: *Anabaena siamensis* Antarikanonda, *Nova Hedwigia* 41: 345, 1985.
Synonym: *Richelia siamensis* (Antarikanonda) Hindák, *Biologia Bratislava* 55(1): 3, 2000 **Figs 4-5**

Mucilaginous, bright blue-green thallus. Trichomes short, single, straight, distinctly constricted at cross-walls, 30-50 μm long. Cells cylindrical, isodiametric

Table 2. Diacritical characters and key to identification of morphospecies of the genus *Cronbergia*. The main diacritical characters are printed in bold.

	filaments		*cells*			*heterocytes*	*akinetes*			*ecology*
	length [µm]	*Nos of cells*	*width [µm]*	***shape***	*length [µm]*	***[µm]***	***position***	*shape*	***dimensions [µm]***	
siamensis	30-50		2.0-3.0	**cylindrical to rounded barrel shaped**	2.0-5.0	**2-4 in diam.**	**apoh., 3-more in series**	oval to subspherical	**5-10 in diam.**	**rice fields, Thailand**
paucicellularis	10-80(120)	– 35	2.5-3.5	**spherical to widely barrel shaped**	2.5-5.0	**3-5 × 3-3.5**	**parah. 1-4 in a row**	cylindrical to oval	**6-25 × 4.5-6**	**pools with vegetation, SW Slovakia**
planctonica	– 72	– 30	2.3-4.3	**± quadratic to elongated – cylindrical**	1.8-6.8	**3-5.9 × 2.3-3.2**	**parah. 1-2 in a row**	cylindrical to oval	**5.1-7.7 × 2.6-3.4**	**plankton in brackish waters, Sweden**
strain PCC 7417	– 600	> 60	3.6-5.4	**barrel-shaped**	± 3.6-5.4	**5-9.2 × 4.8-7.1**	**apoh., in series**	barrel-shaped	**4-11.2 × 4.5-10**	**isolated from soil in greenhouse, Sweden**

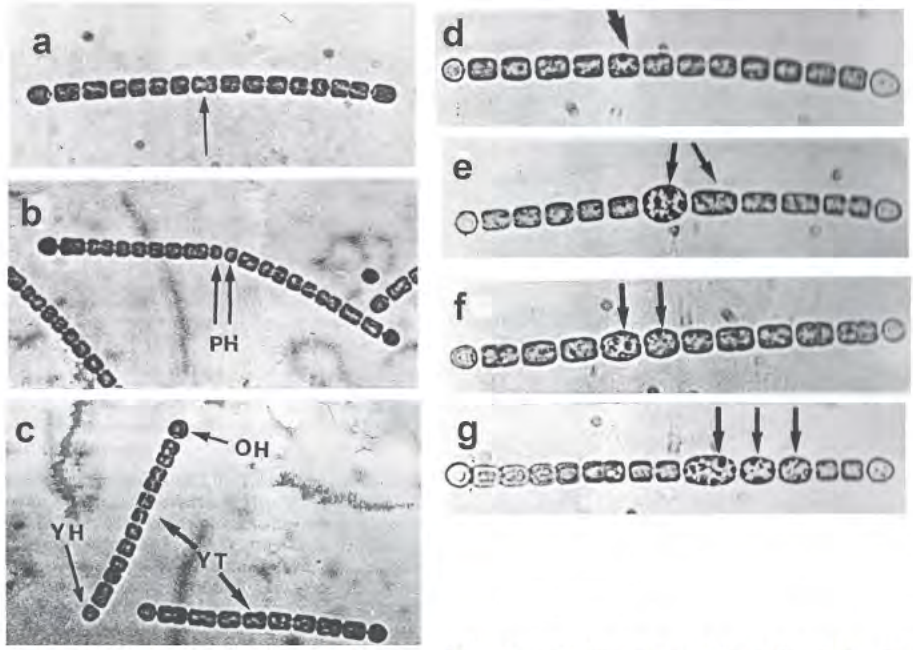

Fig. 4. *Cronbergia siamensis*, **a-c**: Trichomes with heterocyte formation. **d-g**: Akinete formation (From Antarikanonda 1985, type strain of "*Anabaena siamensis*").

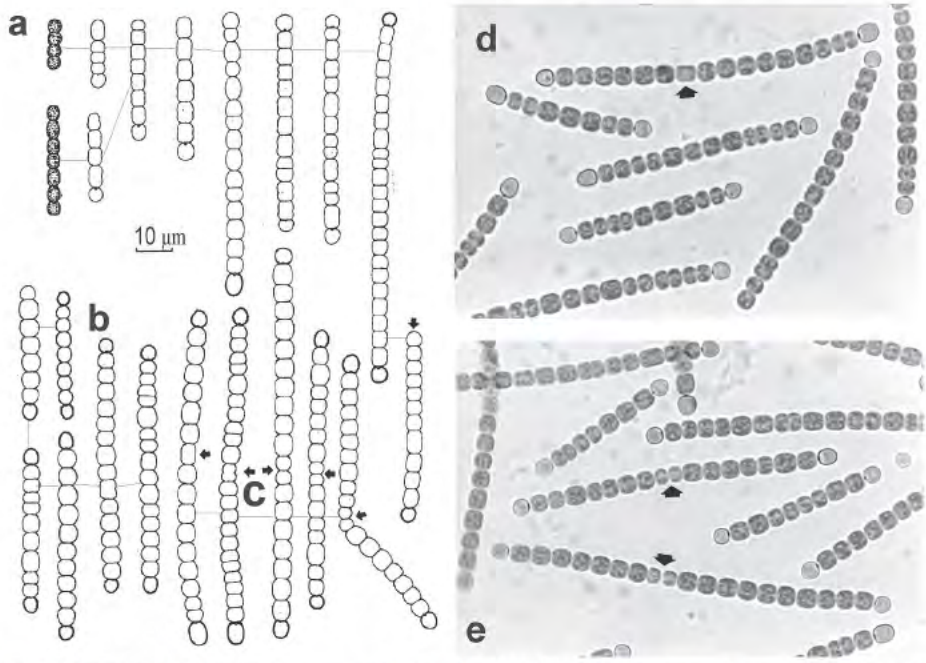

Fig. 5. *Cronbergia siamensis*, type strain SAG B11.82. Arrows in **c, d, e**: trichomes with developing heterocytes (From Hindák, 2000).

or longer than wide, without aerotopes, 2-5 × 2-3 μm. Heterocytes spherical or subspherical, terminal on both ends of a trichome, 2-4 μm in diameter. Akinetes are distant from heterocytes in series up to 3 (or more) in a row, oval to subspherical, 5-10 μm in diameter.

Occurrence: On moist soils and in paddy fields in Bangkok, Thailand.

Note: The type (reference) strain from *Anabaena* (= *Cronbergia*) *siamensis* exists (SAG B11.82 = CCALA 756) and the verification of generic characters is possible. Accession number in GenBank is GO389643. Hindák (2000, 2001, 2003, 2008) also identified as "*Richelia siamensis*" one population from marshes of central European Slovakia, with a similar morphology. However, later studies indicated that this population has to be described as a new species, *Cronbergia paucicellularis*.

Cronbergia paucicellularis sp. nov. **Fig. 6**

Diagnosis: *Fila sine vaginis conspicuis, libera, curta, recta vel paulum curvata, solitaria; trichomata paucicellularia (4-25-35-cellularia), 10-80(120) μm longa, ad dissepimenta*

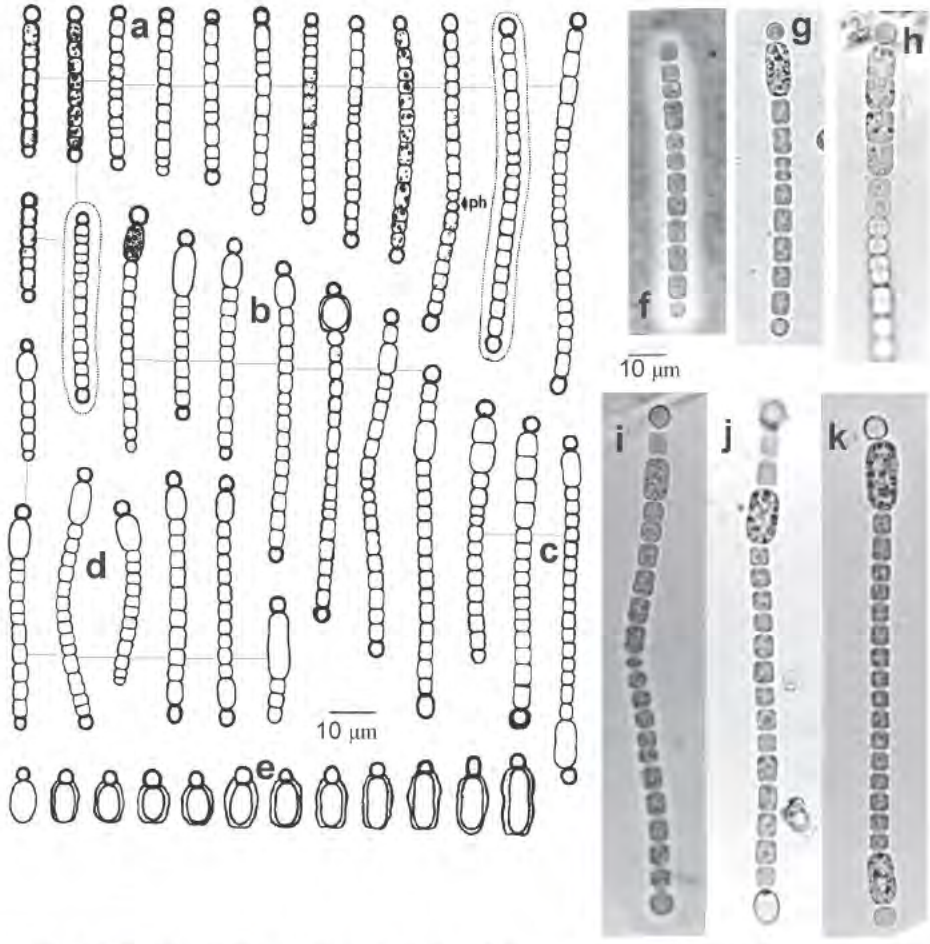

Fig. 6. *Cronbergia paucicellularis*, natural population. **b-d, g-k**: Akinete formation. **e**: form variability of akinetes.

valde constricta, cum heterocytis terminalibus, ad unum vel ambos apices evolutis, subsymmetrica. Cellulae cyanoprocaryoticae, plus minusve sphaericae, subsphaericae vel breviter barriliformes, contentu pallide aerugineo, sine aerotopis, 2.5-5 × 2.5-3.5 µm. Heterocytae solitariae, terminales, sphaericae, vel breviter ellipsoidales, 3.5 × 3-3.5 µm. Akineta ad heterocytas terminales evoluta, solitaria, vel ad quattuor in series ordinata, cylindrica, vel plus minusve ovalia, cum exosporio ad 1.5 µm crasso, rare de heterocytis cum 1-2 cellulis, pallide aeruginosis, paucim remota, (6)9-15(25) × 4.5-5(6) µm. – **Habitatio**: Periphytice metaphyticeque in stagnis prope Šúr, Slovakia occidentalis, aestate 1995 collecta (locus classicus). – **Holotypus**: figura nostra 6; since it was impossible to preserve an original specimen showing the features of the taxon (cf. ICBN, Art 37.5). (Because the original material is lost, we prefer the typification according to original figure.)

Trichomes solitary, free living in metaphyton, straight or slightly curved, symmetric, 4-25(35)-celled, 10-80(120) µm long, with one terminal heterocyte at one or both ends. Sheaths or enveloping mucilage not observed. Vegetative cells spherical, subspherical to short barrel-shaped, pale or bright blue-green, without aerotopes, 2.5-5 × 2.5-3.5 µm. Heterocytes in old trichomes terminal on both ends of trichomes, solitary, spherical to short barrel-shaped or broad oval, 3-5 × 3-3.5 µm. Akinetes arise facultatively aside heterocytes on both ends of trichomes, solitary, cylindrical or ± oval with a thick (1-1.5 µm) brown exospore, 2 up to 4 in a row, rarely separated from a heterocyte by 1-2 pale blue-green cells, (6)9-15(25) × 4.5-5(6) µm. **Occurrence**: In the metaphyton of a small pool in the Slovakian National Nature Reserve Šúr near Bratislava, and in backwaters "Číčovské mrtvé rameno" (the Číčov oxbow) near the Danube River, S Slovakia.

Cronbergia planctonica (Cronberg) Komárek, Zapomělová *et* Hindák **comb. nov.**

Basionym: *Cylindrospermum planctonicum* Cronberg, *Algological Studies* 109: 205, 2003 **Fig. 7**

Trichomes planktic, solitary, straight, blue-green, without sheaths, up to 30-celled, up to 72 µm long. Cells ± quadratic to elongated cylindrical, up to more than twice longer than wide, sometimes barrel-shaped, without aerotopes, 1.8-6.8 × 2.3-4.3 µm. Heterocytes terminal, solitary, spherical to elongated, cylindrical-oval, 3-5.9 × 2.3-3.2 µm, or they arise intercalarly in pairs. The intercalary heterocytes develop from a symmetric division of the cell in the middle of a trichome. Akinetes are adjacent to terminal heterocytes, sometimes in pairs, on both ends of a trichome, cylindrical and rounded at the ends, or ± oval, yellow on the surface, 5.1-7.7 × 2.6-3.4 µm. The trichomes break between the intercalary proheterocytes, which grow out and form new terminal heterocytes.

Occurrence: Planktic in water blooms in brackish water bays of Östhammarsfjärden, Uppland, Sweden. The "*Cylindrospermum* sp.", found on the western coast of Jylland (Denmark) by Grøntved (1960) belongs evidently to this species.

It is possible that also the strain PCC 7417 represents a new species of *Cronbergia.* This strain is identified as "*Cylindrospermum* sp." in the Pasteur Culture Collection and requires further studies. However, we describe here its morphology on the basis of the culture (cf. its phylogenetic position in Fig. 3).

Cronbergia sp.? ["*Cylindrospermum* sp." strain PCC 7417] **Fig. 8**

Description from cultures: Mats of densely and irregularly agglomerated trichomes. Trichomes slightly and irregularly bent or coiled, up to 600 µm long, but usually distinctly shorter, often disintegrating into immotile, few-celled

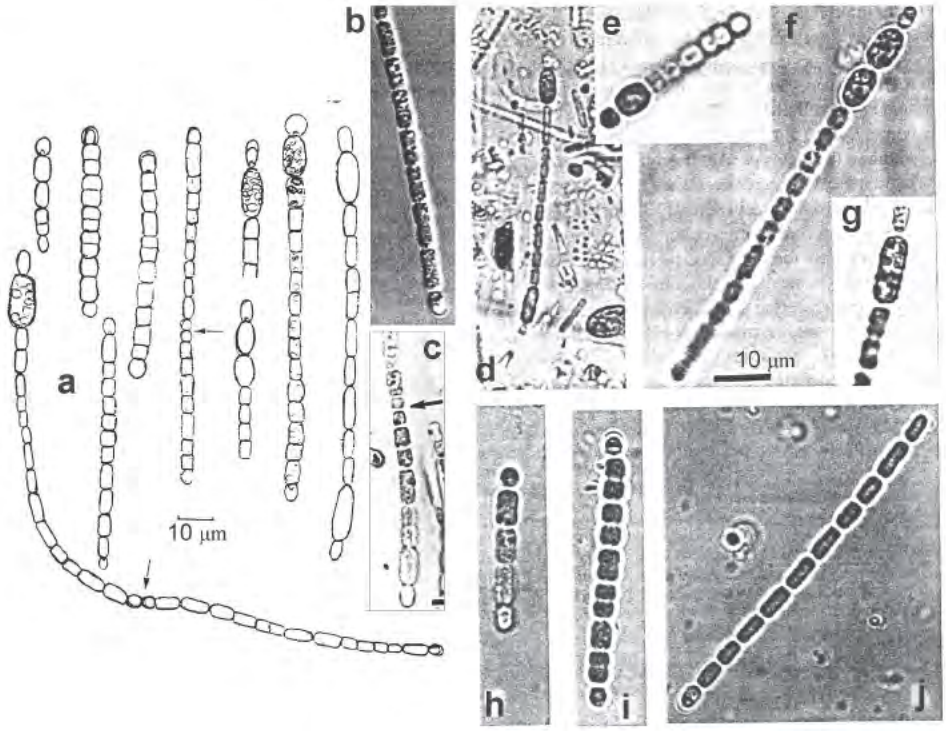

Fig. 7. *Cronbergia planctonica*, natural population; arrows = heterocyte formation. (From Cronberg 2003, as *Cylindrospermum planctonicum.*)

segments, without distinct sheaths, constricted at cross-walls. Cells cylindrical, ± quadratic or slightly longer or shorter than wide, with blue-green, often distinctly granulated content, less frequently almost barrel-shaped, 3.6-5.4 µm wide. Heterocytes develop terminally, solitary, exceptionally in pairs, very rarely intercalary, spherical to mostly oval, cylindrical-oval, 5-9.2 × 4.8-6.8(7.1) µm. Akinetes (ripen) observed rarely (on solid media), usually arise in rows, (4)5-11.2 × (4.5)5.8-7.5(10) µm, with ± homogeneous content, ± spherical, barrel-shaped up to cylindrical, sometimes the whole trichome changes in a series of akinetes. The trichomes often break into short segments, in which develop later the polar heterocytes.

Occurrence (data from PCC): Isolated from soil in greenhouse, Stockholm, Sweden, 1972.

Diacritical features with related genera (Fig. 2)

Cylindrospermum Kützing *ex* Bornet *et* Flahault: Phylogenetically closely related to *Cronbergia,* the 16S rRNA gene sequence similarity varies from 96.2 to 96.4% in different strains (Tab. 1). The most distinct phenotypic character that is common for both genera are the terminal heterocytes. This relation with *Cylindrospermum* was stressed particularly by Cronberg (2003). However, *Cronbergia* has shorter trichomes (only up to 80-120 µm) and akinetes develop

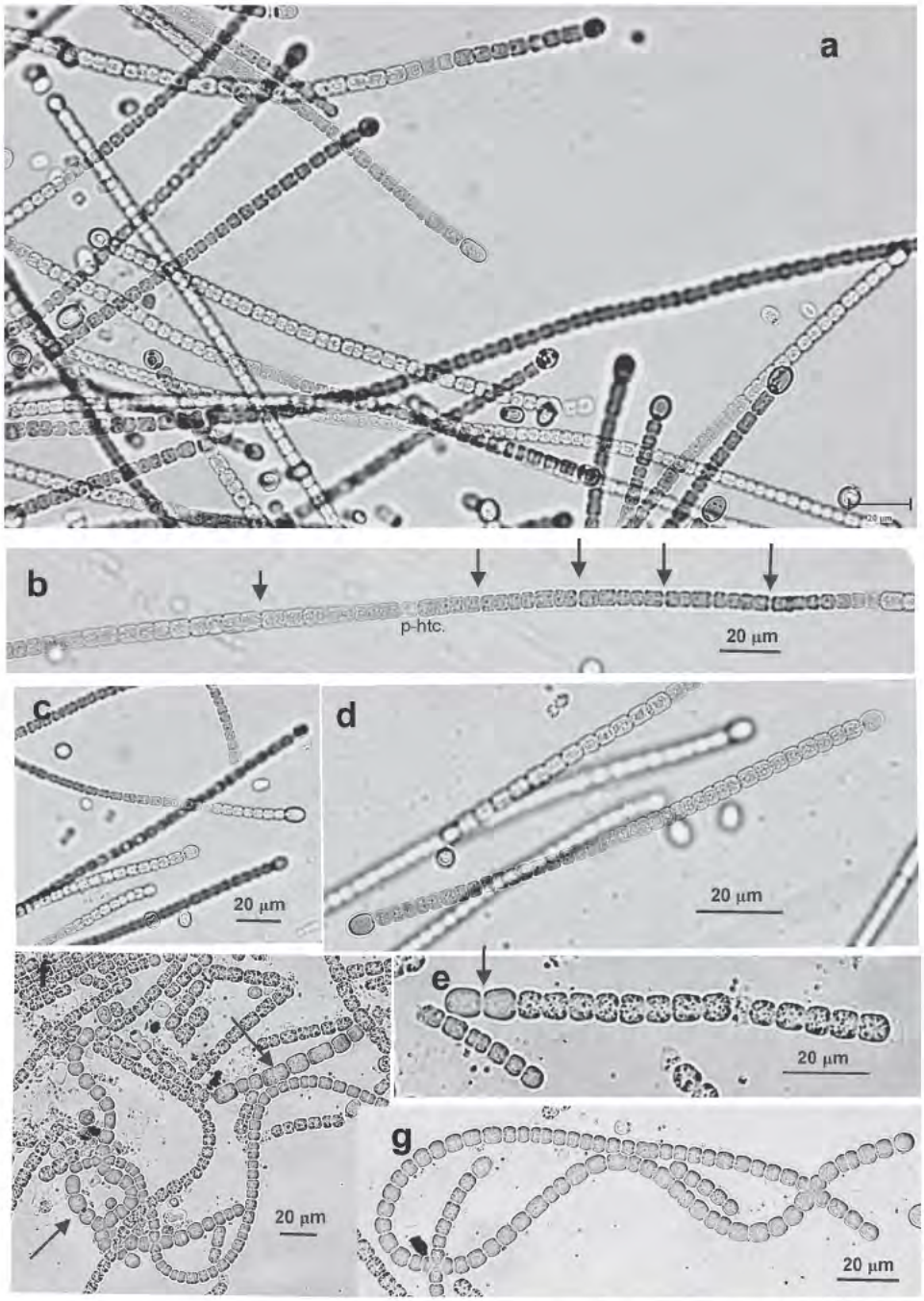

Fig. 8. "*Cylindrospermum/Cronbergia*" strain PCC 7417: **a, c-d**: Filaments from liquid medium. **b**: Disintegrating trichome and intercalar proheterocyte. **e**: Formation of akinetes. **f, g**: Rows of apoheterocytic akinetes.

either in paraheterocytic or paraheterocytic-distant position (separated by 1-4 vegetative cells from the heterocytes), or apoheterocytically (*C. siamensis*). This variability in position of akinetes does not occur in *Cylindrospermum*, which forms symmetrical trichomes with strictly paraheterocytic akinetes. The main phenotypic and biological difference between both genera is the strategy of heterocyte development, which is in principle intercalary in *Cronbergia*, while terminal in *Cylindrospermum* (cf. Schwabe 1970). The little known tropical species of *Cylindrospermum* with torulose, short trichomes (*C. breve* Welsh, *C. bourrellyi* Komárek) may also belong to the morphological complex of *Cronbergia*.

Richelia J. Schmidt: The terminal large spherical heterocytes at the ends of short trichomes and particularly their origin, which is probably intercalary from one mother cell, are the most distinctive features of this genus; these can be similar to *Cronbergia*. It was the main reason why the *Cronbergia*-morphotypes (including the original strain of "*Anabaena siamensis*") were classified to *Richelia* by Hindák (2000, 2003, 2008). Although no 16S rRNA sequences are available for *Richelia*, its generic identity with *Cronbergia* is improbable. *Richelia* is a tropical endobiotic cyanobacterium growing exclusively endophytically (inside) in frustules of planktic oceanic diatoms (*Hemiaulus, Rhizosolenia* and few others; Desikachary, 1959). Its akinetes are wider than long, develop irregularly paraheterocytically in rows, but their position towards heterocytes varies irregularly resembling more the genus *Nodularia* (Komárek & Komárková, 2002). The morphology and ecology of *Richelia* is therefore very specific. Nevertheless, its relation to *Cronbergia* is to be definitely solved by molecular methods.

Anabaenopsis (Wołoszyńska) Miller: This is another genus with more or less free-floating trichomes, which have heterocytes in a terminal position. However, this location of heterocytes is secondary, because they develop obligatorily in pairs in an intercalary position after mirror-like, asymmetrical division of neighbouring vegetative cells. The trichomes later disintegrate between the newly developed heterocytes. The intercalary development of heterocytes in pairs is therefore seemingly similar as in *Cronbergia*, but the process of their differentiation in *Cronbergia* from one (sometimes narrowed) vegetative cell after a symmetrical division of the mother cell is distinctly different (Figs 1, 2). Also the phylogenetic position (Fig. 3) of both types is markedly distant one from another (94.96% sequence similarity of the 16S rRNA gene; cf. Tab. 1).

Cylindrospermopsis Seenayya *et* Subba Raju: The terminal heterocytes develop only from terminal cells (= difference from *Cronbergia* or *Anabaenopsis*), but (in contrast to *Cylindrospermum*) after an asymmetrical division of the mother cell, similarly to *Anabaenopsis* (Jeeji Bai *et al.* 1977). *Cylindrospermopsis* is also characterized by subsymmetric trichomes. Similarly to *Cronbergia,* the akinetes are oval and joined or slightly distant from the terminal heterocytes, sometimes in short rows. Nevertheless, *Cronbergia* is clearly separated from this genus by a distant phylogenetic position (Fig. 3).

Nostoc Geoffrey *in* Linneaus *ex* Bornet *et* Flahault: This genus differs substantially morphologically from *Cronbergia* by apoheterocytic formation of akinetes and the development of single heterocytes in both intercalary and terminal positions. However, the 16S rRNA gene sequence similarity of typical *Nostoc*-strains of *N. calcicola* and *N. muscorum* to *Cronbergia* is 96.1-96.6% (Tab. 1). This means that the bacteriological generic criterium (95% of 16S rRNA gene sequence

similarity) concerns here several very distant genera and can be accepted only with corrections; its application must necessarily be combined with other evaluation methods.

Anabaena Bory *ex* Bornet *et* Flahault: This is another genus whose morphology, particularly the strategy of heterocyte formation, is distinctly different from the *Cronbergia*-type. Both genera are also clearly separated in both the Bayesian and Neighbour Joining phylogenetic trees (Fig. 3). However, the 16S rRNA gene sequence similarities to *Cronbergia* are 94.7-96.6% in some typical *Anabaena*-strains (Tab. 1), and the separation of both genera is similar as in the case of *Nostoc*.

DISCUSSION

Modern cyanoprokaryotic (cyanobacterial) taxonomy is based on a combined evaluation of genetic, cytomorphological, biochemical and ecophysiological characters (polyphasic approach), with emphasis on molecular markers. The method of 16S rRNA gene sequencing is considered as the basic method to characterize taxa at the generic level (Stackebrandt & Goebel, 1994; Castenholz, 2001). Revised genera should have other diacritical markers without transitional forms (autapomorphic features). This evaluation process is surely the most prospective method in the modern reorganization of the cyanobacterial classification system, but we meet many problems in the consistent application of polyphasic classification methods.

Recently, numerous genera whose phylogenetic positions were in good agreement with morphological and ecological characters (*Halothece, Microcystis, Planktothrix, Aphanizomenon, Cylindrospermum, Cylindrospermopsis, Cuspidothrix* and others) have already been re-defined by the combined methodological procedure (Suda *et al.,* 2002; Rajaniemi *et al.,* 2005; Margheri *et al.,* 2008; Komárek, 2010). Many traditional genera appear to be polyphyletic and heterogeneous, and their taxonomic revision is the subject of recent studies (*Anabaena/Dolichospermum/Sphaerospermopsis, Nostoc, Trichormus* and others; cf. Rajaniemi *et al.,* 2005; Řeháková *et al.,* 2007; Zapomělová *et al.,* 2009, 2010). Moreover, numerous "small" groups of morpho- and ecotypes exist, which do not evidently belong to any of the revised clusters. They have special and unique characteristics eliminating them from the known generic entities, but their definition by commonly used genetic and/or morphological criteria is complex. *Cronbergia* belongs also to these enigmatic perturbances in cyanobacterial diversity and taxonomic classification.

The whole group of several related *Cronbergia*-species (*C. siamensis, C. paucicellularis,* and *C. planctonica*) shows a quite unique character, which does not occur in any other cyanobacteria, i.e. the differentiation of heterocytes intercalarly in pairs from one mother vegetative cell. The phylogenetic affiliation of *Anabaenopsis,* whose heterocyte differentiation is the most similar to *Cronbergia* (pairs of intercalary heterocytes but arising from two adjacent vegetative cells after their asymmetric division), is markedly distant from *Cronbergia* (Figs 1, 2). According to the genetic evaluation (16S rRNA gene sequences), *Anabaenopsis* has a more distant position from *Cronbergia* (Fig. 3) than, e.g., *Nostoc* and *Anabaena*. *Cronbergia* represents therefore a

phylogenetically and morphologically unique cluster with one very special biological character, which is separated from all other heterocytous types.

Our phylogenetic trees were based on relatively long sequences of the 16S rRNA gene (1443 bp) and the significance of these results can thus be considered high. *Cronbergia siamensis* SAG B11.82 was markedly separated from other heterocytous genera except the *Cylindrospermum* strains, especially the strain *Cylindrospermum* sp. PCC 7417. However, the identification of this strain is problematic; it has more characters identical with *Cronbergia,* and it surely belongs rather to this new genus than to *Cylindrospermum*. The development of intercalary heterocytes in pairs was not clearly found in this strain. In spite of important morphological differences between *Cronbergia* and *Cylindrospermum*, sequences of more strains from the morphological complex of *Cronbergia* need to be analyzed to verify their genetic consistency or diversity. Moreover, other genomic regions need to be analysed to clarify the situation of similar types of cyanobacteria.

In terms of bacteriological taxonomy, *Cronbergia* does not correspond to the main criterion of genus definition, since the percentage of 16S rRNA gene sequence similarity to the most related genera is not less than the required 95% *(Cylindrospermum, Nostoc, Anabaena)* (Tab. 1). Unification with the genus *Cylindrospermum* could be considered, however there is no hesitation about the existence also of the related genera *Nostoc* and *Anabaena,* nor about the separation of *Cronbergia* from them. *Cronbergia* versus *Cylindrospermum* has a similar position as, e.g., *Cyanospira* versus *Anabaenopsis* (cf. Fig. 3). From recent experience therefore it follows that strict application of the 95% limit of 16S rRNA gene sequence similarity for genera and particularly 97-98% for species (Wayne *et al.,* 1987; Stackebrandt & Goebel, 1994) can itself hardly be used obligatorily. Similarity values below this limit can be considered as the criterion for the separation of cyanobacterial clusters at the generic level, but a similarity of about and over 95% can be only one indicating feature, which has to be combined with other criteria, e.g., with a distinct autapomorphic or another molecular or cytological markers (cf. Ferris *et al.,* 2003; Johansen & Casamatta 2005; Ward, 2006; and others). Besides, multi-locus analysis of several housekeeping genes could help to resolve the phylogenetic and taxonomic status of the *Cronbergia* complex, as well as other above-mentioned problematic genera. This is, however, hampered by a lack of comparative genetic data from most of the related nostocacean genera. As mentioned above, the relationships of *Cronbergia* with the genus *Richelia* (type species *R. intracellularis*) should be clarified by molecular methods.

Another apparent confusion in the case of *Cronbergia* is the formation of akinetes, which is in a few aspects contradictory to the other principal criteria of cyanoprokaryotic taxonomy of nostocacean types. The paraheterocytic or apoheterocytic origin of akinetes is in coincidence with the phylogenetic delimitation of the majority of the revised cyanoprokaryotic genera. However, three very similar morphospecies with a common important biological marker (type of heterocyte formation) exist within the group of *Cronbergia* that differ not only in ecology and distribution, but also just in the strategy of akinete formation. Their akinetes have more or less similar morphology, but in the type species (*C. siamensis*) they develop in the middle of trichomes between two heterocytes, while in the other two morphospecies they develop paraheterocytically aside the heterocytes or slightly distant from them, separated by several vegetative cells. Akinetes develop successively up to 4 in rows, but in *C. siamensis* also "all vegetative cells develop into akinetes in older cultures" (Antarikanonda, 1985).

The same formation of akinetes was observed also in the most related strain "*Cylindrospermum/Cronbergia*" sp. PCC 7417. The irregular origin of akinetes in rows (combined paraheterocytical and apoheterocytical development) is known also in *Nodularia.* In *Cronbergia,* the para- or apoheterocytic formation of akinetes seems to vary and not to be coincident with the unique character of heterocyte development.

At least two important features (percentage of 16S rRNA gene sequence similarity and the polymorphic type of akinete formation) are in disagreement with the main taxonomic principles for establishment of a new genus. On the contrary, the special cluster (genus) of heterocytous cyanoprokaryotes in the phylogenetic tree is supported mainly by the unique strategy of heterocyte development, the common morphology of filaments and also by the fact that this cluster cannot be classified into any other genus without other serious reservations. The proposal of a new genus is, therefore, the only possible solution. It follows from all recent results that such clusters of nostocacean cyanoprokaryotes represent always special genetic and generic units after a combined evaluation (Komárek, 2010).

From the modern taxonomic evaluation of cyanoprokaryotic diversity it also follows that the regulation and unification of any common criteria for various taxa (genera, species) is impossible. Cyanoprokaryotes are phylogenetically very old bacterial organisms and the diversification processes during their long existence occurred in different ways in different genotypes and in different ecological situations. It is therefore difficult to apply the same criteria in all groups. The combined polyphasic approach (as wide as possible) is surely necessary for the re-evaluation of cyanobacterial diversity, but evidently various characters have different values and importance in different cyanobacterial clades, for whose characterization we must also use different diacritical criteria.

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