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Molecular and phylogenetic characterization of two species of the genus *Nostoc* (Cyanobacteria) based on the *cpcB-IGS-cpcA* locus of the phycocyanin operon

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ABSTRACT

Traditionally, the taxonomy of the genus *Nostoc* is based on morphological and physiological characters. The extreme morphological variability of the *Nostoc* species, due to their life cycle and environmental conditions, hampers the correct identification of the individual species. This is also one of the reasons for the disputed taxonomic positions and relationships between the genera *Anabaena–Aphanizomenon* as well as between *Anabaena–Nostoc*. Therefore, it is necessary to use additional markers for development of a polyphasic classification system of order *Nostocales*. In light of this, we here present the first molecular and phylogenetic characterization of two species of the genus *Nostoc* (*Nostoc linckia* and *Nostoc punctiforme*) based on the *cpcB-IGS-cpcA* locus of the phycocyanin operon. The phylogenetic position of these two species within order *Nostocales* as well as within division Cyanobacteria has been determined. Our results indicate that genus *Nostoc* is heterogeneous. Analysis of the IGS region between *cpcB* and *cpcA* showed that *Nostoc* and *Anabaena* are distinct genera. Reported molecular and phylogenetic data will be useful to solve other problematic points in the taxonomy of genera *Aphanizomenon, Anabaena* and *Nostoc*.

Key words: *Cyanobacteria*, *Nostoc*, phycocyanin operon, IGS, phylogeny

Introduction

Cyanobacteria are autotrophic organisms that perform oxygenic photosynthesis. During their long and slow evolution, they have achieved huge diversity both in morphology and genetics, ranging from simple unicellular organisms to complex filamentous organisms (Whitton, 1992). These characteristics make it difficult to resolve their phylogenetic relationships and elucidate taxonomic classification (Liu et al., 2003). The morphological taxonomy and its derived numerical taxonomy, however, are based primarily on morphology and developmental characteristics. According to Rippka et al. (1979) and Rippka (1988), Cyanobacteria can be divided into five orders based on phenotypic characteristics: *Chroococcales* /I/, *Pleurocapsales* /II/, *Oscillatoriales* /III/, *Nostocales* /IV/, and *Stigonematales* /V/. *Nostoc*, the dominating genus in terrestrial symbiotic systems, is classified into subsection IV cluster I together with the genera *Anabaenopsis, Cyanospira, Aphanizomenon, Anabaena, Nodularia, Cylindrospermum, Cylindrospermopsis* and *Scytonema* (Wilmotte & Herdman, 2001). Traditionally, the taxonomy of *Nostoc* species has been based on morphological and physiological observations (Vagnoli et al., 1992). The extreme morphological flexibility of *Nostoc* species, which is influenced by the life cycle stage and environmental conditions (Vagnoli et al., 1992; Mollenhauer et al., 1994; Dodds et al., 1995), makes identification and taxonomy based on morphology alone problematic. This is also a reason for the unresolved taxonomic positions and relationships between the genera *Anabaena – Aphanizomenon* as well as between *Anabaena – Nostoc*. Therefore, supplementary genetic information should be incorporated to give a polyphasic classification system for the order *Nostocales* (Rasmussen & Svenning, 2001). Recently, many authors have used the genetic background as a "start line" for characterization and taxonomic evaluation of

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different taxa. In several studies *16S rRNA* gene has successfully been used for cyanobacterial phylogeny including *Nostocales* species (Giovannoni et al., 1988; Wilmotte, 1994; Nelissen et al., 1994; Nelissen et al., 1996; Lyra et al., 1997; Lyra et al., 2001; Gugger et al., 2002; Rajaniemi et al., 2005; Arima et al., 2012). Although the 16S rRNA molecule contains variable regions (Woese, 1987), it is too well conserved for studying species identity (Fox et al., 1992; Arima et al., 2012) or intraspecies variation (Ward et al., 1992). For this purpose more suitable is the non-coding *IGS* region between *cpcB* and *cpcA* subunits of the phycocyanin operon. (Neilan et al., 1995; Manen & Falquet, 2002; Teneva et al., 2005). The *IGS* region together with the flanking subunits β (cpcB) and α (cpcA) as well as *IGS* region alone can be used as a marker for subgenus identification of Cyanobacteria (Neilan et al., 1995; Bolch et al., 1996; Bolch et al., 1999) even in environmental samples (Baker et al., 2001; Baker et al., 2002).

Analysis of the internal transcribed spacer (*ITS*) region between the *16S* and *23S* rRNA genes has been used to discriminate between different cyanobacterial species and for phylogenetic analysis as well (Gugger et al., 2002). Sequence analysis of structural genes such as *nifH, petH, nrtP* and *groESL* has also been used for classification and phylogenetic analysis of Cyanobacteria (Henson et al., 2002; Arima et al., 2012). Recently, denaturing gradient gel electrophoresis (DGGE) has been applied to the identification of Cyanobacteria (Ferris & Ward, 1997; Nübel et al., 1997; Nübel et al., 2000; Ramsing et al., 2000).

The aim of this study was to determine the phylogenetic position of *Nostoc linckia* and *Nostoc punctiforme* within order *Nostocales* as well as within Cyanobacteria using the *cpcB-IGS-cpcA* locus of the phycocyanin operon.

Materials and Methods

Species and culturing conditions

Two species of the genus *Nostoc* (Cyanobacteria) were studied: *Nostoc linckia* (Roth.) Born et Flah – kept in PACC (Plovdiv Algal Culture Collection) under No 5085 and *Nostoc punctiforme* (Kütz) Har – kept in PACC under No 8646.

Prior to genomic DNA isolation, blue-green algae were grown intensively under sterile conditions as described by Dilov et al. (1972). A Z-nutrient medium was used for culturing (Staub, 1961). Cultures were synchronised by altering light/dark periods of 16/8 hours. The temperature was 33°C and 22°C during the light and dark period, respectively. The culture medium was aerated with 100 litre of air per one litre of medium, adding 1% CO_2 during the light cycle. The period of cultivation was 14 days.

DNA extraction, amplification and sequencing

Fresh algal mass was extracted according to the xanthogenate-SDS (XS) extraction protocol (Tillett & Neilan, 2000) with slight modifications. Modifications were concerned with the amount of algal mass, which was increased from 20 to 40 mg per isolation and with the volume of resuspension buffer (TER – 10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8; 100 μg/mL RNAse A), which was increased from 50 to 75 μL.

The *IGS* and flanking coding regions were amplified using the primers PCβF (5'-GGCTGCTTGTTTACGCGACA-3') and PCαR (5'-CCAGTACCACCAGCAACTAA-3') (Neilan et al., 1995). PCR was done using ReadyToGo Beads (Amersham Biosciences, Uppsala, Sweden) where the final mixture contained 1.5 U of *Taq* DNA polymerase, 10 mM Tris HCl pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 5 pmol of each of the two primers, 50 ng genomic DNA and water to a final volume of 25 μl. Amplification was done in a Biometra T3-thermocycler using the following programme: preheating for 5 minutes at 94°C, followed by 40 cycles of 10 seconds at 94°C, 20 seconds at 55°C and 40 seconds at 72°C. The final step was 10 minutes at 72°C. All PCR reactions were analysed by electrophoresis in a 1.5% agarose gel in 1x Tris-Acetate-EDTA Buffer (TAE) with GeneRuler™ 100 bp DNA Ladder Plus as the size marker (Fermentas GmbH, St. Leon-Rot, Germany). All gels were stained with ethidium bromide and photographed under UV trans-illumination. After visualizing the bands under UV light, bands of interest were cut out of the gel with a sterile blade and placed in a sterile 2 mL centrifuge tube. The DNA from PCR amplification was purified using a QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). In the majority of cases, the purified DNA was cloned into a plasmid, using TOPO TA Cloning® Kit (Invitrogen Life Technologies Inc, Carlsbad, CA, USA). The clones were checked for the predicted size of inserts by a broth checking procedure, which comprised PCR amplification of 2 μL of bacterial suspension using the specific primers mentioned above. PCR started with a 10 min pre-heating period at 94°C, followed by 30 cycles of amplification with an annealing temperature of

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55°C. Gel electrophoresis of the PCR products was done as described above. Selected plasmids with inserts were isolated and purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega GmbH, Mannheim, Germany). Plasmid DNA was lyophilised and 1-2 μg DNA were sent for sequencing (MWG Biotech AG, Ebersberg bei München, Germany). Alternatively, the DNA purified from the PCR product was lyophilised and at least 140 ng DNA, along with the specific primers, were sent to sequencing directly without prior cloning.

Phylogenetic analysis

Nucleotide sequences obtained from DNA sequencing were compared with sequence information available in the National Center for Biotechnology Information (NCBI) data base using BLAST. Based on previously published sequences, the regions of subunit ß (*cpcB*), IGS and subunit α (*cpcA*) of the phycocyanin operon were determined. DNA sequences of *cpcB* and *cpcA* were translated into amino acid sequences and compared to data available in the NCBI data base using BLAST X and Protein–Protein BLAST. Multiple pair-wise alignment was done using the MEGALIGN option in CLUSTAL W, which is part of the DNAStar software. Further, MEGALIGN (DNAStar) and PHYLIP (Phylogeny Inference Package) were used to phylogenetically analyse the *cpcB* and *cpcA* coding regions of the *Nostoc* sequences obtained here, along with NCBI Sequences available for other Cyanobacteria as indicated in Table 1. Analyses comprised the methods of parsimony, maximum likelihood and distance and were used to construct phylogenetic trees.

The sequence alignment was randomly re-sampled 100-fold using SEQBOOT to produce a data set for input into PROTPARS (protein maximum parsimony), ProML (Protein maximum Likelihood), and PROTDIST (JTT-corrected distances). The data produced by PROTDIST were analyzed with NEIGHBOR (Neighbor-Joining method). Majority rule tree topologies were calculated using CONSENSE, and trees were viewed using DRAWGRAM. *Arthrospira sp*. Paracas P2 (AJ401166) was used as an outgroup to root the trees.

CLUSTAL-W was used to perform a multiple pair-wise comparison. The alignment of sequences was converted into a distance matrix, which describes the divergence of the sequences. The phylogenetic tree was based on the distance matrix and was built using the Neighbor Joining Algorithm. The tree has branches of differing length, which are proportional to the divergence.

Table 1. *List of species/strains used for phylogenetic analysis within Cyanobacteria.*

Taxon	Strain	GenBank accession number §
Chroococcales		
Chroococcus dispersus	SA01P31	AJ003184
Microcystis aeruginosa	UWOCC 001	AF195158
Microcystis aeruginosa	UWOCC MR-C	AF195173
Microcystis aeruginosa	EAWAG120a	AJ003173
Microcystis aeruginosa	EAWAG171	AJ003179
Microcystis flos-aquae	UWOCC C3-9	AF195163
Synechococcus sp	PCC 9005	AF223465
Synechococcus sp.	PS685	AF223455
Synechocystis sp.	EAWAG174	AJ003180
Nostocales		
Anabaena circinalis	AWQC019A	AF426003
Anabaena flos-aquae	AWQC264D	AF426008
Anabaena sp.	KAC 16	AY036901
Anabaena lemmermannii	BC Ana 0027	AY886910
Anabaena lemmermannii	BC Ana 0031	AY886914
Anabaena lemmermannii	BC Ana 0034	AY886917
Aphanizomenon flos-aquae	CCAP 1401/1	AJ243971
Aphanizomenon sp.	TR183	AY036900
Aphanizomenon sp.	KAC15	AF364339
Cylindrospermopsis raciborskii	4799	AF426786
Cylindrospermopsis raciborskii	Florida I	AY078438
Cylindrospermopsis raciborskii	Germany2	AF426798
Cylindrospermopsis raciborskii	isolate sds	AF426803
Cylindrospermopsis raciborskii	isolate sds	AF426804
Nodularia harveyana		AF364342
Nodularia spumigena	PCC 7804	AF101452
Nodularia spumigena	nsb105	AF101444
Nodularia spumigena	clone kas 32-pc	AF364343
Nodularia sp.		AJ224915
Nodularia sp.		AJ224916
Nostoc linckia	**PACC 5085**	**AY466120**
Nostoc punctiforme	**PACC 8646**	**AY466131**
Oscilatorialles		
Arthrospira sp.	Paracas P2	AJ401166
Arthrospira sp.	PK	AJ401179
Lyngbya sp.	PCC 7419	AJ401187
Lyngbya arugineo-coerulea	PACC 8601	AY466129
Lyngbya kutzingiana	PACC 5419	AY466121
Planktothrix rubescens	BC-Pla 9307	AJ131820
Planktothrix rubescens	BC-Pla 9303	AJ132279
Oscillatoria sp.	PCC 7515	AJ401185
Oscillatoria sp.	PCC 6304	AJ401186
Phormidium autumnale	PACC 5505	AY466122
Phormidium autumnale	PACC 5511	AY466123
Phormidium autumnale	PACC 5517	AY466124
Phormidium autumnale	PACC 5522	AY466125
Phormidium autumnale	PACC 5527	AY466126
Phormidium autumnale	PACC 5529	AY466127
Phormidium molle	PACC 8140	AY466128
Phormidium bijugatum	PACC 8602	AY466130
Phormidium uncinatum	PACC 8693	AY466132

§ Sequences determined in this study are indicated in bold.

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Based on the multiple pair-wise comparison of the *cpcB-IGS-cpcA* locus of *Nostoc* sequences, all variable positions were identified.

Results and Discussion

The sequence of *Nostoc linckia* studied here contained the 258 nucleotides (nt) of the 3' end of the *cpcB* coding region, 100 nt representing the intergenic spacer region, and 296 nt of the 5' end of *cpcA*. The sequence of *Nostoc punctiforme* contained the 234 nucleotides (nt) of the 3' end of the *cpcB* coding region, and 335 nt of the 5' end of *cpcA*. The obtained sequence of the IGS region of *Nostoc punctiforme* was short and incomplete. Studied regions were identified based on sequence comparisons, using BLASTN and BLAST2 Sequence with *Lyngbya sp*. AJ401187, as well as on the *cpcB* stop- and the *cpcA* start-codon (TAA and ATG, respectively). Determined nucleotide sequences were translated into amino acid sequences and together with others, chosen from the GenBank (Table 1) were used for molecular and phylogenetic analyses.

Molecular and genetic characterization of the cpcB and cpcA coding regions

Since this was the first study of genus *Nostoc* based on *cpcB-cpcA* locus, we decided to determine the variability of the amino acid sequences of β- and α-subunits in different species within order *Nostocales* as well as to compare this variability among the genera. Our data showed higher variability in both β- and α-subunits (15.7% and 34.7% respectively) of *Nostoc* species compared with the values for other genera within *Nostocales* (0 - 4.22% for *cpcB* and 1.07 - 3% for *cpcA*) (Table 2). The relatively high variability in the two conservative subunits determines the heterogeneity of genus *Nostoc*. Since the α-subunit has higher values, it seems that *cpcA* dominates the phylogeny of this genus.

Phylogenetic analysis based on cpcB and cpcA within order Nostocales

It is well known that the variability in the amino acid sequences of the studied species influences their phylogeny. These high indices of variability in *Nostoc* species determine the lack of monophyly in the phylogenetic trees based on *cpcB* and *cpcA* subunits (Figures 1 and 2).

In both phylogenetic trees *Nostoc punctiforme* forms sister clasters with *Anabaena circinalis* (AF426003). In the phylogenetic tree based on *cpcB* it is closely related to *Anabaena lemmermannii* (AY886910) (Figure 1) and grouped in one clade with the same species (AY886910) in *cpcA* (Figure 2).

Nostoc linckia had a similar to *Nostoc punctiforme* topology in the *cpcB* tree and forms a cluster together with two strains of *Anabaena lemmermannii*, one of which again was AY886910 (Figure 1). Likewise our data, the analysis of phylogenetic relationships between *Nostoc* and *Anabaena* based on 16S rDNA showed that *Nostoc* species form a cluster interspersed with members of the genus *Anabaena* (Svennimg et al., 2005).

The phylogenetic position of *Nostoc linckia* based on *cpcA* clustered this species in one monophyletic clade containing *Nodularia* species (Figure 2). This topology correlates with the position of *Nostoc linckia* in the phylogenetic tree based on *cpcA* within Cyanobacteria (Figure 5) and other investigations (Lyra et al., 2001).

In both phylogenetic reconstructions (Figures 1 and 2) the members of genera *Nodularia* and *Cylindrospermopsis* form monophyletic clusters. In contrasts, the members of *Anabaena* and *Aphanizomenon* are heterogeneous and intermixed within the order *Nostocales*. Similar topology was obtained in other phylogenetic analyses based on 16S rDNA, *ITS1*, *rpoB* and *rbcLX* genes (Gugger et al., 2002; Rajaniemi et al., 2005; Svenning et al., 2005).

Table 2. *Variability of the amino acid sequences of cpcB and cpcA subunits within order Nostocales.*

Genus	cpcB			cpcA		
	Number of compared positions	Substitutions	Variability (%)	Number of compared positions	Substitutions	Variability (%)
Nostoc	76	12	15.7	72	25	43.7
Anabaena	71	2	2.81	65	1	1.53
Aphanizomenon	71	2	2.81	93	1	1.07
Cylindrospermopsis	67	0	0	79	1	1.26
Nodularia	71	3	4.22	66	2	3.00

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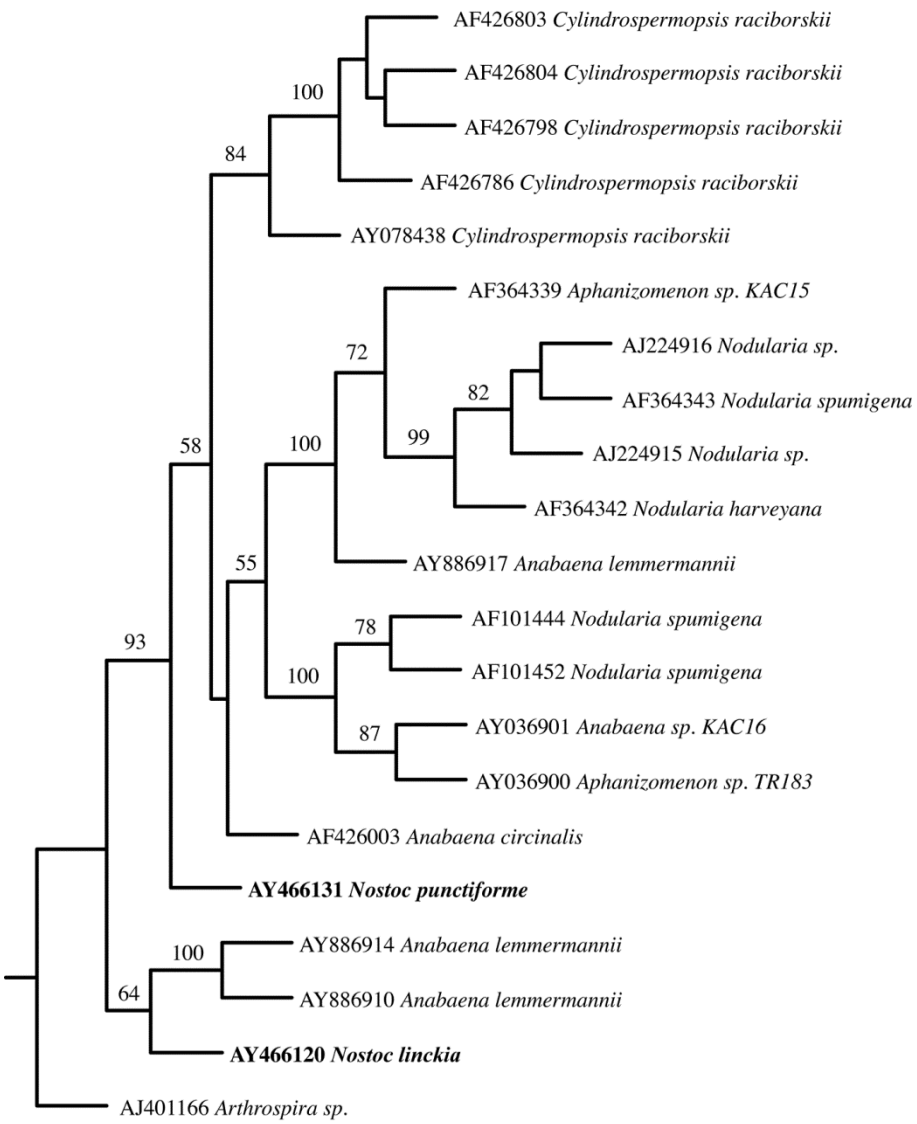

Figure 1. *Amino acid parsimony tree obtained from phylogenetic analysis (PHYLIP) of parts of the cpcB coding region within order Nostocales. Figures at branch points indicate the bootstrap support for each branch estimated from a bootstrap consensus analysis using 100 replications.*

Svenning et al. (2005) showed that the clade formed from *Anabaena* is intermixed and *Aphanizomenon* is deeply nested within *Anabaena*, which becomes paraphyletic if *Aphanizomenon* is maintained. Gugger et al. (2002) suggested that *Anabaena* and *Aphanizomenon* belong to one and the same genus.

The division of *Nostoc* and *Anabaena* into two distinct genera has been debated for some time (Rippka, 1988; Zehr et al., 1997; Tamas et al., 2000). *Nostoc* and *Anabaena* are traditionally separated based on morphological characteristics and life cycle differences (Rippka, 1988; Wilmotte, 1994; Turner, 1997; Tamas, et al., 2000). 16S rDNA studies have shown that *Nostoc* and *Anabaena* are closely related. However, these studies were unable to clearly differentiate between the two genera (Giovannoni et al., 1988; Wilmotte, 1994; Turner, 1997). Likewise, studies using partial (359 bp) *nifH* sequences found *Nostoc* and *Anabaena* to be very closely related (Turner, 1997; Zehr et al., 1997; Tamas, et al., 2000). Additionally, Tamas et al. (2000) suggest that *Nostoc* and

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Anabaena should be merged into a single genus. Henson et al. (2002) suggest the opposite: that *Nostoc* and *Anabaena* should be remaining as separate genera.

Since the present data about the phylogenetic position of the genera *Anabaena–Aphanizomenon* and *Nostoc–Anabaena* cannot resolve the problematic taxonomy of these genera, we decided to analyze the IGS regions of several species. As already was noted, the IGS region between *cpcB* and *cpcA* of the phycocyanin operon can be used as a marker for identification of genera and species within Cyanobacteria. Data from the pair distance analysis of the IGS sequences showed high similarity values between *Anabaena* and *Aphanizomenon* (Figure 3). The identity between the IGS sequences of *Aphanizomenon* and *Anabaena* species was ranging from 84.8% to 96.3%. For comparison, the identity of this region between species within one genus is 85.4% - 100%.

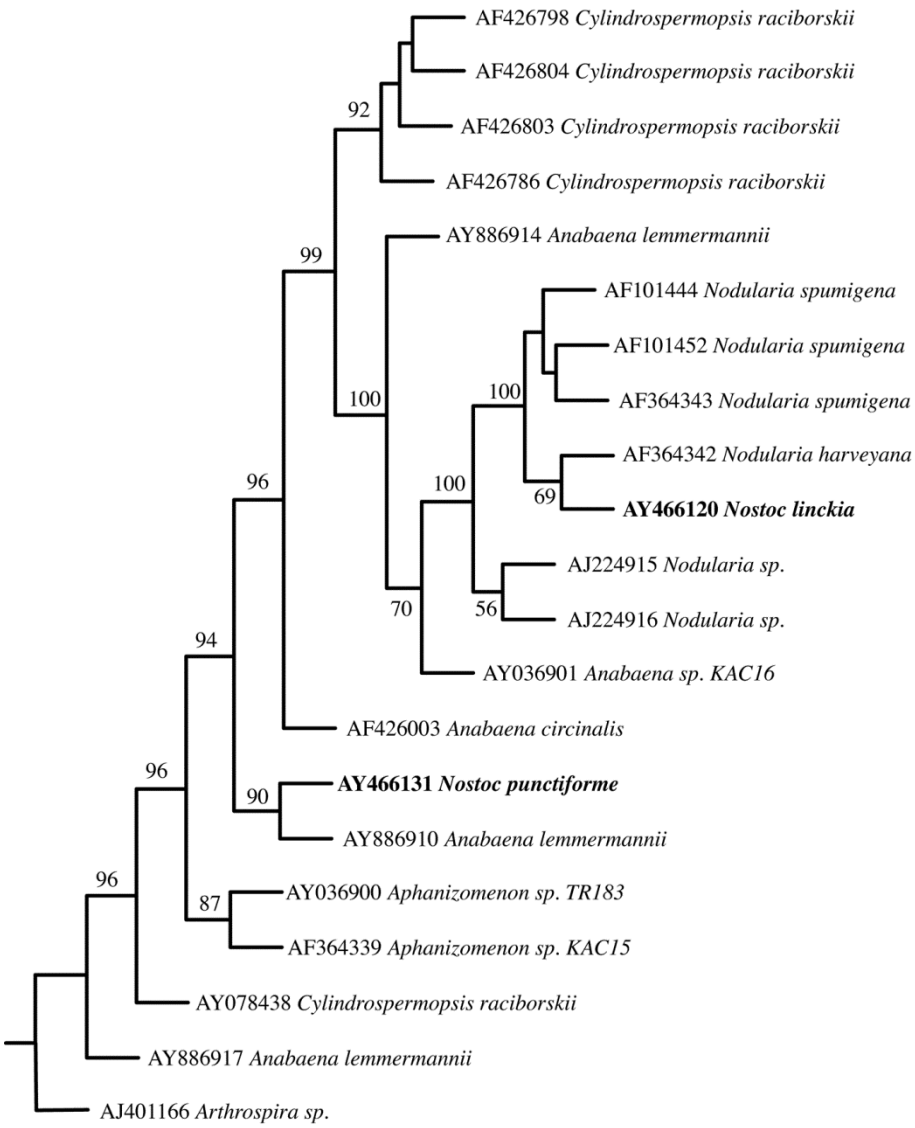

Figure 2. *Amino acid parsimony tree obtained from phylogenetic analysis (PHYLIP) of parts of the cpcA coding region within order Nostocales. Figures at branch points indicate the bootstrap support for each branch estimated from a bootstrap consensus analysis using 100 replications.*

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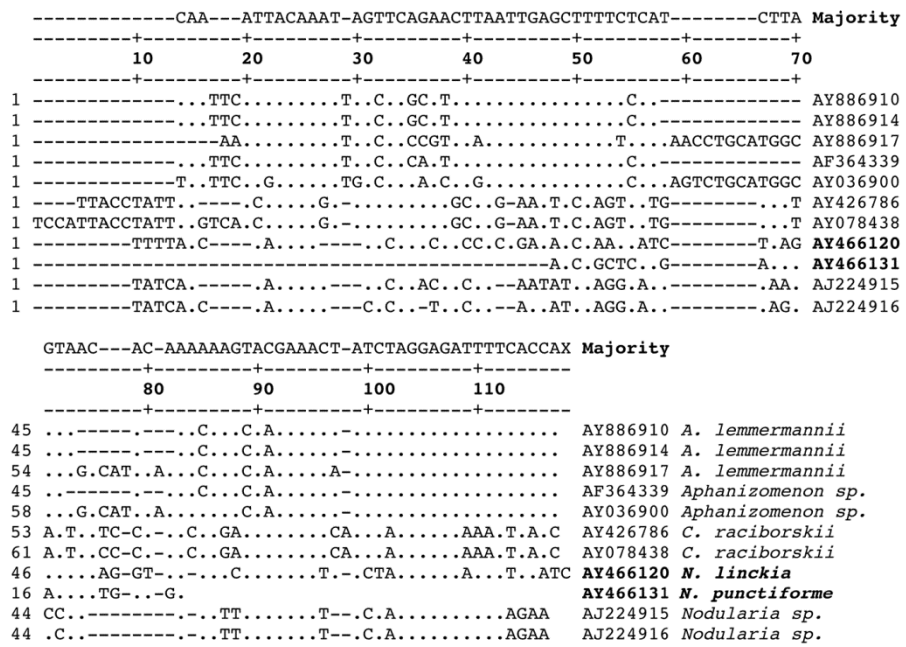

Figure 3. *Multiple alignment (DNAStar) of sequences of the IGS region of some Nostocales species. The different lengths of the IGS region are noted. Dash (-) represents on alignment gap, period (.) represents sequence identical to that of the majority sequence generated by the program.*

For the members of the other genera these values were: 40.4% - 47.6% (for *Anabaena-Nostoc*), 42.4 - 51.2 (for *Anabaena-Cylindrospermopsis*), and 35.4 - 45.1 (for *Anabaena-Nodularia*). These data indicate that a revision of the taxonomic position of *Anabaena* and *Aphanizomenon* within *Nostocales* is required.

Phylogenetic analysis based on cpcB and cpcA within division Cyanobacteria

To determine the position of the studied *Nostoc* species within the division, we have performed several phylogenetic analyses. ML- NJ- and parsimony trees were generated using the protein sequences of each coding subunit (*cpcB* and *cpcA*). Since the topology of the phylogenetic trees was similar in Figures 4 and 5 are presented only the parsimony trees.

The topology of *Nostocales* in the phylogenetic tree based on cpcB showed 3 clades (Figure 4). The first clade [1] includes two *Anabaena* species (*A. circinalis* AWQC019A and *A. flos-aquae* AWQC264D). *Synechococcus sp*. was also clustered in this group, which is normal taking in account that the orders *Chroococcales* and *Oscillatoriales* are polyphyletic. This topology was supported by the maximal bootstrap value of 100%.

The second clade [2] was not coherent and it is divided into three subclades – 2A, 2B and 2C. It contains several *Oscillatoriales* (genus *Phormidium*). *Nodularia harveyana* and *Aphanizomenon flos-aquae* form the first subclade [2A]. The topology of the species forming subclade 2B (*Anabaena, Aphanizomenon, Nodularia*) was similar to the previous phylogenetic tree (Figure 1) and supported again with a bootstrap value of 100% (Figure 4). Subclade 2C consisted of *Cylindrospermopsis raciborskii* strains with *Synechococcus* sister to the subclade was supported with a bootstrap value of 59%. This topology confirmed the obtained monophyly of *Cylindrospermopsis*.

Subclade 3, which is supported by bootstrap values of 100% include the investigated species (*Nostoc linckia* and *Nostoc punctiforme*). These two species are closely related and linked as a sister group to *Planktothrix rubescens* and *Lyngbya aerugineo-coerulea*. In the distance tree based on the partial 16S rRNA sequences presented by Rudi et al. *Nostoc* and *Planktothrix* were clustered in sister clades and had similar to the presented here topology (Rudi et al., 1997). The performed phylogenetic analysis confirmed that *Anabaena* and *Aphanizomenon* are polyphyletic and heterogeneous genera.

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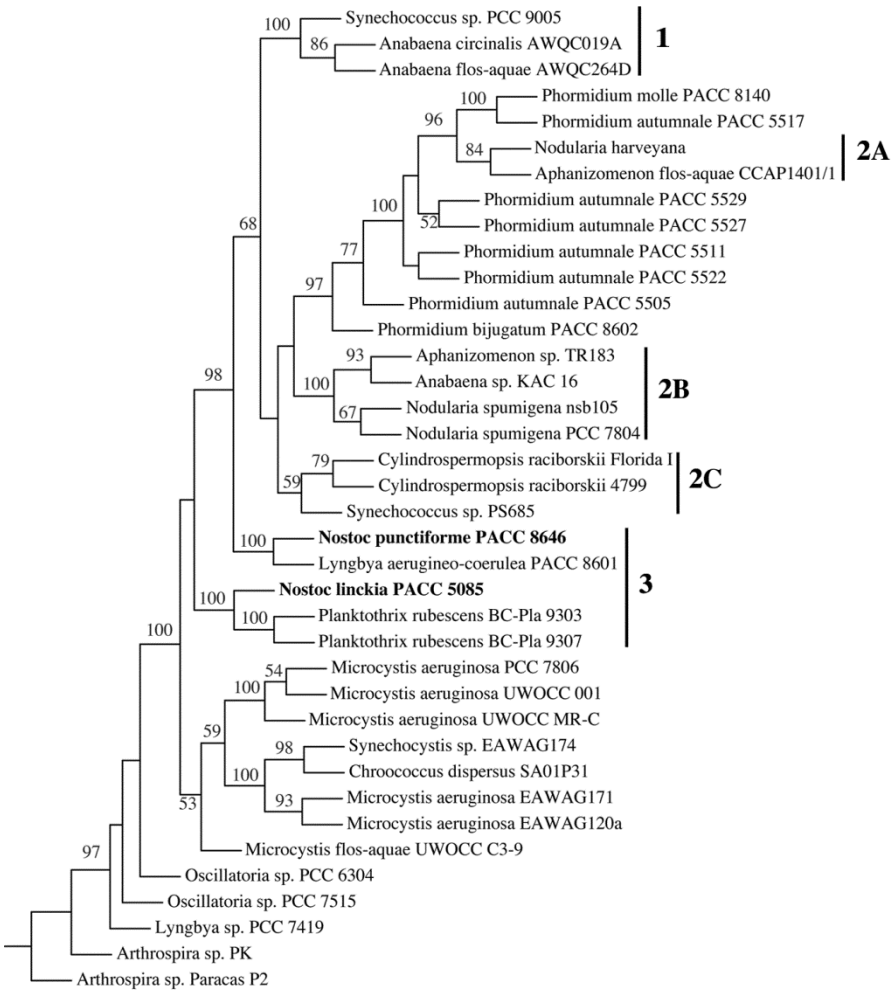

Figure 4. *Phylogenetic parsimony tree based on partial amino acid sequences of the cpcB region within Cyanobacteria. Figures at branch points indicate the bootstrap support for each branch estimated from a bootstrap consensus analysis using 100 replications.*

In the phylogenetc tree based on *cpcA*, the members of order *Nostocales* are divided into 2 clades – 1A and 1B (Figure 5). The first clade [1A] contained *Nostoc punctiforme* and all *Anabaena*, *Aphanizomenon* and *Cylindrospermopsis* species included in the phylogenetic analysis. *Nostoc punctiforme* is closely related to *Anabaena sp*. KAC16 and *Cylindrospermopsis raciborskii,* which topology was supported with a high bootstrap value (76%). Similar topology of genera *Anabaena, Cylindrospermopsis* and *Nostoc* was obtained in a distance tree based on alignment of partial *16S rRNA* sequences from 47 cyanobacterial species (Nelissen et al., 1996).

The second clade [1B] contained *Nostoc linckia* and members of genus *Nodularia*. This phylogenetic position repeat the topology observed in the phylogenetic tree based on cpcA within *Nostocales*. Heterogeneous species of *Chroococcales* and *Oscillatoriales* (Ishida et al., 1997) are intermixed within this clade as well.

Phylogenetic analyses of *Nostoc* species based on *petH, nrtP* and *groEL* genes generated the same phylogenic trees as the trees based on the *16S rRNA* gene sequences (Arima et al., 2012). Hence, these genes are not suitable to recognize distinct species. In addition, Arima et al. (2012) investigated the phylogeny of *Nostoc commune* using a combination of molecular and chemotaxonomic approach.

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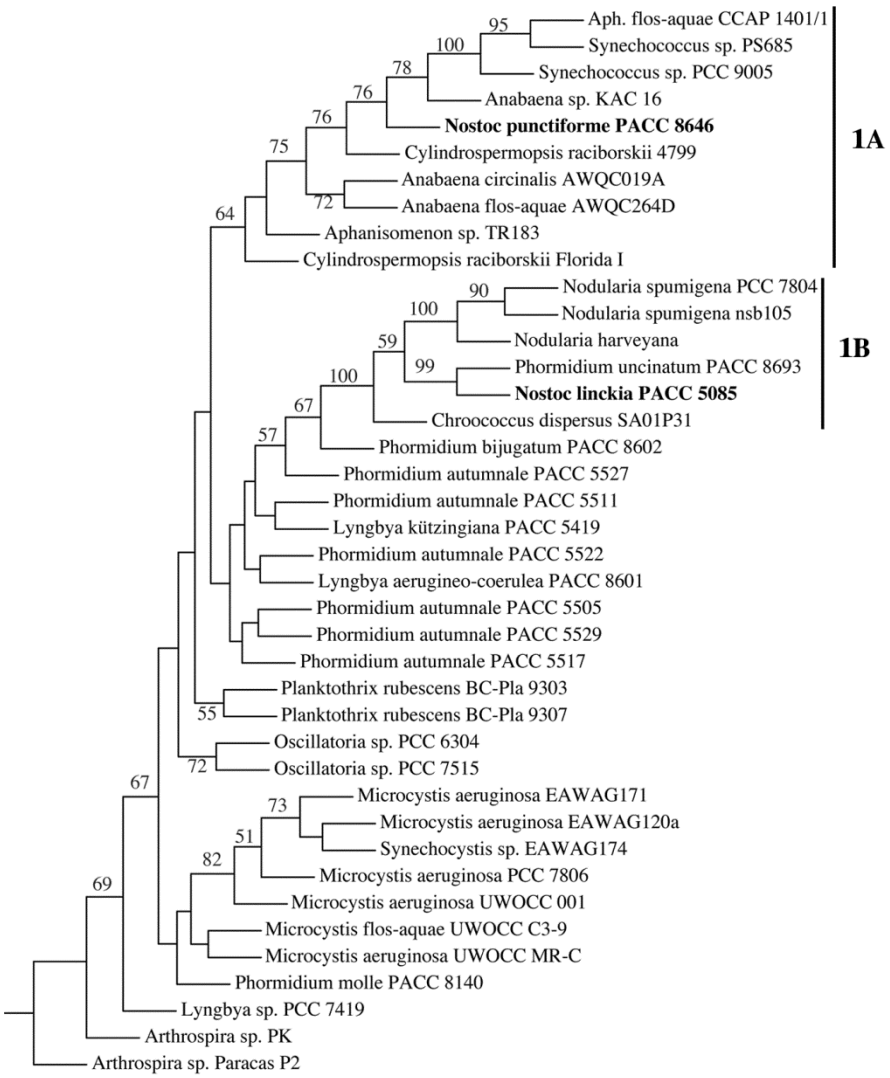

Figure 5. *Phylogenetic parsimony tree based on partial amino acid sequences of the cpcA region within Cyanobacteria. Figures at branch points indicate the bootstrap support for each branch estimated from a bootstrap consensus analysis using 100 replications.*

To discriminate the terrestrial cyanobacterium *N. commune* from the other *Nostoc* species the authors studied the presence and/or absence of specific genes involved in the carotenogenesis pathways and genes related to the structure of the extracellular matrix (*wspA* gene). This chemotaxonomic approach was suggested as a valid tool to characterize and distinguish different *Nostoc* strains.

To elucidate the phylogenetic relationships among cyanobacteria a combination of phylogenomic and signature proteins has been also proposed (Gupta & Mathews, 2010). Sixty-five proteins specific for all of the sequenced *Nostocales* species (*Nostoc, Anabaena, Nodularia*) have been identified. These unique signature proteins were suggested as useful tools for identification of cyanobacterial species/strains and for understanding cyanobacterial phylogeny and taxonomy (Gupta & Mathews, 2010).

Conclusion

The present study indicates that genus *Nostoc* is heterogeneous and the *cpcB-IGS-cpcA* gene sequences are suitable markers for distinguishing *Nostoc* species or

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strains. Analysis of the IGS region between cpcB and cpcA shows that *Nostoc* and *Anabaena* are distinct genera, while the taxonomic status of the genera *Aphanizomenon* and *Anabaena* is still unclear since their similarity is up to 96.3%. Therefore, additional analyses and revision are required.

References

Arima H, Horiguchi N, Takaichi S, Kofuji R, Ishida K, Wada K, Sakamoto T. 2012. Molecular genetic and chemotaxonomic characterization of the terrestrial cyanobacterium *Nostoc commune* and its neighboring species. FEMS Microbiol. Ecol., 79(1): 34-45.

Baker JA, Entsch B, Neilan BA, McKay DB. 2002. Monitoring changing toxigenicity of a cyanobacterial bloom by molecular methods. Appl. Environ. Microbiol., 68(12): 6070-6076.

Baker JA, Neilan BA, Entsch B, McKay DB. 2001. Identification of cyanobacteria and their toxigenicity in environmental samples by rapid molecular analysis. Environ. Toxicol., 16(6): 472-482.

Bolch CJ, Blackburn SI, Neilan BA, Grewe PM. 1996. Genetic characterization of strains of cyanobacteria using PCR-RFLP of the *cpcBA* intergenic spacer and flanking regions. J. Phycol., 32(3): 445-451.

Bolch CJ, Orr PT, Jones GJ, Blackburn SI. 1999. Genetic, morphological and toxicological variation among globally distributed strains of *Nodularia* (Cyanobacteria). J. Phycol., 35(2): 339-355.

Dilov Chr, Bozkova M, Staev S. 1972. Prakticeskij blok dlja kultivirovanija mikrovodoroslej v laboratornych uslovijach [A practical block for cultivation of microalgae in laboratory conditions]. Dokl. Akad. Selskoch. Nauk. Bolg., 4: 169–175.

Dodds WK, Gudder GA, Dodds D. 1995. The ecology of *Nostoc*. New Phytol., 31: 2-18.

Ferris MJ, Ward DM. 1997. Seasonal distribution of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. Appl. Environ. Microbiol., 63(4): 1375-1381.

Fox GE, Wisotzkey JD, Jurtshuk P Jr. 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol., 42(1): 166-170.

Giovannoni SJ, Turner S, Olsen GJ, Barns S, Lane DJ, Pace NR. 1988. Evolutionary relationships among cyanobacteria and green chloroplasts. J. Bacteriol., 170(8): 3584-3592.

Gugger M, Lyra C, Henriksen P, Coute A, Humbert JF, Sivonen K. 2002. Phylogenetic comparison ot the cyanobacterial genera *Anabaena* and *Aphanizomenon*. Int. J. Syst. Evol. Microbiol., 52(5): 1867-1880.

Gupta RS, Mathews DW. 2010. Signature proteins for the major clades of Cyanobacteria. BMC Evol. Biol., 10: 24.

Henson B, Watson L, Barnum S. 2002. Molecular differentiation ot the heterocystous cyanobacteria, *Nostoc* and *Anabaena*, based on complete *Nif*D sequences. Curr. Microbiol., 45(3): 161-164.

Ishida T, Yokota A, Sugiyama J. 1997. Phylogenetic relationships of filamentous cyanobacterial taxa inferred from 16S rRNA sequence divergence. J. Gen. Appl. Microbiol., 43(4): 237-241.

Liu XJ, Chen F, Jaing Y. 2003. Differentiation of *Nostoc flagelliforme* and its neighboring species using fatty-acid profiling as a chemataxonomic tool. Curr. Microbiol., 47(6): 467-474.

Lyra C, Hantula J, Vainio E, Rapala J, Rouhiainen L, Sivonen K. 1997. Characterization of cyanobacteria by SDS-PAGE of whole-cell proteins and PCR/RFLP of the 16S rRNA gene. Arch. Microbiol., 168(3): 176-184.

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(2): 513-526.

Manen JF, Falquet J. 2002. The *cpcB-cpcA* locus as a tool for the genetic characterization of the genus *Arthrospira* (Cyanobacteria) evidence for horizontal transfer. Int. J. System. Evol. Microbiol., 52(3): 861-867.

Mollenhauer D, Büdel B, Mollenhauer R. 1994. Approaches to species deliminations in the genus *Nostoc* VAUCHER 1803 ex BORNET et FLAHAULT 1888. Algol. Studies, 75: 189-209.

Neilan BA, Jacobs D, Goodmann A. 1995. Genetic diversity and phylogeny of toxic cyanobacteria determined by DNA polymorphisms within the pycocyanin locus. Appl. Environ. Microbiol., 61(11): 3875-3883.

Nelissen B, de Baere R, Wilmotte A, de Wachter R. 1996. Phylogenetic relationships of nonaxenic filamentous cyanobacterial strains based on 16S rRNA sequence analysis. J. Mol. Evol., 42(2): 194-200.

Nelissen B, Wilmotte A, Neefs J-M, de Wachter R. 1994. Phylogenetic relationships among filamentous helical cyanobacteria investigated on the basis of 16S ribosomal RNA gene sequence analysis. Syst. Appl. Microbiol., 17(2): 206-210.

Nübel U, Garcia-Pichel F, Clavero E, Muyzer G. 2000. Matching molecular diversity and ecophysiology of benthic cyanobacteria and diatoms in communities along a salinity gradient. Environ. Microbiol., 2(2): 217-226.

Nübel U, Garcia-Pichel F, Muyzer G. 1997. PCR primers to amplify 16S rRNA genes from cyanobacteria. Appl. Environ. Microbiol., 63(8): 3327-3332.

Rajaniemi P, Hrouzek P, Kastovska 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(1): 11-26.

Ramsing NB, Ferris MJ, Ward DM. 2000. Highly ordered vertical structure of *Synechococcus* populations within the one-millimeter- thick photic zone of a hot spring cyanobacterial mat. Appl. Environ. Microbiol., 66(3): 1038-1049.

Rasmussen U, Svenning M. 2001. Characterization by genotypic methods of symbiotic *Nostoc* strains isolated from five species of *Gunnera*. Arch. Microbiol., 176(3): 204-210.

Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY.

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1979. Generic assignments, strain histories and properties of pure cultures of Cyanobacteria. J. Gen. Microbiol., 111: 1-61.

Rippka R. 1988. Recognition and identification of cyanobacteria. Methods Enzymol., 167: 28-67.

Rudi K, Skulberg OM, Larsen F, Jakobsen KS. 1997. Strain characterization and classification of oxyphotobacteria in clone cultures on the basis of 16S rRNA sequences from the variable regions V6, V7, and V8. Appl. Environ. Microbiol., 63(7): 2593-2599.

Staub R. 1961. Ernährungsphysiologisch- antokologische Untersuchungen an der planktonischen Blaualga *Oscillatoria rubescens* DC. Schweiz Z. Hydrol. 23: 82-198.

Svenning MM, Eriksson T, Rasmussen U. 2005. Phylogeny of symbiotic cyanobacteria within the genus *Nostoc* based on 16S rDNA sequence analyses. Arch. Microbiol., 183(1): 19-26.

Tamas I, Svircev Z, Andersson S. 2000. Determinative value of a portion of the *nif*H sequence for the genera *Nostoc* and *Anabaena* (cyanobacteria). Curr. Microbiol., 41(3): 197-200.

Teneva I, Dzhambazov B, Mladenov R, Schirmer K. 2005. Molecular and phylogenetic characterization of *Phormidium* species (Cyanoprokaryota) using the *cpcB-IGS-cpcA* locus. J. Phycol., 41(1): 188-194.

Tillett D, Neilan BA. 2000. Xantogenate nucleic acid isolation from cultured and environmental Cyanobacteria. J. Phycol., 36(1): 251-258.

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

Vagnoli L, Magheri MC, Allotta G, Materassi R. 1992. Morphological and physiological properties of symbiotic cyanobacteria. New Phytol., 120: 43-249.

Ward DM, Bateson MM, Weller R, Ruff-Roberst AL. 1992. Ribosomal RNA analysis of microorganisms as they occur in nature. Adv. Microb. Ecol., 12: 219-286.

Whitton BA. 1992. Diversity, ecology, and taxonomy of the cyanobacteria. – In: Mann NH & Carr NG (eds), Photosynthetic prokaryotes, Plenum, p. 1-51.

Wilmotte A, Herdman M. 2001. Phylogenetic relationships among the cyanobacteria based on 16S rRNA sequences. – In: Boone DR, Castenholz RW & Garrity GM (eds), Bergey's manual of systematic bacteriology, 2nd edn., Springer, p. 487-514.

Wilmotte A. 1994. Molecular evolution and taxonomy of the cyanobacteria. – In: Bryant DA (ed), The molecular biology of Cyanobacteria, Kluwer, p. 1-25.

Woese CR. 1987. Bacterial evolution. Microbiol. Rev., 51(2): 221-271.

Zehr J, Mellon T, Hiorns W. 1997. Phylogeny of cyanobacterial *nifH* genes: evolutionary implications and potential applications to natural assemblages. Microbiology, 143(4): 1443-1450.