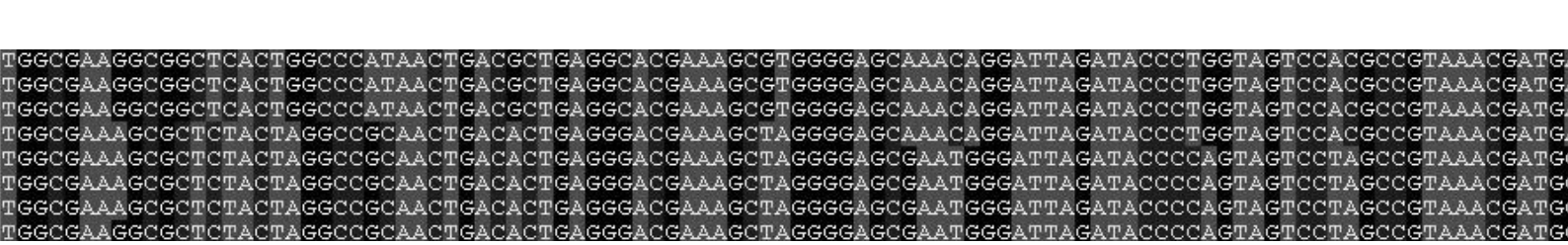

University of South Bohemia in České Budějovice
Faculty of Science

MSc thesis

Phylogeny of heterocytous Cyanobacteria (Nostocales and Stigonematales)

Jana Korelusová

2008

Supervisor: RNDr. Jan Kaštovský, Ph.D.

MSc. thesis

Korelusová, J. (2008): Phylogeny of heterocytous cyanobacteria (Nostocales and Stigonematales), MSc. Thesis, in English. - 33pp., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation

16S rDNA sequences from 23 heterocytous cyanobacteria were obtained and phylogenetic tree from these sequences and sequences available in GenBank was constructed. Relationships between traditional taxonomy and phylogenetic clusters were discussed.

Prohlašuji, že svoji diplomovou práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.
Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své diplomové práce, a to v nezkrácené podobě elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách.
V Českých Budějovicích 24.4.2008

Jana Korelusová

Poděkování

Na tomto místě bych chtěla poděkovat všem, co mě podpořili psychicky nebo fyzicky při mojí práci. Především děkuji Hanysovi, svému školiteli, za potřebné rady, finanční zázemí a přátelskou atmosféru, které umožnily se práci věnovat. Z odborného hlediska děkuji panu profesorovi Komárkovi, za neustálé rozšiřování obzorů a zájmu o taxonomii sinic. Dále pak děkuji lidem z laboratoře molekulární fylogenetiky, kteří mi pomohli se trochu zorientovat v této rozsáhlé problematice a také všem, kteří mi poskytli nějaké kmeny sinic použité pro tuto práci. Rodině a všem kamarádům děkuji za podporu v tom, co dělám. Doufám, že Markétě a Kristýně budu moci někdy vrátit takovou službu, jakou je oprava mojí angličtiny!

Table of contents

1. Introduction

1.1. Phylogeny of cyanobacteria

Taxonomic classification is a method to obtain a review of the studied organisms in their whole variation possibilities and their relations. It is a way to understand the biodiversity and to estimate its dimensions. Unfortunately, there are no definite methods of classification. Nowadays, methods are improving and new methods appear. Hence, the system of Cyanobacteria is constantly in process. The classification is discussed and revised (e.g. Komárek and Anagnostidis 1986, 1989, Anagnostidis and Komárek 1988, 1990, Turner 1997, Castenholz 2001). New data from ultrastructure studies, ecological analyses and particularly from a molecular biology have considerably changed the cyanobacterial taxonomy in last years. Modern combined approach allows greater recognition and more exact definition of the width of cyanobacterial diversity.

Classification of cyanobacteria is still being resolved by easily applicable morphological and ecological features. New scientific period yields also new methods - mainly in molecular biology. Unfortunately, in spite of big amount of new molecular data, there is a lot of misinformation and errors. The data are cumulated in on-line databases (NCBI, The European ribosomal RNA database) not providing an easy survey. Misidentifications are common and data about taxonomy and ecology are missing. Even known mistakes are not corrected. In most cases, new information is added beside the wrong one making the whole situation more complicated. Despite this fact, on-line databases offer a huge supply of under-used or unused information (but a critical view is needed for using these data).

Confusion in cyanobacterial nomenclature is caused also by an effort to place nomenclature of Cyanobacteria under the rules of International Code of Nomenclature of Bacteria (Stanier 1978). Primarily, only botanical code was valid, bacteriological code was validated later and used simultaneously. Taxa validly described under the bacteriological code are summarized in Bergey's manual of Systematic Bacteriology (Castenholz 2001), where 63 form-genera are listed. This means that most of the Cyanobacteria still do not have valid bacteriological name. Up to date, total number of validly described cyanobacterial genera under botanical code is 255 (Komárek and Hauer 2004). This number is only a small part of worldwide diversity (especially many of the very diverse tropical taxons remain unexplored). Only 101 genera have their 16S rDNA sequenced. Molecular data seem to be very important for taxonomy and further phylogenetic investigations, revealing necessity of separation of polyphyletic taxa into a number of narrower monophyletic genera or cryptogenera. Acquirement of suitable 16S rDNA cyanobacterial sequence is limited by the ability of cyanobacterium to grow in culture or by natural occurrence in monotypic colonies allowing DNA isolation. These conditions are usually hardly achievable and therefore so few types are sequenced so far.

Currently used classification system divides Cyanobacteria into five sections (Section I: order Chroococcales (Wettstein 1924 emend. Rippka *et al*. 1979), Section II: order Pleurocapsales (Geitler

1925 emend. Waterbury and Stanier 1978), Section III: order Oscillatoriales (Elenkin 1934), Section IV: order Nostocales (Geitler 1925 emend. Castenholz 1989), Section V: order Stigonematales (Geitler 1925)). Phylogenetic analyses of Cyanobacteria based on 16S rDNA were carried out by several research groups (Nelissen et al. 1996, Ishida et al. 1997, Honda et al. 1999, Turner et al. 1999, Garcia-Pichel et al. 2001) and indicated that Chroococcales (I) and Oscillatoriales (III) were polyphyletic. In 2001, the monophyly of Pleurocapsales (II) was denied (Ishida et al. 2001). Heterocytes forming Nostocales (IV) and Stigonematales (V) were found to be monophyletic (Giovannoni et al. 1988, Wilmotte et al. 1994, Nelissen et al. 1996, Turner 1997, Turner et al. 1999, Wilmotte and Herdman 2001, Lyra et al. 2001, Gugger and Hoffmann 2004).

Nowadays, scepticism to resolve branching of big clusters by sequencing of 16S rDNA has appeared (Casamatta et al. 2005). Giovannoni et al. (1988) suggested that all branches on the basis diverged in a very short interval of evolutionary distance. Later authors hypothesized that this topology may reflect evolutionary invention of oxygenic photosynthesis, which allowed an explosive radiation of the cyanobacteria in a short time span. No clearly resolved relationships among the clusters might be explained by this rapid radiation or by recombination within 16S rDNA sufficient to scramble phylogenetic signal (e.g. Yap et al. 1999, Boucher et al. 2004, Miller et al. 2005, Morandi et al. 2005). By this theory, reconstruction of phylogeny of the whole cyanobacteria using only 16S rDNA is impossible, whereas partial problems of monophyletic groups can be explained adequately by this means. Relationships within a big monophyletic group (e.g. heterocytous cyanobacteria) can be confused by a lot of “loner” sequences. Their unstable position in the tree can be caused by their relatively large evolutionary distances from the other investigated cyanobacteria and consecutive attraction to unrelated sequences ("long branches attraction"). The origin of these false attractions is probably a combination of different factors and it is linked to the fact that false similarities might arise between groups without real phylogenetic affinities by chance. The absence of organisms at intermediate levels of relatedness prevent from inferring which identities are real and which occurred accidentally (Ludwig et al. 1998). Causal grouping of unrelated sequences distinctively lowers bootstrap values all over the tree. Short partial sequences disable usage of some positions of alignment in analyses and therefore also decrease the support of single clusters.

Cyanobacterial genomes reveal a complex evolutionary history, which cannot be represented by a single strictly bifurcating tree for all genes or even most genes (Zhaxybayeva et al. 2006). Horizontal or lateral gene transfer (HGT), potentially followed by recombination with or replacement of resident homologs, is now recognized as a major force shaping evolutionary histories of both prokaryotes and eukaryotes (e.g. Koonin et al. 2001). For example, genes for tRNA synthetases (also in Cyanobacteria) are known to have complex evolutionary histories involving multiple HGT events (Wolf et al. 1999, Zhaxybayeva et al. 2006). 16S rDNA gene was considered the most suitable for phylogenetic analyses providing information not affected by horizontal gene transfer. However, it was denied by Miller et al. (2005), who found cyanobacterium with incorporated bacterial loop in its 16S rDNA. Cyanobacteria

can contain multiple rrn operons and intra-genomic sequence heterogeneity of the 16S rDNA genes, which is inconsistent with phylogenetic use of 16S rDNA for this group (Iteman et al. 2002, Lokmer 2007).

Despite these facts, 16S rDNA gene remains for its availability the most used and useful tool in molecular phylogeny at the genus level.

1.2. Heterocytes forming cyanobacteria

All the cyanobacteria with heterocytes studied so far evolved from a common ancestor (Gugger and Hoffmann 2004). Monophyly of the heterocyte forming cyanobacteria is supported by 16S rDNA gene sequences (Giovannoni et al. 1988, Wilmotte et al. 1994, Nelissen et al. 1996, Turner 1997, Turner et al. 1999, Wilmotte and Herdman 2001, Lyra et al. 2001), *nif*D sequences (Henson et al. 2004), *nif*H sequences (Zehr et al. 1997), *gyr*B, *rpo*C1, *rpo*D1 (Seo et al. 2003) and RFLP and genomic fingerprinting (Lyra et al. 2001). Heterocytous cluster corresponds to a homogenous genotypic lineage well supported by the bootstrap analysis and consists of Nostocales and Stigonematales (subsection IV and V of Bergey's Manual, Castenholz 2001). The first subgroup consists of filamentous heterocytous cyanobacteria dividing always in a plane at right angles to the long axis of the trichome and therefore they are uniseriate and lack true branching. They are grouped in Subsection IV according to the proposed bacteriological classification (Rippka et al. 1979, Castenholz 2001) and in the order Nostocales in the traditional classification system (Komárek and Anagnostidis 1989). The second subgroup consists of filamentous heterocytous cyanobacteria in which longitudinal and oblique cell division occurs in addition to transverse cell division resulting in periodic true branching in all genera and in multiseriate trichomes (two or more rows of cells) in some genera. They are classified in subsection V (formerly order Stigonematales) (Rippka et al. 1979, Anagnostidis and Komárek 1990, Castenholz 2001). Analyses of new Stigonematales strains show that the true branching cyanobacteria are polyphyletic and should be separated into at least two major groups based on the branching. The first group is characterised by T-branching and the second group by Y-branching (Anagnostidis and Komárek 1990, Gugger and Hoffmann 2004).

A complex study of grouping within heterocytous cluster has not been performed so far, but there are some papers dealing with the phylogeny and taxonomy of some particular taxons showing that the traditional classification needs to be revised (e.g. Rajaniemi et al. 2005, Sihvonen et al. 2007) Many genera were found to be polyphyletic: *Anabaena, Aphanizomenon, Trichormus* (Lyra et al. 2001, Gugger et al. 2002, Iteman et al. 2002, Rajaniemi 2005), *Tolypothrix, Calothrix* (Sihvonen et al. 2007). Furthermore, the evolutionary distances among cyanobacteria morphologically identified as *Calothrix* suggest that they belong to at least five different genera. Correlation between the genetic grouping and morphology was found. However, the morphology alone seems to be insufficient for distinguishing different 16S rDNA clusters. Also higher taxa such as family Rivulariaceae in the

botanical code and subsection IV.II in the proposed bacterial classification (Rippka et al. 1979, Castenholz 2001) appeared not to be monophyletic (Sihvonen et al. 2007). *Anabaena, Aphanizomenon, Trichormus* and *Nostoc* strains examined by Rajaniemi et al. (2005) consistently formed six clusters in the analyses of 16S rDNA, *rpo*B and *rbc*LX gene with strain of different genera intermixed. Furthermore, the separation of the genera *Nostoc* and *Anabaena* has also been discussed in recent years (Tamas et al. 2000, Henson et al. 2002). In the other hand, some morphologically defined taxa seem to be in good correspondence with molecular data: *Cylindrospermopsis* (Saker and Neilan 2001, Dyble et al. 2002, Neilan et al. 2003), *Nodularia* (Lehtimaki *et al*. 2000, Laamanen 2001, Moffitt *et al*. 2001), *Anabaena circinalis* (Beltran and Neilan 2000). In general, it can be concluded that cyanobacterial phylogenetic studies have demonstrated that genetic relationships conflict with the morphological classification (Lyra et al. 2001, Iteman et al. 2002, Gugger and Hoffmann 2004).

1.3. Aims of the thesis

The main aim of this work was to reconstruct the evolution of heterocytous cyanobacteria by means of molecular phylogeny. Large phylogenetic studies dealing with the phylogenetic relationships of whole orders or phylum were executed many years ago (Turner 1997, Honda et al. 1999, Ishida et al. 2001, Litvaitis 2002, Gugger and Hoffmann 2004, Henson et al. 2004). Since that time, number of sequences in GenBank has increased exponentially. The higher number of sequences, the more information, but also the more errors and taxa misidentification and that is the main reason why scientists focused on smaller analyses dealing with phylogenies involving only a single genera or few related taxa (e.g. Bolch et al. 1999, Boyer et al. 2002, Casamatta et al. 2005, Rajaniemi et al. 2005, Sihvonen et al. 2007, Marquardt and Palinska 2007, Palinska and Marquardt 2008, Papaefthimiou et al. 2008). Most of the recent works are interested only in water bloom forming cyanobacteria, representing only a small part of cyanobacterial diversity. Progress in phylogeny tends toward sequencing of whole genomes and building phylogenetic trees from such a large datasets (Zhaxybayeva et al. 2006, Shi and Falkowski 2008). As in 1988 only 29 cyanobacterial 16S rDNA sequences were available (Giovannoni et al. 1988), nowadays 13 whole genomes of cyanobacteria are freely accessible (Shi and Falkowski 2008). Despite all modern options and large gaps in knowledge of 16S rDNA gene, it remains the most extensively used phylogenetic tool. Every new sequence adds additional information and helps to improve cyanobacterial tree topology. Many genera still remain without any sequence and therefore each new sequence is important (particularly when representing non-sequenced genus or non-sequenced cluster of some genera). Thus, the next aim of this work was to get new sequences of important heterocytous genera to fill the holes in tree topology.

In terms of constructed phylogenetic tree, I tried to resolve these problems:

- What are the relationships between molecularly defined clusters and their morphological and ecological characteristics?
- Which level of traditional taxonomy matches the molecular phylogeny? (from species to orders)
- Is number of planes of a cell division a good taxonomic characteristic? (branching vs. non-branching; Nostocales vs. Stigonematales)
- Is it possible, that true and false branching of cyanobacteria have common morphological and phylogenetic origin?

2. Material and methods

Strains used for this work were collected by the author or collectors named in Tab.1. Some strains were purchased from culture collections. The strains collected by the author were isolated by successive purifying during cultivation on 1.5% agar plates with BG11 medium (Stanier et al. 1971). Strains were maintained under artificial light with 12-12 light/dark regime and temperature of 22˚C.

Tab.1 Strains used for 16S rDNA sequencing and their origin. Papua-New Guinea strains were collected by J. Korelusová and K. Mohlová in 2006.

Strain	**Origin**
Brasilonema sp. PAP144b	Wet stones, waterfall in Ohu, lowland tropical rain forest, Papua New Guinea (PNG)
Brasilonema sp. PAP148	Wet stones, waterfall in Ohu, lowland tropical rain forest, PNG
Brasilonema sp. PAP153	On the bark of coconut palm, Ohu, lowland tropical rain forest, PNG
Brasilonema sp. PAP158	Wet stones, waterfall in Ohu, lowland tropical rain forest, PNG
Brasilonema sp. PAP144a	Wet stones, waterfall in Ohu, lowland tropical rain forest, PNG
Scytonema sp. PAP148	Wet stones, waterfall in Ohu, lowland tropical rain forest, PNG
Fischerella sp. PAP92	On stone in fresh water creek, Wannang, lowland tropical rain forest, PNG
Parthasarthiella sp. PAP155	Wet stones, waterfall in Ohu, lowland tropical rain forest, PNG
Parthasarthiella sp. PAP156	Wet stones, waterfall in Ohu, lowland tropical rain forest, PNG
Fischerella sp. HAN	Soil, India (collected by Adhikary)
Stigonema mamillosum SM	Wet stones near a lake, South Norway (collected by J. Korelusová, 2007)
Calothrix sp. PAP153	On the bark of coconut palm, Ohu, lowland tropical rain forest, PNG
Tolypothrix elenkinii CCALA 195	Wet stone wall, Traunstein, Austria, CCALA culture colection
Tolypothrix elenkinii CCALA 10071	Soil, CCALA culture colection
Tolypothrix distorta CCALA 194	Soil, flower pot, Netherlands, CCALA culture colection
Tolypothrix tenuis CCALA 197	Basin, Botanical garden of Charles University, Prague, Czech Republic, CCALA culture colection
Tolypothrix sp. TOM	Soil (collected by Tomáš Hauer)
Hassallia sp. ŠKALOUD	Soil (collected by Pavel Škaloud)
Hassallia byssoidea CCALA 823	Granitic rock, Znojmo, Czech Republic, CCALA culture colection
Cylindrospermum sp. 10	Soil, culture collection of Alena Lukešová
Cylindrospermum sp. 16	Soil, culture collection of Alena Lukešová
Microchaete sp. PAP148	Wet stones, waterfall in Ohu, lowland tropical rain forest, PNG
Nostoc sp. PAP81	On nude roots of a tree near a creek, Wannang, lowland tropical rain forest, PNG

2.1. DNA isolation and PCR amplification

Total genomic DNA was isolated from cultured cyanobacterial cells using Invisorb® Spin Plant Mini Kit following the protocol and stored in -20˚C. Living cells were previously broken up in a mini-

beadbeater using a mixture of 0.1, 0.5 and 1.0 mm diameter glass beads. 16S rRNA gene and associated 16S-23S ITS region from the genomic DNA of strains were amplified by PCR using the oligonucleotide primers: primer 1 (CTC TGT GTG CCT AGG TAT CC) (Wilmotte et al. 1993) and primer 2 (GGG GAA TTT TCC GCA ATG GG) (Nübel et al. 1997). Amplification was performed in a Biometra® T3 thermocycler using 25 µl reactions containing 10-20 ng of genomic DNA, 50 pmol of each oligonucleotide primer, 200 µM dNTP, 10x *Taq* reaction buffer and 1 unit of *Taq* DNA polymerase. Reactions were cycled with an initial denaturation step in 95°C for 5 min, followed by 35 cyc1es of DNA denaturation in 94°C for 1 min, primers annealing in 55°C for 45 s, strand extension in 72°C for 2 min and a final extension step in 72°C for 10 min. PCR products were analysed on 1 % agarose gels.

2.2. Sequencing of 16S rRNA

PCR products of expected size (approximately 1600 base pairs in length) were purified from the gel using QIAquick gel extraction kits (Qiagen) and sequenced directly by cycle sequencing using the BigDye™ Big Dye Terminator Cycle Sequencing V3.1 (Perkin-Elmer). Primers used for the cycle sequencing of 16S rRNA were: the primer 1 and primer 2 (used for PCR amplification) and the internal primers- primer 5 (TGT ACA CAC CGG CCC GTC) (Wilmotte et al. 1993), primer 6 (GAC GGG CCG GTG TGT ACA) (reverse complement of primer 5), primer 7 (AAT GGG ATT AGA TAC CCC AGT AGT C) (Nübel et al. 1997), primer 8 (AAG GAG GTG ATC CAG CCA CA) (Wilmotte et al. 1993). The cycle sequencing reaction started in 94°C for 1 min followed by 30 cycles of the following: 30s in 94°C, 30s in 50°C, and 4 min in 60°C. Both strands were sequenced, so that each region was available in at least two independent reads. The 16S rRNA sequenced fragments were assembled into contigs using the software EditSeq™ and SeqMan™ II software (DNAStar, Madison, WI, USA).

2.3. Selection of sequences for analyses

For the phylogenetic analysis all available 16S rDNA sequences of heterocytous cyanobacteria were downloaded from the GenBank. Shorter sequences were included only in preliminary analysis (Neighbor Joining (NJ) method of building trees). In further analyses, short sequences (less than 1000 b) were used only if representing a separate cluster or insufficiently sampled genera. From well-supported clusters, only a few sequences covering the generic diversity were chosen for further analyses and some problematic loner sequences with low bootstrap support were excluded. Only generic level was used for construction of phylogenetic relationships thus only genera are discussed below. Species designation is used only if generic level seems to be insufficient and division into more genera according to species is proposed.

2.4. Alignment and phylogenetic analyses

The nucleotide sequences of 16S rRNA acquired in this study and related ingroup and outgroup sequences obtained from the GenBank (www.ncbi.nlm.nih.gov) were aligned by MAFFT (Katoh et al. 2005) and ambiguous or hypervariable sites were removed using BioEdit 7.0.4.1 (Hall 1999). Alignment was analysed by Maximum parsimony (MP), Neighbour-Joining (NJ) and Maximum Likelihood (ML) methods, using PAUP*4.0b10 (Swofford 2002), Mega version 4 (Tamura et al. 2007) and PHYML Online (Guindon et al. 2005) with each topology verified using 500 bootstrap replications. Trees were edited using TreeView version 1.6.6 (Page 1996) and rooted using 16S rDNA sequence of *Bacillus subtillis*.

ML trees were constructed by PHYML Online (Guindon et al. 2005) using default settings with GTR model for nucleotide substitutions with discrete gamma distribution in 4 categories; all parameters (gamma shape, proportion of invariants) were estimated from the dataset. ML bootstrap support was computed in 500 replicates. MP analyses were performed using PAUP*4.0b10 (Swofford 2002) and Mega software (Tamura et al. 2007). MP trees were generated using heuristic search constrained by random sequence addition with TBR as a branch-swapping method and 500 bootstrap replicates, gaps were excluded from the analysis. NJ trees were constructed using Mega (Tamura et al. 2007) with 500 bootstrap replications.

3. Results and discussion

23 sequences of partial 16S rDNA and partial 16S-23S ITS of heterocytous cyanobacteria were obtained (Tab.1). These sequences together with 601 sequences of the 16S rDNA of 38 genera of heterocytous cyanobacteria from the GenBank (225 sequences of *Nostoc* and 201 sequences of *Anabaena* and *Aphanizomenon*) were used for the phylogenetic analysis.

Tab. 2 Sequences of 16S rDNA obtained in this work divided by clusters they belong to (see Fig.1).

Cluster H1 *Brasilonema* sp. PAP144b *Brasilonema* sp. PAP148 *Brasilonema* sp. PAP153 *Brasilonema* sp. PAP158 *Brasilonema* sp. PAP144a *Scytonema* sp. PAP148	**Cluster H2** *Calothrix* sp. PAP153 *Tolypothrix elenkinii* CCALA 195 *Tolypothrix elenkinii* CCALA 10071
Cluster H3 *Fischerella* sp. PAP92 *Parthasarthiella* sp. PAP155 *Parthasarthiella* sp. PAP156 *Fischerella* sp. HAN	**Cluster H5** *Tolypothrix distorta* CCALA 194 *Tolypothrix tenuis* CCALA 197 *Tolypothrix* sp. TOM *Hassallia* sp. ŠKALOUD *Hassallia byssoidea* CCALA 823 *Cylindrospermum* sp. 10 *Cylindrospermum* sp. 16 *Microchaete* sp. PAP148 *Nostoc* sp. PAP81
Cluster H4 *Stigonema mamillosum* SM	

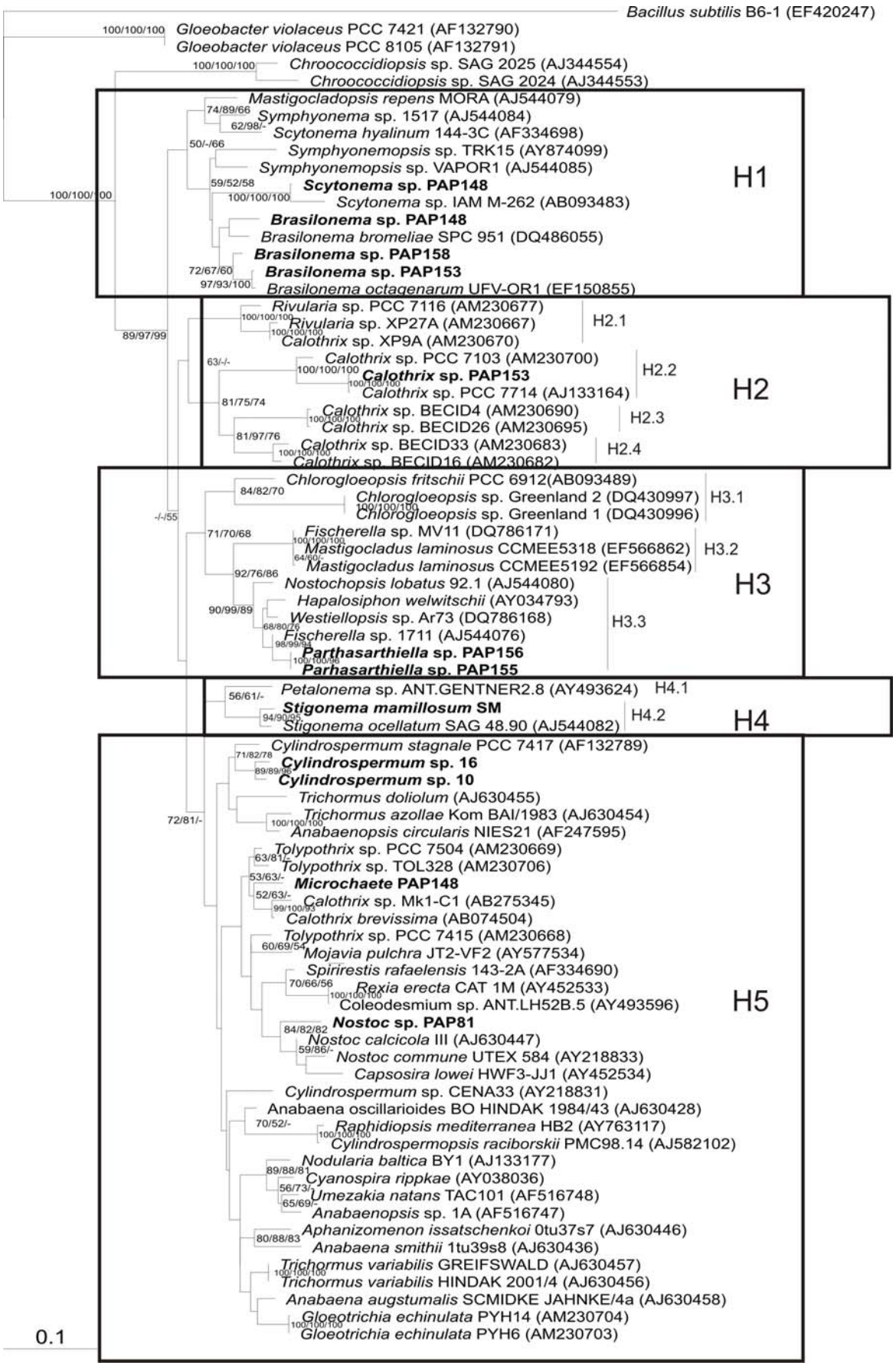

Fig.1 Maximum Likelihood tree based on 16S rDNA (1453 bp) showing the clustering of studied heterocytous cyanobacteria (sequences obtained in this work are in bold). Numbers near nodes indicate bootstrap values over 50% for ML, NJ, MP analyses. Clusters discussed in the text are marked H1-H5. (Strains of *Bacillus*, *Gloeobacter* and *Chroococcidiopsis* are outgroup taxa.)

Monophyletic origin of heterocytous cyanobacteria was supported by this work and five well-supported evolutionary lineages (clusters H1-H5) within heterocytous cyanobacteria were found (Fig.1). Unfortunately, not all of the relationships within these large clusters are well supported by the bootstrap values. Even the big clusters are in conflict with the existing taxonomic system:

- **Cluster H1** gathers strains of cyanobacteria with double false branching (traditional Nostocales, Scytonemataceae: genera *Scytonema, Brasilonema*) and strains with Y-type of true branching (Stigonematales, Nostochopsaceae and Mastigocladaceae: genera *Mastigocladopsis, Symphyonema, Symphyonemopsis*) disclaiming traditional separation of true and false branching. Traditional separation of true and false branching cyanobacteria is denied by multiple origin of true branching in phylogeny as well as by common intermixed clustering of those morphotypes. For better investigation of the relationships within this cluster, all available strains from cluster H1 were used to construct new phylogenetic tree (Fig.3), revealing 6 well-supported subclusters.
- **Cluster H2** consists of *Calothrix* and *Rivularia* strains making them monophyletic group strictly separated from other members of Rivulariaceae (except two *Tolypothrix elenkinii* strains and symbiotic genus *Richelia*).
- **Cluster H3** includes most of the true branching heterocytous cyanobacteria and divides them into tree main subclusters: basal H3.1 with *Chlorogloeopsis* species and two sister subclusters- H3.2 (*Fischerella, Mastigocladus*) and H3.3 (*Westiellopsis, Hapalosiphon, Nostochopsis, Mastigocladus, Fischerella*). This clustering shows polyphyletic origin of some genera.
- **Cluster H4** is represented by probably the first sequences of *Stigonema* and *Petalonema*, which again demonstrate common clustering of true and false branching strains.
- **Cluster H5** consists of the main part of traditional Nostocales (Microchaetaceae, some Rivulariaceae and Nostocaceae) with two strains traditionally included in Stigonematales (*Umezakia* and *Capsosira*).

3.1. Cluster H1

(Symphyonema, Symphyonemopsis, Mastigocladopsis, Scytonema, Brasilonema)

Although subsections IV and V were considered to be true taxonomic divisions till lately, Wilmotte and Herdman (2001) suggested that their delimitation does not reflect the evolutionary relationships within the heterocytous lineage. This idea was proved by Henson et al. (2004) and Gugger and Hoffmann (2004). Later Fiore et al. (2007) reported surprisingly close clustering of false branching genus *Brasilonema* and *Symphyonemopsis* sp. VAPOR1, cyanobacterium with true branching.

However, *Symphyonemopsis* sp. VAPOR1 was not considered to be a typical or reference strain of *Symphyonemopsis,* hence common grouping with false branching *Brasilonema* species was not explained, only nostocalean and stigonematalean close relationship was confirmed.

This grouping is interesting because of the morphological similarities of true or false branches creation. In both cases branching can begin in sheathed trichomes by a local weakness in the sheath. This allows the initial bulging out of a trichome as a loop which eventually breaks (double false branching, Fig.2a) in other case the plane of division shifts in some trichome cells of the trichome and the true Y-branching occurs (Fig.2b). All the stigonematalean members included in this cluster perform typical reverse Y-branching (Anagnostidis and Komárek 1990) with low ability to form T-branching (in genus *Mastigocladopsis*, Komárek 1992, Komárek and Hauer 2004). Genera *Symphyonema* and *Symphyonemopsis* are even further able to form false branches (Gugger and Hoffmann 2004).

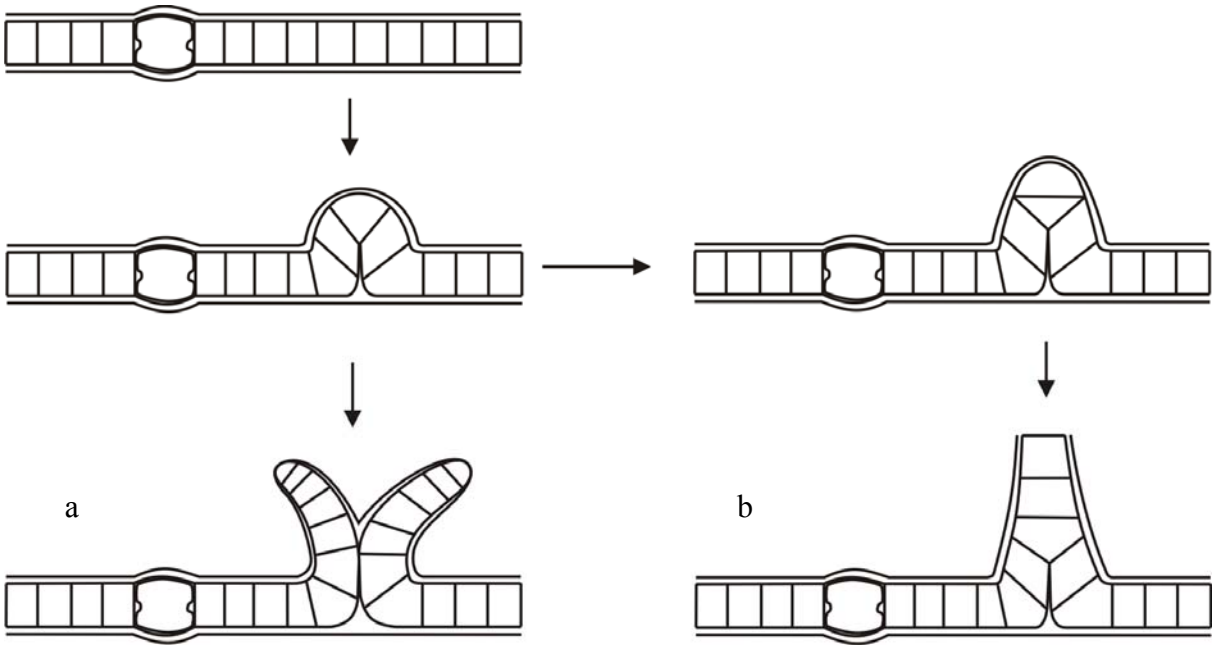

Fig.2 Analogy of development of false branching (a) and Y-type of true branching (b) in cluster H1.

Genus *Brasilonema* is described from tropical part of South and Central America (Fiore et al. 2007). New sequences of *Brasilonema* also appeared in the GenBank (environmental sample from soil from India, sample from leaf surface of rainforest plant from Costa Rica and *Brasilonema roberti-lammi* from central Mexico). On the basis of detection *Brasilonema* species also in samples from Papua-New Guinea (PNG; island in tropical SW Pacific ocean), in samples from tropical glasshouse "Fata Morgana" in Praha (Czech Republic) and from Hawaii Islands, we suggest that this genus is pantropical. Moreover, sequences obtained from PNG strains are intermixed with sequences of Brazilian strains in the phylogenetic tree. It suggests that some *Brasilonema* species from Brazil are closer to some PNG strains than to other strain from the same locality (e.g. 16S rDNA of strains *B.*

octagenarum UFV-OR1 and *B. octagenarum* UFV-E1 from Brazil have more than 99% of gene similarity with *Brasilonema* sp. PAP153 from PNG (Fig.3). Morphological evaluation of *Brasilonema* species has not been published yet, therefore subgeneric classification of PNG strains is impossible.). Phylogeny of some taxa correlates with their geographic distribution and allows us to trace the history (Dyble et al. 2002). In this case, diversification of genotypes was proceeding to their distribution throughout the world (the same genotypes occur in different parts of the world) and therefore genus *Brasilonema* is considered to be ancient.

Genus *Scytonema* is clearly divided into two clusters: H1.1 (*S. hyalinum* cluster), H1.2 (*S. hofmannii* cluster). This corresponds with the previous theory separating genus *Scytonema* on the basis of morphological data (Bornet and Flahault 1887, Bourrelly 1985), in which genus *Myochrotes* should include *Scytonema* morphotypes with narrowed central part of filament and widening ends and genus *Scytonema* comprises of morphotypes with tapering ends and without any tapering at the full filament length.

This analysis proposes that Y-type branching evolved minimally two times in cluster H1 (Fig.3): first in *Symphyonemopsis* species and second time in *Symphyonema* and *Mastigocladopsis* species. It is also possible that evolution of branching in *Symphyonema* and *Mastigocladopsis* were two independent events. In this case, switching from *Scytonema*-type false branching into Y-type true branching might be caused by small mutation that appeared more times during the evolution.

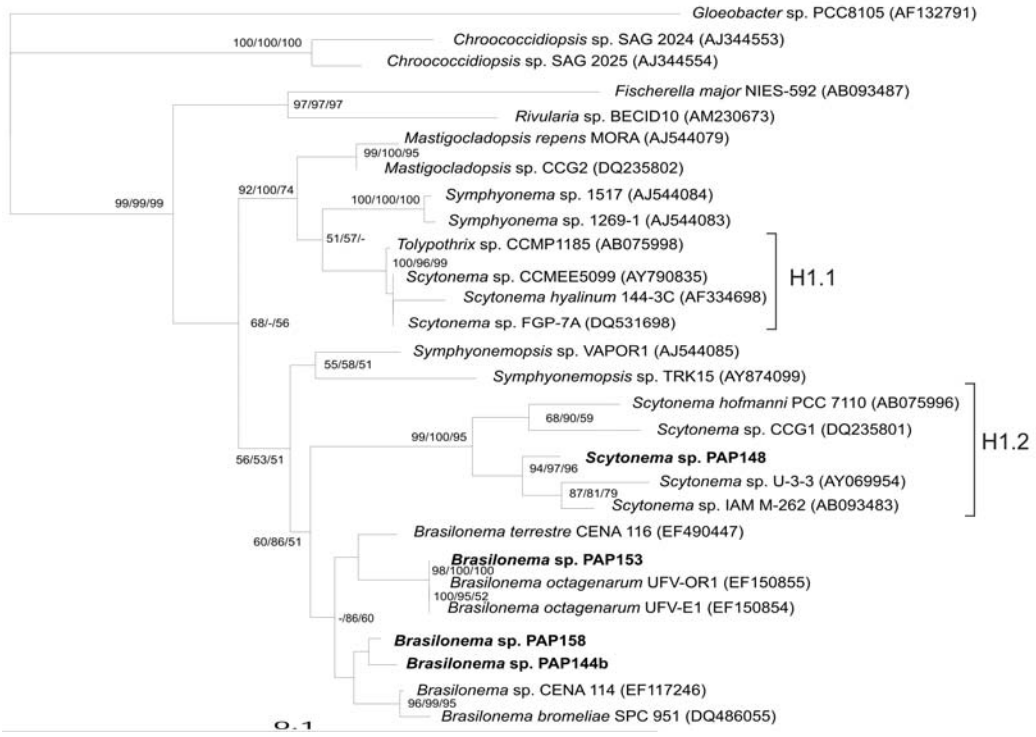

Fig.3 Maximum Likelihood tree based on 16S rDNA (1230 bp) showing the clustering of cyanobacteria from cluster H1 (sequences obtained in this work are in bold). Numbers near nodes indicate bootstrap values over 50% for ML, NJ, MP analyses. Clusters discussed in the text are marked H1.1-H1.2. (strains of *Gloeobacter*, *Chroococcidiopsis Fischerella* and *Rivularia* are outgroup taxa)

3.2. Cluster H2

(Rivularia, Calothrix, Tolypothrix, Richelia)

With increasing number of sequences in the GenBank, better sampling for molecular analyses is possible. Artefacts of insufficient sampling are noticeable in many works - e.g. Wilmotte and Herdman (2001), where sequence of akinetes forming *Calothrix* PCC 7507 (Rippka et al. 1979) clustered with *Cylindrospermum stagnale* and *Nostoc* species. This was interpreted as clustering of akinetes forming cyanobacteria and the strain PCC 7507 was described as "clearly different genus". In paper of Sihvonen et al. (2007) as well as in this work, the PCC 7507 strain represents basal member of cluster H2.1 with other *Calothrix* strains (Fig.1). Akinetes forming cyanobacteria are distributed among other heterocytous cyanobacteria without akinetes, suggesting artificial nature of Wilmotte's and Herdman's interpretation.

Strains of cluster H2 have recently been discussed in work of Sihvonen et al. (2007), where clusters 1-4 correspond to clusters H2.1-H2.4 in this work. Different genus (*Rivularia*) is included in cluster H2.1. Genus *Rivularia* (forming spherical colonies) is separate monophyletic group according to Sihvonen et al. (2007), whereas in this work, six *Calothrix* strains (Calothrix sp. PCC 7507, Calothrix sp. CCMEE 5058, Calothrix sp. CCMEE 5059, Calothrix sp. BECID14, Calothrix sp. XP9A, Calothrix sp. ANT.PROGRESS2.4, data not shown) clustered with all so far molecularly studied *Rivularia* strains. Two of the *Calothrix* sequences were obtained as "*Rivularia"* from work of Sihvonen et al. (2007) but they were submitted by the same author to the GenBank still labelled as *Calothrix* (*Calothrix* sp. BECID14, *Calothrix* sp. XP9A) – it can suggest, that they were primary described as *Calothrix* and consequently after phylogenetic analysis they were transferred to the genus *Rivularia* without morphological reasons. Ambiguous identification of the same strains to different genera is not rare and creates confusion. Those *Calothrix* strains might be originally colonial *Rivularia* forms misidentified because of disintegration during the cultivation.

3.2.1. *Calothrix* vs. *Tolypothrix*

Traditional separation of genera *Calothrix* and *Tolypothrix* is based on the basal-apical ratio. *Calothrix* is more tapered towards the ends of the filaments and (contrary to *Tolypothrix*) sometimes forms akinetes. Unfortunately, molecular phylogenetic studies revealed inconsistencies in this division. Tapering strains seem to form number of independent clusters, and moreover, some of these clusters contain both *Calothrix* strains and *Tolypothrix* strains (Fig.6, cluster H2 and H5.5). Sihvonen et al. (2007) reported that *Calothrix brevissima* (AB074504) clustered with *Tolypothrix* strains and also mentioned that strain BECID 4 (molecularly belonging to one of the *Calothrix* clusters) corresponded morphologically to *Tolypothrix* (cluster H2.2, not shown). There is one more *Calothrix* strain (*Calothrix* sp. Mk1-C1) clustering with *Tolypothrix* strains from the GenBank (Fig.6, cluster H5.5).

Two of the sequences obtained in this work (*Tolypothrix elenkinii* CCALA195, *Tolypothrix elenkinii* CCALA 10071) appeared in cluster H2, sustaining the suspicion of insufficiency and artificiality of traditional *Calothrix* and *Tolypothrix* differentiation. Mitigating circumstance is that morphology of *Tolypothrix elenkinii* does not exactly match typical *Tolypothrix* strains (nor *Calothrix*) thus its special position in phylogenetic tree is not surprising (it has huge mucilaginous sheets, situation of filaments differs).

3.2.2. *Richelia*

Genus *Richelia* is traditional member of the order Nostocales, but molecular analyses prove its affiliation to the genus *Calothrix* (Rivulariaceae) (Fig.6, Foster and Zehr 2006). The 16S rRNA sequences of the symbiotic *Richelia* and the closest *Calothrix* strain are very similar (98.2%). This level of sequence identity is often interpreted as a single species. However, the *het*R and *nif*H DNA nucleotide sequences were only 83% and 91% identical respectively (Foster and Zehr 2006). The low resolution in 16S rRNA sequence analyses can partially be attributed to the low length of the amplification product (359 bp; Nübel et al. 1997). It has been shown that 16S rRNA sequences do not resolve closely related cyanobacterial species or strains (Toledo and Palenik 1997). The marine strain *Calothrix* sp. BECID 30 and *Calothrix* strain symbiotic in marine diatoms (as well as *Richelia*) are the closest sequenced relatives to the genus Richelia (Fig.6, cluster H2). This cluster matches to a branch B of the tree from work of Sihvonen et al. (2007), where *Calothrix* sp. BECID 30 was the only representative of this branch, which gathers marine and symbiotic strains. The possible explanation of the common close clustering of *Richelia* and *Calothrix* is that *Richelia* morphotype with two terminal heterocytes might have evolved from heteropolar *Calothrix* morphotype.

3.3. Cluster H3

(Chlorogloeopsis, Fischerella, Mastigocladus, Nostochopsis, Westiellopsis, Hapalosiphon)

Cluster H3 (Fig.1, 5) consists of heterocytous cyanobacteria traditionally classified as Stigonematales with T and V type of true branching (H3.2, H3.3) and strains assigned to the genus *Chlorogloeopsis* (H3.1).

3.3.1. *Chlorogloeopsis*

Two *Chlorogloeopsis* subclusters exist inside the cluster H3.1. Similarity of 16S rDNA between those subclusters is 91.7–92%. This value is large enough to distinguish two different genera on the basis of molecular data. Unfortunately, morphological data are not available, so I cannot state if those two clusters are phenotypically distinguishable or whether they are representatives of cryptogenera. Nevertheless, first cluster is clearly separated from the other by geography – these five Greenland strains and one strain from near Iceland (PCC 7518) share 99,8% 16S rDNA similarity.

3.3.2. Thermal strains

Second cluster (H3.2) matches the description of *Mastigocladus laminosus* in Kaštovský and Johansen (in press). All the strains assigned to *Mastigocladus laminosus* and 13 strains of *Fischerella* are found in this group. They were all collected from thermal springs and therefore they have to be reclassified to the genus *Mastigocladus* as was suggested by Kaštovský and Johansen (in press).

3.3.3. Subcluster H3.3

The other T and V branching stigonematalean genera are members of a cluster H3.3 (except of *Stigonema* forming separate cluster H4.1 and *Umezakia, Capsosira* clustering with nostocalean strains in H5). Only genera *Fischerella, Westiellopsis, Parthasarthiella, Hapalosiphon* and *Nostochopsis* from this cluster have been sequenced till now. The only thermal strains in cluster H3.3 are *Westiellopsis* AR73 and L32 (phenotypically diverse from strains in *Mastigocladopsis* cluster H3.2) and *Hapalosiphon* strains CCG5, CCG6 (phenotypically similar to *Mastigocladopsis* but ecologically different – occurring as endolithic organisms in geothermal rock of Costa Rica (see NCBI)).

3.3.4. Type of branching in H3.3

Y-branching of *Mastigocladus* was mentioned in literature (Komárek 1992), but Kaštovský and Johansen (in press) found mostly T-type branching in *Mastigocladus*. They found only limited V-type branching in *M. laminosus.* This type of branching is closer in ontogeny to T-type branching than Y-type branching. Therefore in cluster H3, no strain performs typical Y-branching (which remains a typical character of cluster H1).

3.3.5. Nostochopsaceae

Genera *Nostochopsis* as well as *Mastigocladopsis* are members of Stigonematales, Nostochopsidaceae (Geitler 1925, Anagnostidis and Komárek 1990). This family is characterised by intercalar bipored more or less spherical heterocytes, wider than vegetative cells, or lateral unipored heterocytes, attached to the vegetative cells forming the ends of short branches (with 1 or few cells). These two genera differ in type of true branching and from this work it is clear, that they are separated and belong to the different clusters (*Mastigocladopsis* with Y branching belongs to cluster H1, *Nostochopsis* with T or V branching belongs to cluster H3). Family Nostochopsaceae is therefore polyphyletic and lateral heterocytes might have evolved at least two times.

3.3.6. Horizontal gene transfer (HGT)

I have found an evidence for within-phylum horizontal gene transfer among true branching heterocytous cyanobacteria in this work. Furthermore, I expect bacterial origin of this transferred part of gene on the basis of previously published analogous gene transfer of another insert from proteobacteria to the same position within 16S rDNA gene (Miller et al. 2005).

Transferred insert corresponds to 31nt polymorphic insert of *Nostochopsis* strains (BB92.1 and 89-45) and *Fischerella* SAG20.27 (found by Gugger and Hoffmann (2004) in the 5' end of 16S rDNA sequences). (Remark: Identity of sequences Fischerella SAG20.27 (=BB98.1) and Nostochopsis BB92.1 was explained by confusion of strains during sequencing. New 16S rDNA sequence of Fischerella SAG20.27 (=BB98.1) was considerably different and clustered with the other Fischerella strains). I found 13 more cyanobacterial strains with the same or nearly the same 31nt insert in this work (Fig.4). These strains were: thermophilic *Fischerella major* NIES-592 (AB093487) branching in cluster H3.2 (*Nostochopsis* strains are branching in cluster H3.3, Fig.5), thermal *Fischerella* CSR and 11 "uncultured bacterial samples" (from hot springs of Yellowstone national park and from Australia) also phylogenetically falling to the cluster H3.2 (probably belonging to the genus *Fischerella/Mastigocladus*, sharing over 98 % similarity of 16S rDNA). All mentioned strains are thermophilic and therefore proposed by Kaštovský and Johansen (in press) to be reclassified into the genus *Mastigocladus*. Occurrence of the same insert in two separated clusters (H3.2, H3.3) can be caused by different reasons:

- The insert can descend from common ancestor and later cease in all evolutionary descendent taxons (all strains in clusters H3.2, H3.3) except of the 16 mentioned strains. This is quite complicated and we would expect some residues in 16S rDNA sequences of sister taxons (these were not observed).
- The insert may evolve more than once independently (very improbable for 31nt long insert).
- The insert evolved (or was acquired from another organism) in one cluster and consequently was transferred to another cluster by horizontal gene transfer (HGT).

Third solution seems to be the less evolutionary demanding and therefore the most probable. HGT of different genes in cyanobacteria was published (e.g. Besendahl et al. 1999) and also HGT of ribosomal DNA was published in related bacterial groups (Yap et al. 1999, Koonin 2001). Miller et al. (2005) found chlorophyll d-producing cyanobacteria (two strains assigned to the genus *Acaryochloris*) that acquired a fragment of the small-subunit rRNA gene encoding a conserved hairpin in the bacterial ribosome from a proteobacterial donor at least 10 million years before the present. I found one more *Acaryochloris* sequence with the same insert in the GenBank. This proteobacterial hairpin is inserted into cyanobacterial 16S rDNA in exactly the same site as above mentioned 31nt insert of *Fischerella/Nostochopsis* strains, but it is quite different (see alignment in Fig.4). I expect bacterial

origin of the *Fischerella/Nostochopsis* insert (as in case of *Acaryochloris* insert) despite of the absence of bacterial sequence identical with *Fischerella/Nostochopsis* insert. Cyanobacteria and some bacteria share identical sequences – approximately 20 nt in front of and about 20 nt behind the position of the insert. This may make recombination of DNAs of different genera easier. The insert matches to the helix 6 of the secondary structure model for the 16S rDNA of *Chlorogloeopsis* sp. PCC 7518 (Wilmotte et al. 1993). Function of this loop is unknown. I found only one cyanobacterium without hairpin sequence structure in this site (*Fischerella* sp. 1711).

Recognition of these inserts as examples of HGT is making our most widely used phylogenetic marker (ribosomal DNA) closer to a mosaic of sequence fragments with highly divergent evolutionary histories. The positive fact is that HGT is a weak force in the long run, and no serious challenge to the historical accuracy of the rRNA-based Tree of Life. Furthermore, it does not mean that HGT is unimportant or infrequent within phyla. There are many reasons to expect that within-phylum HGT will be more vigorous and more fruitful than between-phylum exchange. In some cases, members of a phylum are more likely to occupy similar environments, and encounter each other's DNA (Zhaxybayeva et al. 2006). Incongruous to this fact, *Fischerella strains* (cluster H3.2) were collected in thermal springs in contrast to *Nostochopsis* strains, which were cryptoendolithic or from submerged stones from fresh water. These ecological differences may make the HGT difficult.

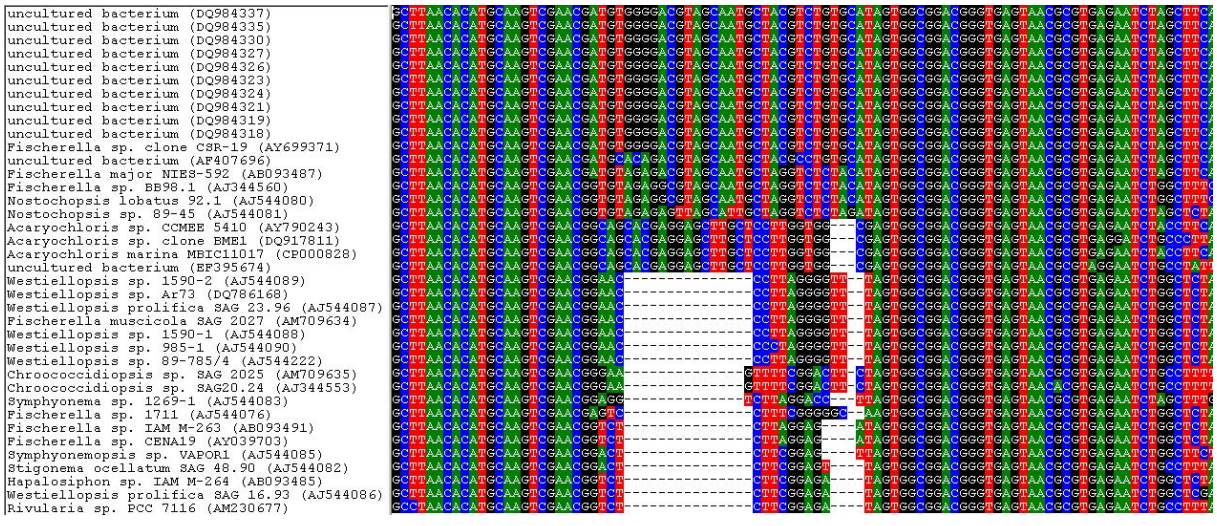

Fig.4 Alignment of 16S rDNA of cyanobacteria with bacterial insert. 16 strains of heterocytous cyanobacteria (line 1-16) have different insert than *Acaryochloris* (chlorophyll d producing cyanobacteria) (line 17-19).

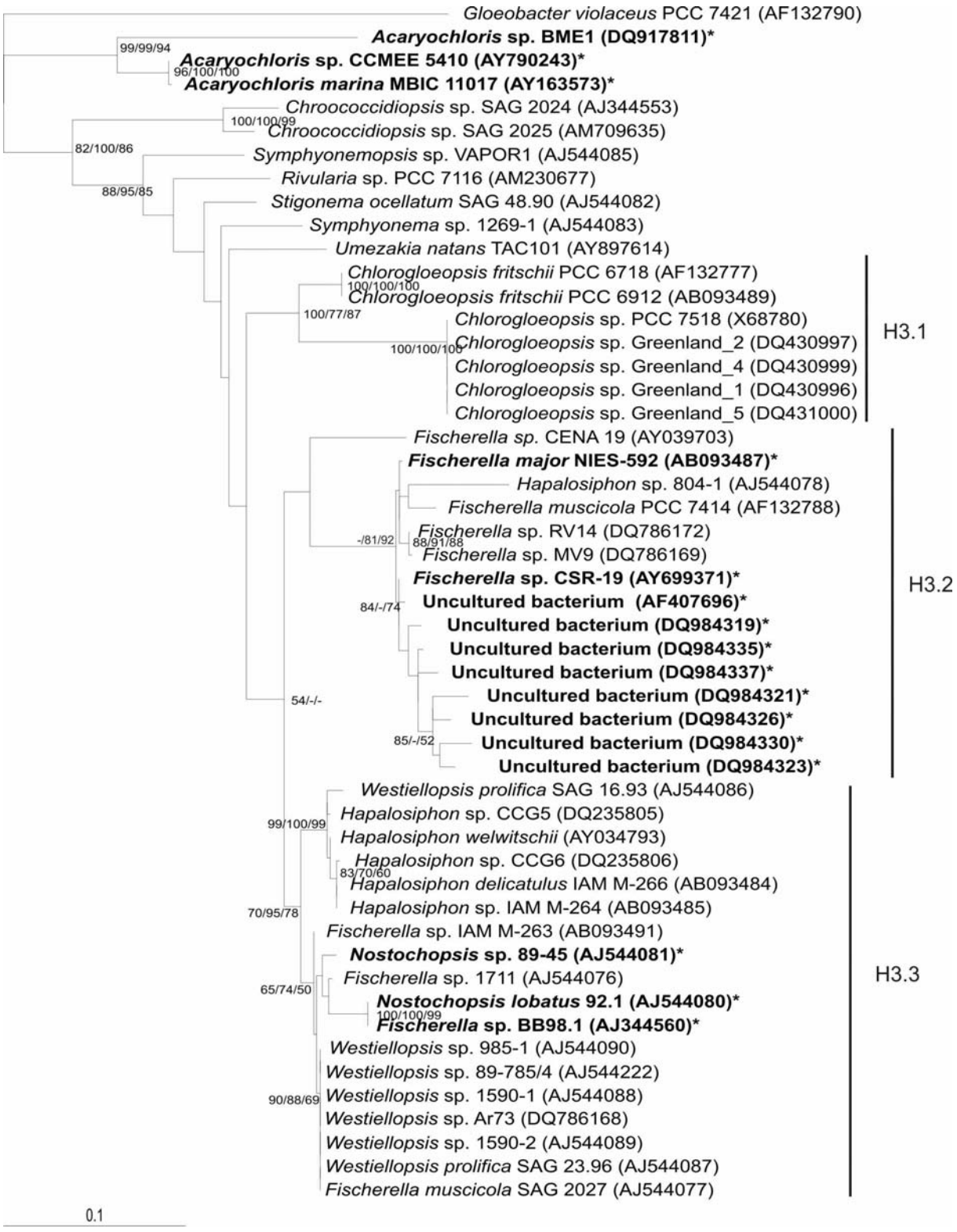

Fig.5 Maximum Likelihood tree based on 16S rDNA (750 bp, 31nt insert excluded from analyses) showing the inner relationships in cluster H3 and polyphyletic distribution of the bacterial insert (strains with the insert in 16S rDNA sequence are in bold, marked with asterisk). Numbers near nodes indicate bootstrap values over 50% for ML, NJ, MP analyses. Clusters discussed in the text are marked H3.1-H3.3.

3.4. Cluster H4

(Stigonema, Petalonema)

The only GenBank sequence of the genus *Stigonema* clustered together with the only existing sequence of the genus *Petalonema,* probably as an artefact of insufficient sampling. Addition of sequence of *Stigonema mamillosum* SM (acquired in this work) divided this cluster into two separate groups. Separation of these groups will be probably deeper with increasing number of sequences. This new sequence denies the speculations that the separate position in phylogenetic tree of *Stigonema ocellatum* SAG 48.90 is only an artefact.

The absence of sequences of *Stigonema* is perhaps caused by difficult and time-consuming cultivation. Only 5 strains of *Stigonema* are available in main world culture collections because of that difficulty. Another problem is a very firm structure of filaments with extracellular mucopolysacharides of their sheaths making the isolation of DNA tough.

Problems may also appear during the sequences gaining. Cyanobacteria can contain multiple rrn operons and ITS region of variable size (Iteman et al. 2002). Furthermore Iteman et al. (2002) as well as Lokmer (2007) provided evidence for intra-genomic sequence heterogeneity of the 16S rDNA genes. As a response to unpredictable position of *Stigonema* in phylogenetic tree (Gugger and Hoffmann 2004), new isolation of DNA from purchased strain SAG48.90 was done. PCR amplification of 16S rDNA and associated 16S-23S ITS of *Stigonema* SAG48.90 repeatedly revealed two bands of different size in gel electrophoresis control. It is caused by at least two copies of ribosomal operon. Larger divergence between these copies is expected in more variable ITS sequence, but differences in 16S rDNA are not excluded. Unfortunately these two bands have not been sequenced yet.

3.5. Cluster H5

(Umezakia, Capsosira and traditional Nostocales (*Anabaena, Anabaenopsis, Aphanizomenon, Calothrix, Coleodesmium, Cyanospira, Cylindrospermopsis, Cylindrospermum, Gloeotrichia, Microchaete, Mojavia, Nodularia, Nostoc, Raphidiopsis, Rexia, Spirirestris, Tolypothrix, Trichormus*))

Cluster H5 gathers main part of traditional Nostocales with some strains dividing in more than one plane. True branching of *Umezakia natans* (phylogenetically member of cluster H5, traditionally member of Stigonematales) is one of the several examples of fission in multiple planes in members of cluster H5. The next representative is *Capsosira lowei* (see Fig.1, 6), also traditional member of Stigonematales. We can further find some strains with dividing in more planes in certain growth forms: *Nostoc* symbiont of the moss *Blasia pussila* in culture (Gorelova et al. 1996), hormogonia of *Rexia erecta* (Casamatta et al. 2006) and *Mastigocladus laminosus* forma *nostocoides* (Kaštovský and

Johansen, in press). These facts raise a question whether or not the cellular division in one or more planes is a valid character for determining phylogenetic relationships. Anyway, none of the mentioned strains dividing in more planes form true branches or they do not form them naturally (the result of a division in more planes is a multiseriate trichome and branches are created only in cultures in *Umezakia natans*).

Large phylogenetic analysis of the cluster H5 revealed at least 14 subclusters but did not allow the relationships among the subclusters to be resolved (Fig.6).

3.5.1. *Nostoc, Anabaena, Aphanizomenon* and *Trichormus*

Genera *Nostoc*, *Anabaena*, *Aphanizomenon* and *Trichormus* are problematic. Each of these genera forms its own subcluster when a majority of available GenBank strains is analysed (*Nostoc* subcluster H5.2 (Fig.6) contains 196 from 225 usable sequences available in GenBank, *Anabaena* and *Aphanizomenon* subcluster H5.10 contains 165 from 201 sequences and *Trichormus* subcluster H5.12 contains 3 from 5 available sequences). The rest of sequences assigned to these genera is dispersed throughout the whole cluster H5 and it does not appear in 3 strictly heteropolar subclusters only (Fig.6; clusters H5.1, H5.5, H5.11). These sequences are sister taxa to the majority of the Nostocalean strains in the most of the subclusters.

It is known that names of many strains in cultures and many sequences in GenBank are wrong (Komárek 2006). Therefore those sources of information are not fully credible. Anyway, I can presume that in this case so many mistakes from so many different sources are improbable. All of the subclusters contain 3-15 sequences from different sources, in original large tree (not shown), assigning them to one of the mentioned genera (*Nostoc*, *Anabaena*, *Aphanizomenon* or *Trichormus*). I concede confusion among those genera for their common features: filamentous character, filaments solitary or in clusters, developing both heterocytes and akinetes, but I presume that organisms assigned to one of those genera share at least characters mentioned above (*Nostoc-Anabaena-Aphanizomenon-Trichormus* (NAAT) morphotype).

The NAAT morphotype occurs throughout the whole cluster H5. This can be explained by the fact, that most of the genera have this universal morphotype in their lifecycle (before it forms structures typical for particular genus) and therefore it cannot be assigned to the correct genus in some life forms without observing the whole cycle. It can be interpreted as a primitive ancestral morphotype also. This morphotype could have differentiated genotypicaly during evolution from one ancestor (causing origin of number of morphologically similar cryptogenera which we are not able to distinguish) and consequently it might have given rise to the other morphotypes (other genera). The result could be a number of subclusters with the originally monophyletic NAAT morphotype separated each from another by new genera making it polyphyletic. The only subclusters without strains with NAAT morphotype are heteropolar subclusters (H5.1, H5.5, H5.11). Strains of these subclusters are well determined by their polarity and therefore substitution with non-polarized genera is unlikely.

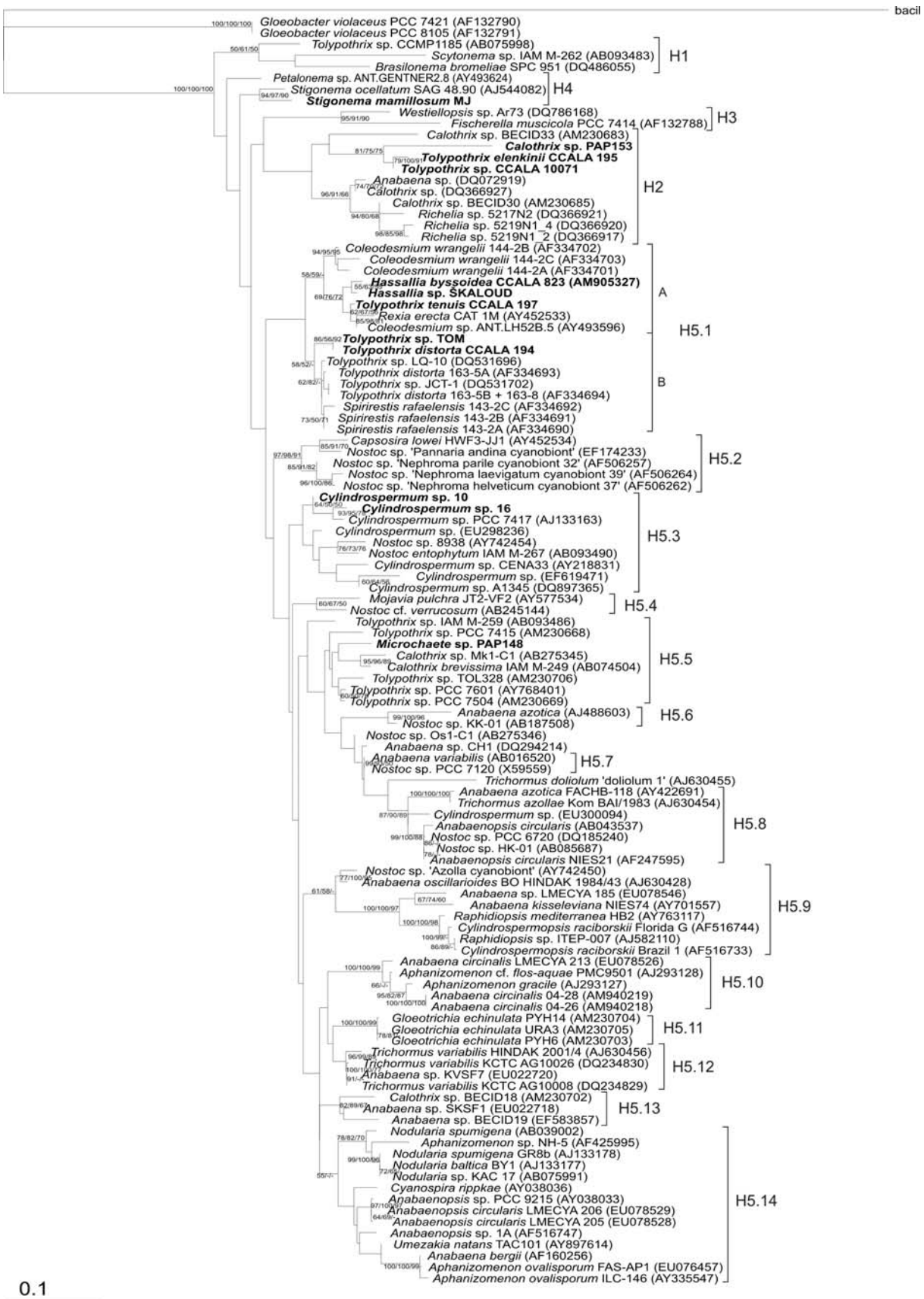

Fig.6 Maximum Likelihood tree based on 16S rDNA (1200 bp) showing the clustering of cyanobacteria from cluster H5 (sequences obtained in this work are in bold). Numbers near nodes indicate bootstrap values over 50% for ML, NJ, MP analyses. Clusters discussed in the text are marked H5.1-H5.14.

3.5.2. Clusters without NAAT morphotype

- **Cluster H5.1** is divided into two subclusters. Any clear taxonomic characters do not support the inner division of the cluster. Genus *Coleodesmium* seems to form two independent units. *Tolypothrix* appears in the group H5.1A and even in H5.1B. Genera *Hassallia*, *Rexia* and *Spirirestris* may be monophyletic, but it is impossible to make any conclusions without better sampling. Similarity of 16S rDNA among strains in cluster H5.1A is higher than 95 % (95,1-99%). Sequence similarity of more than about 95 % is generally enough to place strains into one genus (reviewed by Rosselló-Mora and Amann 2001). These genera are morphologically similar, but there is a question whether morphological differences are substantive enough to divide those types into 3-5 different genera in terms of cluster H5.1A and into 2-3 genera in terms of cluster H5.1B. Further investigation is needed.
- **Cluster H5.5** consists of *Tolypothrix*, *Calothrix* and *Microchaete* strains and thus they form another heteropolar cluster. This cluster (as well as cluster H2) shows intermixed phylogenetic grouping of tapering and non-tapering genera. No sequence of *Microchaete* was published before.
- **Cluster H5.11** is formed by *Gloeotrichia* sequences and seems to be well defined and monophyletic. Position of this cluster is not well defined, and therefore there is a possibility that in future analyses with better sampling it will form a sister group to another heteropolar cluster.

3.5.3. Clusters with NAAT morphotype

- **Cluster H5.2** consists of *Nostoc* strains only. The sequence of *Capsosira lowei* clustering with 196 *Nostoc* strains seems to be an error. *Capsosira lowei* morphologically matches the description of the genus *Nostoc* with the exception of dividing in more planes (which was published for *Nostoc* strain too - Gorelova et al. 2006).
- **Cluster H5.3** does not have big bootstrap support, but it consists of two *Cylindrospermum* subclusters representing probably two distinct genera. *Nostoc* strains occurring in this cluster may be basal, but their position is not well supported in terms of cluster H5.3.
- **Cluster H5.4** represents another *Nostoc* cluster containing sequence of *Mojavia pulchra* (AY577534). Genus *Mojavia* was set apart from the genus *Nostoc* on the basis of ecological and molecular data. It is possible, that all the *Nostoc* strains clustering with *Mojavia* belong to this genus as well.
- **Cluster H5.9** gathers three independent types probably. Interesting is common clustering of genera *Raphidiopsis* and *Cylindrospermopsis*. Morphological features of these genera are similar and the main discriminating traits are a position of akinetes, formation of heterocytes and cylindrospermopsin production. These characters are variable during the life cycle and therefore instable. Affiliations inferred from 16S rRNA gene sequences demonstrated that

Raphidiopsis strains clustered with *Cylindrospermopsis* (Li et al. 2008). By this analysis, *Raphidiopsis* and *Cylindrospermopsis* form intermixed cluster and similarity within all available strains of those two genera is at least 98,9% (enough to transfer them into one genus, even one species). Similarity between different genera is higher then similarity within strains from the same genus in some cases.

- **Cluster H5.10** joins up 16S rDNA sequences of *Anabaena* and *Aphanizomenon.* On the basis of this gene, it is not possible to resolve this complicated relationship.
- Sequences of the genus *Trichormus* are included in **clusters H5.12** and **H5.8** and they probably form at least three separate genera. Because *Trichormus* is a relatively new genus, naming of some GenBank sequences might have been done without accepting separation of genera *Anabaena* and *Trichormus*. Genus *Trichormus* was separated from *Anabaena* on the basis of akinete development and according to the last revision, species *Anabaena variabilis*, *Anabaena azollae* and *Anabaena doliolum* belong to the genus *Trichormus* (Komárek and Anagnostidis 1989, Rajaniemi et al. 2005). Strains assigned to those species are included in **clusters H5.6, H5.7, H5.8** and it is possible that all strains in the mentioned clusters belong to the genus *Trichormus*. The model strain PCC 7120, with whole genome sequence, falls into the cluster H5.7 as a sister taxon to *Anabaena variabilis* (= *Trichormus variabilis*) thus it may be a strain of *Trichormus*.
- **Cluster H5.13** consists of (among others) a *Calothrix* sp. strain BECID18. It is the only sequence of heteropolar cyanobacterium, which appears out of the main polarized clusters (see above clusters H5.1, H5.5, H5.11). It seems to be caused by an error during sequencing (sequence of *Anabaena* sp. BECID19 from the same laboratory is also a part of this cluster, so there is a possibility of contamination).
- There are two very similar genera in **cluster H5.14 –** *Anabaenopsis* and *Cyanospira*. Whether these two genera would eventually need to be combined was discussed by Rippka et al. (2001). Iteman et al. (2002) suggested that they might be assignable to a single genus (in that case *Cyanospira* would lose validity). There are two sequences assigned to the genus *Anabaenopsis* in **cluster H5.8** grouping with some *Nostoc* strains. These sequences surely represent different genus.
- Genus *Nodularia* forms distinct subcluster in terms of cluster **H5.14**. This genus is well defined and all available strains assigned to this genus suggest its monophyly.
- *Umezakia natans* is an interesting cyanobacterium isolated from plankton of a lake in central Japan (the planktic populations with dominant uniseriate, isopolar, not branched trichomes can be confused with *Raphidiopsis*; later stages develop intercalary solitary heterocytes and oval akinetes, sometimes with true branching of T-type). *Umezakia* is the only planktic member of Stigonematales (Komárek 1992). Two sequences of 16S rDNA of *Umezakia natans* TAC101 with similarity only 91.4% exist in the GenBank. First sequence (AY897614; Kellmann et al.

2006) corresponds to ordinary cyanobacterial sequences but the second one (AF516748, Neilan et al. 2003) has only 89% similarity with the nearest cyanobacterial 16S rDNA sequence and 87% similarity with nearest bacterial ribosomal DNA. Additional investigation revealed hybrid character of this sequence (first 720 nucleotides match with cyanobacterial 16S rDNA and the rest of the sequence matches with bacterial 16S rDNA, Fig.7). Kellmann et al. (2006) suggested that sequence AF516748 from previous work (Neilan et al. 2003) might have been a "PCR hybrid". In that case, I would presume identity of the parts of sequences corresponding to cyanobacterial 16S rDNA in AF516748 and AY897614. Incongruously with this presumption, similarity between those parts of sequences is only 97,4%, as well as similarity of second part of the AF516748 sequence with closest bacterial DNA is only 97%. Furthermore the first part of sequence AF516748 is more similar to another cyanobacterial strains (98-99% similarity with *Aphanizomenon ovalisporum* (EU076457) and *Anabaena bergii* (AF160256)) than to the second *Umezakia* sequence (AY897614). Neither Kellmann et al. (2006) nor Neilan et al. (2003) used cloning during sequencing. Hence, some copies of the 16S rDNA might have been omitted (in work of Iteman et al. (2002), more than one copy of rrn operon was revealed in all tested strains). In terms of this fact, sequences of 16S rDNA of *Umezakia natans* (AF516748 and AY897614) seem to be two different copies of the same gene and thus evidence of horizontal gene transfer from bacterial genome was possible in sequence AF516748. This report, together with the previous one (see H3, section HGT) and work of Miller et al. (2005), lower the evolutionary value of 16S rDNA.

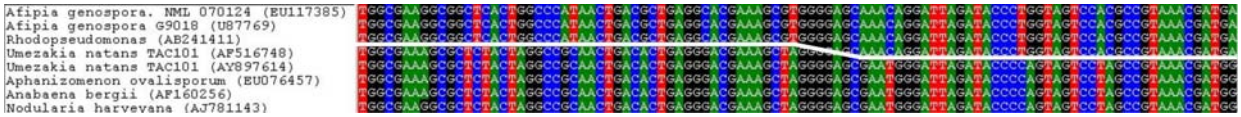

Fig.7 Part of an alignment of 16S rDNA of two different *Umezakia natans* sequences with similar sequences of cyanobacteria (*Aphanizomenon ovalisporum, Anabaena bergii, Nodularia harveyana*) and bacteria (*Afipia genospora* and *Rhodomonas* sp.). First part of sequence of *Umezakia natans* (AF516748) matches with cyanobacterial 16S rDNA and the second part matches with bacterial 16S rDNA.

3.6. Evolution of heterocyte-forming cyanobacteria (Fig.8)

The closest living relative to heterocyte-forming cyanobacteria is *Chroococcidiopsis* (Fewer et al. 2002). Representatives of this genus are able to fix atmospheric nitrogen under anaerobic conditions (Rippka et al. 1979) and they are known to differentiate specialised cells under nitrogen limiting conditions (Billi and Grilli-Caiola 1996), helping them to survive periods of nitrogen limitation and desiccation. Fewer et al. (2002) suggested that the developmental and physiological processes underlying the formation of survival cells in *Chroococcidiopsis* may be related to heterocyte differentiation. *Chroococcidiopsis*-like form can be therefore considered to be common ancestor for heterocytous cyanobacteria.

With a respect to unicellular aggregating form of this ancestor, the next evolutionary stadium (first heterocytous cyanobacterium) should be aggregated amorphous cluster of cells or pseudofilaments forming heterocytes. We can actually find this morphotype within existing cyanoflora – genus *Chlorogloeopsis*. *Chlorogloeopsis* is dividing in more than one plane as well as *Chroococcidiopsis*. The filamentous nature of this organism is often unclear, except in the hormogonia. Hormogonia (dividing in one plane) are composed of short chains of cylindrical or barrel-shaped cells that enlarge to become spherical cells after they loose their motility. Heterocytes develop in both intercalary and terminal positions when levels of combined nitrogen are low. Growth continues with cell division in more than one plane, so that multiseriate trichomes develop (Rippka et al. 1979). *Chlorogloeopsis*-like life cycle enable multiple origins of polyseriate and branching filaments alternating in phylogeny with uniseriate non-branching morphotypes and relatively fast diversification of heterocyte-forming cyanobacteria. This is because of the evolutionary possibility to outweigh either aggregated form with ability to divide in more than one plane, or hormogonium-form with intercalary or terminal heterocytes.

Modern *Chlorogloeopsis* registered many changes in 16S rDNA despite of probably primitive and conserved cell-packets and aggregated stages in its life cycle. In spite of that controversy, I suppose that *Chlorogloeopsis*-like morphotype was a primitive form in the evolution of heterocytous cyanobacteria and this form was maintained only as a sister taxon in the group H3 (Fig.1, 8).

Pros and cons:

- ⊕ All heterocyte-forming cyanobacteria are in principle branching or non-branching filamentous forms in contrast to hypothetical *Chroococcidiopsis*-like ancestor with aggregated unicellular cell organisation. Therefore *Chlorogloeopsis*-like organism is a transitional form enabling an evolution of the filamentous morphotypes.
- ⊕ Some heterocytous cyanobacteria (particularly in cluster H5, Fig.1, 6) are able to divide in more than one plane even though it is not a typical characteristic for the phylogenetic cluster they belong to (e.g. *Umezakia* and *Capsosira*- traditional members of Stigonematales – in cluster with

non-branching Nostocales (Fig.1, 6, cluster H5); *Stigonema* emerging distantly from other true branching cyanobacteria in separated cluster (Fig.1 cluster H4); hormogonia of *Rexia* dividing in two planes clustering with other Microchaetaceae dividing in one plane (Fig.1, 6, cluster H5, Casamatta et al. 2006); some strains of *Nostoc* which were observed to undergo cellular division in more planes in cultures (Gorelova et al. 1996), *Mastigocladus laminosus* forma *nostocoides*, in which most typical phenotype shows some indication of branching, similar to the branching of the genus *Chlorogloeopsis* (Kaštovský and Johansen, in press.)). It can manifest suppressed ancestor's ability to divide in more planes exhibited in some special conditions or retained in some life forms (not complicated multiple invention of division in more planes).

⊕ It is possible to find growth forms of *Glorogloeopsis*, which would enable (by outweighing in life cycle) evolution of each of the clusters H1-H5 (hormogonia with terminal and intercalary heterocytes – H1, H2, H4.1, H5, aggregated clusters of cells dividing in more planes – H3, H4.2, some genera in H.5).

⊕ If we do not expect a primitive ancestor, basal branching of relatively morphologically derived clusters is possible.

⊖ Contrary to the proposed *Chlorogloeopsis*-like ancestor theory, *Chlorogloeopsis* is not branching as the most basal cluster within heterocytous cyanobacteria. It is not claimed that the common ancestor of heterocytous cyanobacteria is *Chlorogloeopsis* itself, but this ancestor is considered to be a life form resembling the genus *Chlorogloeopsis* – morphologically similar and with similar life cycle. This organism (or its sequence) is not available. It may be conserved only as morphotypes derived from its life stages up to now. Maintenance of a life form resembling the hypothetical ancestor in derived branch (cluster H3.1) is similar to polyphyletic appearance of the same morphotypes in different branches (e.g. heteropolar morphotype in H2 and H5) and is common in cyanobacterial phylogeny.

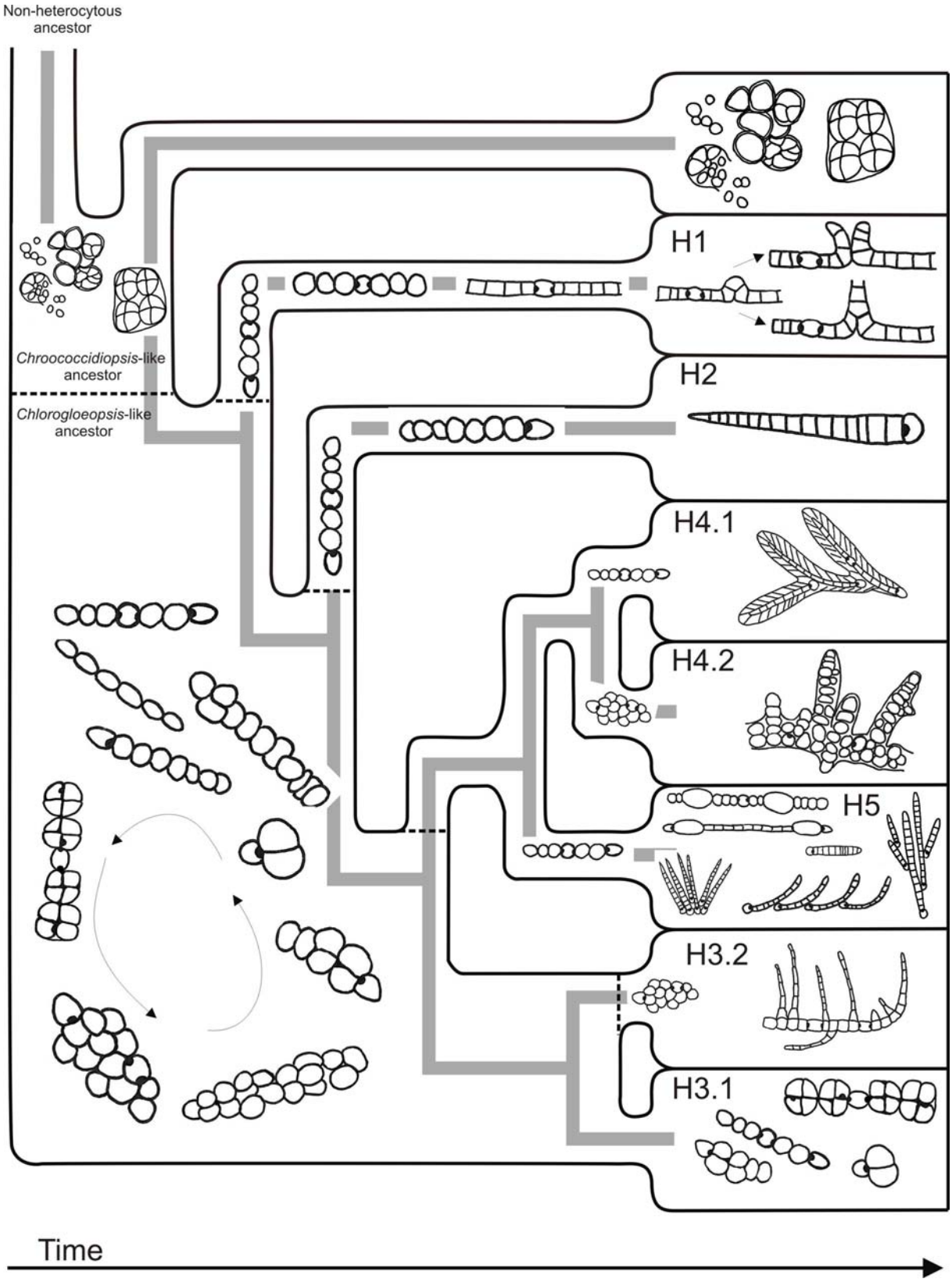

Fig.8 Proposed evolution of heterocyte-forming cyanobacteria showing simplified morphology of main clusters. Ancestor's life cycle enable evolution of each from in clusters H1-H5.

4. Conclusions

Traditional taxonomy does not exactly match with phylogenetic relationships revealed by the 16S rDNA gene. Differences are noticeable on the level of species, genera and even orders. This work confirms the monophyly of heterocytous cyanobacteria, but in agreement with previous studies, it points out the polyphyly of Nostocales and Stigonematales. According to this work, true branching appeared at least three times in evolution of cyanobacteria (clusters H1, H3, H4). It is also very important to distinguish cell division in more than one plane from formation of true branches. Cell division in more planes without forming true branches (resulting in multiseriate filaments) seems to appear more times in cluster H5 and it may demonstrate abilities of an ancestor to divide in this way. T-type branches are typical for groups H3 and H4, Y-branches appear in cluster H1. True branching of *Umezakia* - member of cluster H5 - is doubtful (it is the only existing strain, isolated from single natural sample and branching only in culture).

This thesis revealed a number of unique and important results:

Genus *Brasilonema* described from South America was suggested to be pantropical (according to records from Mexico, tropical glasshouse in Praha, Hawaii, India, Costa Rica, Papua-New Guinea). It is considered to be an ancient genus because its phylogeny does not correlate with geographical distribution.

Division of heteropolar cyanobacteria (genera *Tolypothrix* and *Calothrix*) according the basal-apical ratio was found to be insufficient. Some strains corresponding to the description of the genus *Tolypothrix* clustered with typical *Calothrix* strains and *vice versa*.

Horizontal gene transfer (HGT) of a part of 16S rDNA was detected in 16 cyanobacterial strains. Bacterial origin of the insert was suggested on the basis of analogous proteobacterial gene transfer of another insert to the same position within 16S rDNA (but donor of the insert was not detected). HGT was proposed also in case of *Umezakia* 16S rDNA sequence but this suggestion needs further investigation.

Sequence of *Stigonema mamillosum* was obtained. Its position in phylogenetic tree confirmed division of T-branching cyanobacteria into two clusters (H3 and H4). This division was unclear and validity of the only *Stigonema* sequence (*Stigonema ocellatum* SAG 48.90) was speculated.

5. References

Anagnostidis, K. & Komárek, J. 1988. Modern approach to the classification system of cyanophytes. Teil 3- Oscillatoriales. *Arch. Hydrobiol./Algolog. Stud.* **50-53**:327–472.

Anagnostidis, K. & Komárek, J. 1990. Modern approach to the classification system of cyanophytes. Teil 5- Stigonematales. *Arch. Hydrobiol./Algolog. Stud.* **59**:1–73.

Beltran, E. C. & Neilan B. A. 2000. Geographical segregation of the neurotoxin-producing cyanobacterium *Anabaena circinalis. Appl. Environ. Microbiol.* **66**:4468–74.

Besendahl, A., Qiu, Y.-L., Lee, J., Palmer. J. D. & Bhattacharya, D. 1999. The cyanobacterial origin and vertical transmission of the plastid $tRNA^{Leu}$ group-I intron. *Curr. Genet.* **37**:12–23.

Billi, D. & Grilli-Caiola, M. 1996. Effect of nitrogen limitation and starvation on *Chroococcidiopsis* sp. (Chroococcales). *New Phytol*. **133**:563–71.

Bolch, C. J. S., Orr, P. T., Jones, G. J. & Blackburn, S. I. 1999. Genetic, morphological, and toxicological variation among globally distributed strains of *Nodularia* (cyanobacteria). *J. Phycol.* **35**:339–55.

Boyer, S. L., Johansen, J. R. & Flechtner, V. R. 2002. Phylogeny and genetic variance in terrestrial *Microcoleus* (Cyanophycae) species based on sequence analysis of the 16S rRNA gene and associated 16S-23S ITS region. *J. Phycol*. **38**:1222–35.

Bornet, J. B. E. & Flahault, C. G. M. 1887. Révision des Nostocacées hétérocystées. *Annales des Sciences Naturelles, Botanique.* **7**:51-129.

Boucher, Y., Douady, C. J., Sharma, A. K., Kamekura, M. & Doolittle, W. F. 2004. Intragenomic heterogenity and intergenomic recombination among holoarcheal rRNA genes. *J. Bacteriol.* **186**:3980–90.

Bourrelly, P. 1985. Les algues d'eau douce. Tome III. N. Boubée & Cie., Paris. 606 pp.

Casamatta, D. A., Johansen, J., R., Vis, M. L. & Broadwater, S. T. 2005. Molecular and morphological characterization of ten polar and near-polar strains within the Oscillatoriales (Cyanobacteria). *J. Phycol*. **41**:421–38.

Casamatta, D. A., Gome, S. R. & Johansen, J. R. 2006. *Rexia erecta* gen. et sp nov and *Capsosira* lowei sp nov., two newly described cyanobacterial taxa from the Great Smoky Mountains National Park (USA). *Hydrobiologia*. **561**:13–26.

Castenholz, R. W. 1989. Subsection IV. Order Nostocales. *In* Staley, J. T., Bryant, M. P., Pfennig, N. & Holt, J. G. [Eds.] *Bergey's Manual of Systematic Bacteriology,* Vol. 3. Williams & Wilkins, Baltimore, Hong Kong, London, Sydney, pp. 1780–93.

Castenholz, R. W. 2001. Phylum BX. Cyanobacteria. *In* Boone, D. R. & Castenholz, R. W. [Eds.] *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1. Springer, New York, pp. 473–87.

Dyble, J., Paerl, H. W. & Neilan, B. A. 2002. Genetic characterization of *Cylindrospermopsis raciborskii* (Cyanobacteria) isolates from diverse geographical origins based on *nifH* and *cpcBA*-IGS nucleotide sequence analysis. *Appl. Environ. Microbiol*. **68**:2567–71.

Elenkin, A. A. 1934. Ob osnovnych principach sistemy Cyanophyceae. *Sov. Bot*. **1934**:51–83.

Fewer, D., Friedl, T. & Büdel, B. 2002. *Chroococcidiopsis* and heterocyst-differentiating cyanobacteria are each other's closest living relatives. *Mol. Phylogenet. Evol*. **23**:82–90.

Fiore, M. F., Sant'Anna, C. L., Azevedo, M. T. D., Komárek, J., Kaštovský, J., Sulek, J. & Lorenzi, A. S. 2007. The cyanobacterial genus *Brasilonema*, gen. nov., a molecular and phenotypic evaluation. *J. Phycol*. **43**:789–798.

Foster, R. A. & Zehr, J. P. 2006. Characterization of diatom–cyanobacteria symbioses on the basis of *nifH*, *hetR* and 16S rRNA sequences. Environmental Microbiology. **8**:1913–1925.

Garcia-Pichel, F., López-Cortés, A. & Nübel, U. 2001. Phylogenetic and morphological diversity of cyanobacteria in soil desert crusts from the Colorado plateau. *Appl. Environ. Microbiol*. **67**:1902–10.

Geitler, L. 1932. Cyanophyceae. *In Rabenhorst's Kryptogamen flora*. Akademische Verlagsgesellschaft, Leipzig, pp. 1-1196.

Geitler, L. 1925. Synoptische Darstellung der Cyanophyceen in morphologischer und systematischer Hinsicht. *Beihefte zum Botanischen Centralblatt.* **41:**163–294.

Giovannoni, S. J., Turner, S., Olsen, G. J., Barns, S., Lane, D. J. & Pace, N. R. 1988. Evolutionary relationships among cyanobacteria and green chloroplasts. *J. Bacteriol*. **170**:3584–92.

Gorelova, O. A., Baulina, O. I., Shchelmanova, A. G., Korzhenevskaya, T. G. & Gusev, M. V. 1996. Heteromorphism of the cyanobacterium *Nostoc* sp., a microsymbiont of the *Blasia pusilla* moss. *Microbiology*. **65**:719–26.

Guindon, S., Lethiec, F., Duroux P. & Gascuel, O. 2005. PHYML Online – a web sever for fast maximum likelihood-based phylogenetic inference. *Nucleic Acids Res*. **33**:557–9.

Gugger, M., Lyra, C., Suominen, I., Tsitko, I., Humbert, J.-F., Salkinoja-Salonen, M. S. & Sivonen, K. 2002. Cellular fatty acids as chemotaxonomic markers of the genera *Anabaena*, *Aphanizomenon*, *Microcystis*, *Nostoc* and *Planktothrix* (cyanobacteria). *Int. J. Syst. Evol.Microbiol.* **52**:1007–15.

Gugger, M. F. & Hoffmann, L. 2004. Polyphyly of true branching cyanobacteria (Stigonematales). *Int. J. Syst. Evol.Microbiol*. **54**:349–57.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. **41**:95–8.

Henson, B. J., Watson, L. E., Barnum, S. R. 2002. Molecular differentiation of the heterocytous cyanobacteria, *Nostoc* and *Anabaena*, based on complete NifD sequences. *Curr. microbiol*. **45**:161–4.

Henson, B. J., Hesselbrock, M., Watson, L. E. & Barnum, S. R. 2004. Molecular phylogeny of the heterocytous cyanobacteria (subsection IV and V) based on nifD. *Int. J. Syst. Evol. Microbiol.* **54**:493–7.

Honda, D., Yokota, A. & Sugiyama, J. 1999. Detection of seven major evolutionary lineages in cyanobacteria based on the 16S rRNA gene sequence analysis with new sequences of five marine *Synechococcus* strains. *J. Mol. Evol.* **48**:723–39.

Ishida, T., Yokota, A. & Sugiyama, J. 1997. Phylogenetic relationships of filamentous cyanobacterial taxa inferred from 16S rRNA sequence divergence. *J. Gen. Appl. Microbiol*. **43**:237–41.

Ishida, T., Watanabe, M. M., Sugiyama, J., Yokota, A. 2001. Evidence for polyphyletic origin of the members of the orders of Oscillatoriales and Pleurocapsales as determined by 16S rDNA analysis. *FEMS Microbiol. Lett*. **201**: 79-82.

Iteman, I., Rippka, R., Tandeau de Marsac, N., Herdman, M. 2002. rDNA analyses of planktonic heterocystous cyanobacteria, including members of the genera *Anabaenopsis* and *Cyanospira*. *Microbiology*. **148**:481–96.

Kaštovský, J. & Johansen, J. R. 2008. *Mastigocladus laminosus* (Stigonematales, Cyanobacteria): phylogenetic relationship to the soil-inhabiting genera of the order, and taxonomic implications for the genus. *Phycologia*. In press.

Katoh, K., Kuma, K., Toh, H. & Miyata, T. 2005. MAFFT version 5: Improvement in accuracy of multiple sequence alignement. *Nucleic Acids Res*. **33**:511–8.

Kellmann, R., Mills, T. & Neilan, B. A. 2006. Functional modeling and phylogenetic distribution of putative cylindrospermopsin biosynthesis enzymes. *J. Mol. Evol.* **62**:267–80.

Komárek, J. 1992. Diversita a moderní klasifikace sinic (Cyanoprocaryota) [Diversity and modern classification of Cyanobacteria (Cyanoprokaryota)]. Inaugural dissertation, not published.

Komárek, J., Anagnostidis, K. 1986. Modern approach to the classification systém of cyanophytes. Teil 2- Chroococcales. *Arch. Hydrobiol./Algolog. Stud.* **43**:157–226.

Komárek, J., Anagnostidis, K. (1989). Modern approach to the classification system of cyanophytes. Teil 4- Nostocales. *Arch. Hydrobiol./Algolog. Stud.* **56**:247–345.

Komárek J. & Hauer T. 2004. CyanoDB.cz - On-line database of cyanobacterial genera. http://www.cyanodb.cz.

Komárek, J. 2006. Cyanobacterial taxonomy: Current problems and prospects for the integration of traditional and molecular approaches. *Algae*. **21**:349–75.

Koonin, E. V., Makarova, K. S. & Aravind, L. 2001. Horizontal gene transfer in prokaryotes: Quantification and classification. *Annu. Rev. Microbiol*. **55**:709–42.

Laamanen, M. J., Gugger, M. F., Lehtimäki, J. M., Haukka, K. & Sivonen, K. 2001. Diversity of toxic and nontoxic *Nodularia* isolates (Cyanobacteria) and filaments from the Baltic sea. *Appl. Environ. Microbiol.* **67**:4638–47.

Lehtimäki, J., Lyra, C., Suomalainen, S., Sundman, P., Rouhiainen, L., Paulin, L., Salkinoja-Salonen, M. & Sivonen, K. 2000. Characterization of *Nodularia* strains, cyanobacteria from brackish waters, by genotypic and phenotypic methods. *Int. J. Syst. Evol. Microbiol.* **50**:1043–53.

Li, R., Wilhelm, S. W., Carmichael, W. W. & Watanabe, M. M. 2008. Polyphasic characterization of water bloom forming *Raphidiopsis* species (cyanobacteria) from central China. *Harmful Algae*. **7**:146-53.

Litvaitis, M. K. 2002. A molecular test of cyanobacterial phylogeny: interferences from constraint analyses. *Hydrobiologia.* **468**:135–45.

Lokmer, A. 2007. Polyphasic approach to the taxonomy of the selected oscillatorian strains (Cyanobacteria). Msc. thesis, Faculty of Biological Sciences, University of South Bohemia, Czech Republic. 40 pp.

Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J., Bachleitner, M. & Schleifer, K. H. 1998. Bacterial phylogeny based on comparative sequence analysis (review). *Electrophoresis*. **19**:554–68**.**

Lyra, C., Suomalainen, S., Gugger, M., Vezie, C., Sundman, P., Paulin, L. & Sivonen, K. 2001. Molecular characterization of planktic cyanobacteria of *Anabaena*, *Aphanizomenon*, *Microcystis* and *Planktothrix* genera. *Int. J. Syst. Evol. Microbiol.* **51**:513–26.

Marquardt, J., Palinska, K. A. 2007. Genotypic diversity of cyanobacteria assigned to the genus *Phormidium* (Oscillatoriales) from different habitats and geographical sites. *Arch. Microbiol.* **187**:397–413.

Miller, S. R., Augustine, S., Olson, T. L., Blankenship, R. E., Selker, J. & Wood, A. M. 2005. Discovery of a free-living chlorophyll d-producing cyanobacterium with a hybrid proteobacterial/cyanobacterial small-subunit rRNA gene. *Proc. Natl. Acad. Sci*. **102**:850–5.

Moffitt, M. C., Blackburn, S. I. & Neilan, B. A. 2001. rRNA sequences reflect the ecophysiology and define the toxic cyanobacteria of the genus *Nodularia*. *Int. J. Syst. Evol. Microbiol.* **51**:505–12.

Morandi, A., Zhaxybayeva, O., Gogarten, J. P. & Graf, J. 2005. Evolutionary and diagnostic implications of intragenomic heterogeneity in the 16S rRNA gene in *Aeromonas* strains. *J. Bacteriol*. **187**:6561–4.

NCBI Sequence Viewer v20. 2006. http://www.ncbi.nlm.nih.gov/entrez/viewer

Neilan, B. A., Saker, M. L., Fastner, J., Törökné, A. & Burns, B. P. 2003. Phylogeography of the invasive cyanobacterium *Cylindrospermopsis raciborskii*. *Mol. Ecol.* **12**:133–40.

Nelissen, B., DeBaere, R., Wilmotte, A. & DeWachter, R. 1996. Phylogenetic relationships of nonaxenic filamentous cyanobacterial strains based on 16S rRNA sequence analysis. *J. Mol. Evol*. **42**:194–200.

Nübel, U., Garcia-Pichel, F., & Muyzer, G. 1997. PCR primers to amplify 16S rRNA genes from cyanobacteria. *Appl. Environ. Microbiol*. **63**:3327–32.

Palinska, K. A. **&** Marquardt, J. 2008. Genotypic and phenotypic analysis of strains assigned to the widespread cyanobacterial morphospecies *Phormidium autumnale* (Oscillatoriales). *Arch. Microbiol.* **189**:325–35.

Page, R. D. M. 1996. TreeView: An application to display phylogenetic trees on personal computers. *Computer Appl. Biosci*. **12**:357–8.

Papaefthimiou, D., Van Hove, C., Lejeune, A., Rasmussen, U. & Wilmotte A. 2008. Diversity and host specificity of *Azolla* cyanobionts. *J. Phycol*. **44**:60–70.

Rajaniemi, P., Hrouzek, P., Kaštovská, K., Willame, R., Rantala, A., Hoffmann, L., Komárek, J. & Sivonen, K. 2005. Phylogenetic and morphological evaluation of the genera *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* (Nostocales, Cyanobacteria). *Int. J. Syst. Evol. Microbiol.* **55**:11–26.

Rippka, R., DeReuelles, J., Waterbury, J.B., Herdman, M. & Stanier, R. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* **111:**1–61.

Rippka, R., Castenholz, R. W., Iteman, I. & Herdman, M. 2001. Form-genus II. *Anabaenopsis* (Woloszynska) Miller 1923. *In* Boone, D. R. & Castenholz, R. W. [Eds.] *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1. Springer, New York, pp. 473–487.

Rosselló-Mora, R. & Amann, R. 2001. The species concept for prokaryotes. *FEMS Microbiol. Rev*. **25**:39–67.

Saker M. L. & Neilan, B. A. 2001. Varied diazotrophies, morphologies, and toxicities of genetically similar isolates of *Cylindrospermopsis raciborskii* (Nostocales, Cyanophyceae) from northern Australia. *Appl. Environ. Microbiol.* **67**:1839–45.

Seo, P.-S. & Yokota, A. 2003. The phylogenetic relationships of cyanobacteria inferred from 16S rRNA, *gyrB*, *rpoC1* and *rpoD1* gene sequences. *J. Gen. Appl. Microbiol*. **49**:191–203.

Sihvonen, L. M., Lyra, C., Fewer, D. P., Rajaniemi-Wacklin, P., Lethimäkki, J. M., Wahlsten, M. & Sivonen, K. 2007. Strains of the cyanobacterial genera *Calothrix* and *Rivularia* isolated from the Baltic Sea display cryptic diversity and are distantly related to *Gloeotrichia* and *Tolypothrix*. *FEMS Microbial. Ecol.* **61**:74–84.

Shi, T. & Falkowski, P. G. 2008. Genome evolution in cyanobacteria: The stable core and the variable shell. *Proc. Natl. Acad. Sci. U. S. A.* **105**:2510–5.

Stanier, R. Y., Kunisawa, R., Mandel, M. & Cohen-Bazire, G. 1971. Purrification and properties of unicellular blue-green algae (order Chroococcales). *Bact. Rev*. **35**:171–205.

Stanier, R.Y., Sistrom, W. R., Hansen, T. A., Whitton, B. A., Castenholz, R. W., Pfenning, N., Gorlenko, V. N., Kondratieva, E. N., Eimhjellen, K. E., Whittenbury, R., Gherna, R. L. & Truper, H. G. 1978. Proposal to place nomenclature of Cyanobacteria (Blue-green-algae) under rules of International Code of Nomenclature of Bacteria. *Int. J. Syst. Bacteriol*. **28**:335–6.

Swofford, D. L. 2002. PAUP*: Phylogenetic Analysis Using Parsimony (and other Methods). Sinauer Associates, Sunderland.

Tamas, I., Svircev, Z., Andersson, S. G. E. 2000. Determinative value of a portion of the *nif*H sequence for the genera *Nostoc* and *Anabaena* (Cyanobacteria). *Curr. microbiol*. **41**:197–200.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**:1596–9.

The European ribosomal RNA database. http://bioinformatics.psb.ugent.be/webtools/rRNA.

Toledo, G. & Palenik, B. 1997. *Synechococcus* diversity in the California Current as seen by RNA polymerase (*rpoC1*) gene sequences of isolated strains. *Appl. Environ. Microbiol.* **63**:4298–303.

Turner, S. 1997. Molecular systematics of oxygenic photosynthetic bacteria. *Plant. Syst. Evol. Suppl.* **11**:13–52.

Turner, S., Pryer, K. M., Miao, V. P. W., Palmer, J. D. 1999. Investigating deep phylogenetic relationships among cyanobacteria and plastids by small submit rRNA sequence analysis. *J. Eukaryot. Microbiol*. **46**:327–38.

Waterbury, J. B. & Stanier, R. Y. 1978. Patterns of growth and development in pleurocapsalean cyanobacteria. *Bacteriol. Rev.* **42**:2–44.

Wettstein, R. 1924. Handbuch der systematischen Botanik.1st ed. Franz Deuticke, Leipzig. 1071pp.

Wilmotte, A., Vanderauwera, G. & Dewachter, R. 1993. Structure of the 16S ribosomal RNA of the termophilic cyanobacterium *Chlorogloeopsis* HTF (*Mastigocladus laminosus* HTF) strain PCC 7518, and phylogenetic analysis. *FEBS Lett*. **317**:96–100.

Wilmotte, A. 1994. Molecular evolution and taxonomy of the cyanobacteria. *In* Bryant, D. [Ed.] *Molecular Biology of Cyanobacteria*. Kluwer, Dordrecht, Netherlands, pp. 1–25.

Wilmotte, A. & Herdman, M. 2001. Phylogenetic relationships among the cyanobacteria based on 16S rRNA sequences. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, pp. 487–493. Edited by D. R. Boone & R. W. Castenholz. New York: Springer.

Wolf, Y. I., Aravind, L. & Koonin, E. V. 1999. Evolution of aminoacyl-tRNA synthetases- analysis of unique domain architectures and phylogenetic trees reveals a complex history of horizontal gene transfer events. *Genome Res*. **9**:689–710.

Yap, W. H., Zhang, Z. & Wang, Y. 1999. Distinct types of rRNA operons exist in the genome of the actinomycete *Thermomonospora chromogena* and evidence for horizontal transfer of an entire rRNA operon. *J. Bacteriol*. **181**:5201–9.

Zehr, J. P., Mellon, M. T. & Hiorns, W. D. 1997. Phylogeny of cyanobacterial *nifH* genes: evolutionary implications and potential applications to natural assemblages. *Microbiology* **143**:1443–50.

Zhaxybayeva, O., Gogarten, J. P., Charlebois, R. L., Doolittle, W. F. & Papke, R. T. 2006. Phylogenetic analyses of cyanobacterial genomes: Quantification of horizontal gene transfer events. *Genome res.* **16**:1099–108.