Rostaniha 17(2): 161–172 (2016)
رستنیها ۱۷(۲): ۱۷۲-۱۶۱ (۱۳۹۵)

A proposal for the unification of two cyanobacterial strains of *Nostoc* as the same species

Received: 20.08.2016 / Accepted: 21.12.2016

Bahareh Nowruzi☒: Assistant Prof., Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran (bahare77biol@yahoo.com, bahareh.nowruzi@srbiau.ac.ir)

Ramezan-Ali Khavari-Nejad: Prof., Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran., Faculty of Biological Sciences, Kharazmi University, Tehran, Iran

Taher Nejadsattari: Associate Prof., Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran

Karina Sivonen: Prof., Division of Microbiology and Biotechnology, Department of Food and Environmental Sciences, University of Helsinki, Finland

David P. Fewer: Prof., Division of Microbiology and Biotechnology, Department of Food and Environmental Sciences, University of Helsinki, Finland

Abstract

The present research aimed to investigate through a polyphasic approach, the differences in morphological and genotypic features of two cyanobacterial strains isolated from paddy fields of northern parts of Iran, belonging to the family *Nostocaceae* (subsect. IV. I). Based on the reliable identification keys, the two strains were identified as *Nostoc ellipsosporum* (Desm.) Rabenh. ex Born. *et* Flah, and *N. muscorum* Ag. ex Born. *et* Flah. Sequencing of cloned bacterial 16S rRNA fragment strongly indicated that, two *Nostoc* strains could not be discriminated by 16S rRNA and ITS genes sequencing and the current morphological classification of both is invalid. Moreover, phylogenetic study of two *Nostoc* strains has demonstrated that, genetic relationships are in conflict with the morphological classification. Besides, after doing DNA-DNA reassociation experiments, it was concluded that, two *Nostoc* strains investigated might possibly be united into one species. Finally, two *Nostoc* species were named as *Nostoc* sp. Bahar_E and *Nostoc* sp. Bahar_M, and have been registered under DDBJ, accession numbers: JF795278 and JF272482, respectively.

Keywords: DNA-DNA hybridization, ITS, morphology, *Nostoc*, 16S rRNA

ROSTANIHA

پیشنهادی برای یکپارچه‌سازی دو سویه سیانوباکتری جنس نوستوک در قالب یک گونه

دریافت: ۱۳۹۵/۰۵/۳۰ / پذیرش: ۱۳۹۵/۱۰/۰۱

بهاره نوروزی☒: استادیار زیست‌شناسی مولکولی سیانوباکتری‌ها، گروه زیست شناسی، دانشگاه آزاد اسلامی، واحد علوم و تحقیقات، تهران، ایران (bahare77biol@yahoo.com, bahareh.nowruzi@srbiau.ac.ir)

رمضانعلی خاوری‌نژاد: استاد زیست شناسی، فیزیولوژی گیاهی، دانشگاه آزاد اسلامی، واحد علوم و تحقیقات، تهران، ایران و دانشکده علوم زیست شناسی، دانشگاه خوارزمی، تهران، ایران

طاهر نژادستاری: دانشیار زیست شناسی، گروه زیست شناسی، دانشگاه آزاد اسلامی، واحد علوم و تحقیقات، تهران، ایران

کارینا سیوونن: استاد گروه میکروبیولوژی و بیوتکنولوژی، دانشکده علوم محیطی و غذایی، دانشگاه هلسینکی، فنلاند

دیوید فیور: استاد گروه میکروبیولوژی و بیوتکنولوژی، دانشکده علوم محیطی و غذایی، دانشگاه هلسینکی، فنلاند

خلاصه

هدف مطالعه حاضر این است که با استفاده از یک سری مطالعات چند منظوره، تفاوت‌های موجود در ویژگی‌های مورفولوژیک و ژنوتیپیک دو سویه سیانوباکتریای جدا شده از شالیزارهای برخی مناطق در شمال ایران که متعلق به تیره *Nostocaceae* (subsect. IV. I) هستند، بررسی شود. براساس کلیدهای شناسایی معتبر، دو سویه سیانوباکتری به اسامی *Nostoc ellipsosporum* (Desm.) Rabenh. ex Born. *et* Flah و *N. muscorum* Ag. ex Born. *et* Flah شناسایی شدند. توالی‌یابی قطعه 16S rRNA کلون شده نشان داد که ژن‌های این قطعه و ITS با نتایج حاصل از تاکسونومی سنتی در دو گونه مذکور تطابق نداشته و رده‌بندی مورفولوژیکی در آن‌ها نامعتبر است. علاوه بر این، مطالعات فیلوژنتیکی دو سویه آشکار کرد که روابط ژنتیکی با رده‌بندی مورفولوژیکی همخوانی ندارد. همچنین، در نتیجه انجام آزمایش‌های هیبریداسیون DNA-DNA، مشخص گردید که دو سویه نوستوک مورد بررسی، احتمالا متعلق به یک گونه هستند. در نهایت دو گونه نوستوک، با نام‌های *Nostoc* sp. Bahar_E و *Nostoc* sp. Bahar_M و کدهای دسترسی JF795278 و JF272482 در بانک ژن ثبت شدند.

واژه‌های کلیدی: مورفولوژی هیبریداسیون DNA-DNA، ITS، 16S rRNA, *Nostoc*

Introduction

Cyanobacteria of the genus *Nostoc* is cosmopolitan in a wide range of terrestrial and aquatic ecosystems (Dembitsky & ezanka 2005). Although, there are vast paddy fields in Iran, very few studies have been carried out with an aim to understand the ecology and taxonomy of heterocystous cyanobacteria of these ecosystems.

The nomenclatural rules are the best formal basis for a taxonomic treatment. Unfortunately, this situation is very unsatisfactory for cyanobacteria (Stanier *et al.* 1978) but ongoing discussion continues within the committee established to unify the necessary principles for cyanobacteria.

16S rDNA sequence data placed the cyanobacteria within the bacteria (Woese 1987) which were supported by many other authors (e.g. Gupta 1998, Ludwig & Klenk 2001). The 16S rRNA gene, the most commonly used marker gene, has a central role in inferring phylogenetic relationships and in identification of cyanobacteria at all taxonomic levels, except between closely related organisms (species and subspecies levels), for which it provided better resolution. A species has to be genetically divergent from its closest relatives. According to the bacteriological approach, at most 70% DNA-DNA similarity or 97% 16S rRNA sequence identity to the closest species is acceptable (Wayne *et al.* 1987).

The Internal Transcribed Spacer between the 16S and 23S rRNA genes (ITS) sequence is more variable than the 16S rRNA gene sequence (Vetrovsky & Baldrian 2013), moreover, it is variable both in length and in the nucleotide sequence (Gurtler & Stanisich 1996). As a result, nucleotide sequences from the ITS were examined.

It is improbable that an organism with completely identical sequences in the variable regions of the genome, such as the ITS, would have great differences in their 16S rRNA gene sequences. Although there are concerns as to the taxonomic relevance of 16S rRNA sequence similarities above 97%, it has also been stated that organisms with 100% identical 16S rRNA sequences are almost certainly conspecific (Fox *et al.* 1992). However, the 16S rRNA gene sequence similarities of bacteria were shown to correlate well with genome relatedness, expressed as DNA: DNA re-association values (Stackebrandt & Goebel 1994). These correlations support the robustness of the 16S rRNA gene-based microbial phylogeny (Konstantinidis & Tiedje 2005).

The present work focused on the combined genetic and phenotypic relationships of two *Nostoc* species. Detailed morphological analyses of these strains were carried out at the time of isolation in order to avoid difficulties in identification. Phylogenetic analysis of 16S rRNA and ITS, were done to discriminate two species and finally DNA-DNA hybridization was used to measure the degree of relatedness between organisms with high 16S rRNA sequence similarity.

Materials and Methods

- Culture conditions, morphological characterization

In 2010, according to the pedological map of the Golestan province (N Iran), five soil types/samples of different textures from paddy fields of Ozineh, Sorkhankolah, Hashemabad, Glin and Yampi were collected (Table 1). Five grams of soil from each sample were weighed and aseptically transferred to sterile Petri dishes with adequate quantities of liquid media $BG11_0$ medium without $NaNO_3$ (Rippka *et al.* 1979). The pH was adjusted to 7.1 after sterilization. The Petri dishes were incubated in a culture chamber at 28° C and were provided with continuous artificial illumination of approximately 50 µmol photon m^{-2} s^{-1} for two weeks (Kaushik 1987). Hormogonia of growing colonies were then placed equidistantly onto an agar surface for purification.

Morphological studies were made by bright-field microscopy and by phase-contrast illumination of 30-day-old cultures using a Leica DM750 P microscope (Leica, Wetzlar, Germany). Cultures were routinely checked for purity by both microscopic examination and by plating on nutrient agar and examining for bacterial colonies.

ROSTANIHA

The following parameters were selected to describe the morphology of heterocytous cyanobacteria: morphological characteristics of vegetative cells (including terminal cell), heterocytes and the shape of the filament and its aggregation in colonies (Rajaniemi *et al.* 2005). The species were identified according to Desikachary (1959).

Two strains of heterocytous cyanobacteria, which were the most frequent strains of paddy fields, were selected to investigate the differences in morphological and genotypic features, through a polyphasic approach.

Table 1. Representing indigenous heterocystous cyanobacteria species identified in five paddy fields of Golestan province (N Iran)

Sorkhankolah	Ozineh	Glin	Hashemabad	Yampi
Anabaena circinalis	-	-	-	-
A. variablilis	Stigonema minutum	Fischerella ambiqua	-	-
Aphanizomenon flosaquae	Anabaena torulosa	Stigonema minutum	-	-
Cylindrospermum majus	-	-	-	-
Fischerella ambiqua	Trichormus khannae	Nostoc punctifoeme	Nodularia harveyana	Calothrix stagnalis
Nodularia harveyana	Nostoc muscorum	N. ellipsosporum	Nostoc ellipsosporum	-
Nostoc ellipsosporum	N. commune	N. spongiforme	-	-
N. muscorum	N. microscopicum	N. muscorum	N. spongiforme	-
N. punctiforme	-	-	-	-
N. spongiforme	N. ellipsosporum	Anabaena oscillarioides	N. muscorum	-
Scytonema ocellatum	Fischerella ambiqua	Scytonema ocellatum	-	-
Stigonema minutum	Nodularia harveyana	Nodularia harveyana	-	-
Trichormus naviculoides	-	Nostoc commune	-	-

ROSTANIHA

- Genomic DNA extraction

Genomic DNA was extracted utilising the E.Z.N.A. SP Plant DNA kit (Omega Bio-tek, Inc., Norcross, GA, USA). The extraction procedure was continued according to the kit's protocol, as supplied by the manufacturer. DNA was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA).

- PCR amplification for comparison of 16S rRNA gene and 16S-23S internal transcribed spacer (ITS) (Nelissen *et al*. 1996, Wilmotte 1994)

Polymerase chain reaction (PCR) amplification of sixteen oligonucleotide primers comprised of one forward primer and a reverse primer (Lepere *et al*. 2000, Edwards *et al.* 1988, Nübel *et al*. 1997) were used for complete amplification of 16S rRNA gene. Internal spacer (ITS) was amplified with primers ITS16CF and ITS23CR. One PCR reaction was comprised of 1 time buffer solution, 0.5 µm forward primer, 0.5 µm reverse primer and 0.5 unit *Taq* polymerase as well as 1 µL template DNA and sterile water in a total volume of 20 µL. The template DNA concentration of two *Nostoc* strains in the reaction accounted for approximately 140 ng. As a positive control for the amplification procedure, genomic DNA of the cyanobacterium *Nostoc* sp. 202 (supplied by the University of Helsinki Cyanobacterial Culture Collection, UHCC) was used, whereas sterile water was used as a negative control. The amplification reactions were conducted in a thermocycler (iCycler; Bio-Rad, Foster city, CA, USA) with the following program: initial denaturation at 94° C for 5 min, 30 cycles comprised of 94° C for 1 min, 51° C for 1 min, 72° C for 1.5 min and 72° C for 7 min. PCR products were checked by electrophoresis on 1% agarose gels (SeaPlaque GTG; Cambrex Corp., East Rutherford, NJ, USA) at 100 V, followed by 0.10 µg mL-1 ethidium bromide (EtBr; Bio-Rad) staining. PCR products were visualized in the gel by UV light utilizing the Molecular Imager Gel Doc XR system (Bio-Rad). The products were purified using the Geneclean Turbo kit

(Qbiogene/MP Biomedicals, Solon, OH, USA) and were quantified with a Nanadrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

- Cloning

It is impossible to directly sequence the PCR products especially at the end of ITS sequence. Cloning was performed by using the TOPO Ta cloning system with the vector 2.1-TOPO (Invitrogen, Carlsbad, USA) according to the manufacture instructions. Ligation reactions were incubated for 30 min at room temperature and transformations were carried out using chemically competent TOPO10 *Eschericha coli* cells. The vector PCR 2.1 TOPO contains Ampicillin and Kanamycin resistance genes and the Lac Z gene, which assisted in the detection and selection of clones with the desired insert. The *lacZ* gene codes for -galactosidas enzyme which hydrolysis e.g. the compound 5-bromo-4-chloro-3-indolyl- -D-galactopyranoside (X-gal) resulting in the release of blue color. To find the correct clones, 40 µml of 50 mg ml^{-1} X-gal solution (promega, Madison, U.S. A.) was spread on Luria-Bertani plates (Sambrook & Russell 2001) containing 50 µmg ml^{-1} of Ampicilin (Sigma-Aldrich, Saint Louis, USA) before plating the transformant cells. Insertion of foreign DNA into the *LacZ* gene during ligation disrupts and inactivates the gene and white colonies are obtained.

- Sequencing and analysis

Sequencing of the complete 16S rRNA gene of two stains was subsequently carried out by the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Life Technologies, Foster city, CA, USA). Reactions for forward and reverse primers were prepared separately. Positive clones (white colonies) of cloning were analysed by sequencing and amplifying the insert using the M13F and M13R primers and sequencing. In this case, positive transformants were amplified by PCR using Primers M13F and M13R and analyzed. Prior to sequencing, cloned PCR products were purified with Amicon Microcon-PCR centrifugal Filter Devices (Millipore, Bedford, USA). BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST) of the complete 16S rRNA gene of two strains were used to identify similar sequences deposited in the GenBank database of NCBI. The 16S rRNA gene sequences obtained in this study as well as reference sequences retrieved from GeneBank were first aligned with CLUSTAL W with the default settings and were then manually edited in BioEdit version 7.0. The positions with gaps as well as undetermined and ambiguous sequences were removed for subsequent phylogenetic analyses. Phylogenetic trees using the maximum likelihood method was constructed by the MEGA version 4.1 (Tamura *et al.* 2007) using the Kimura two parameter method (Kimura 1980). The robustness of the tree was estimated by bootstrap percentages using 1,000 replications. The root of the tree was determined using the 16S rRNA of *Synechococcus* sp. PCC7335 (Fig. 3).

- DNA hybridization experiments

DNA-DNA hybridizations were performed with DNA from both *Nostoc* strains as probes according to Otsuka *et al*. (2001). Percentages of DNA re-association were determined fuorometrically from the extent of hybridization. Each experiment was repeated at least two times.

ROSTANIHA

Results

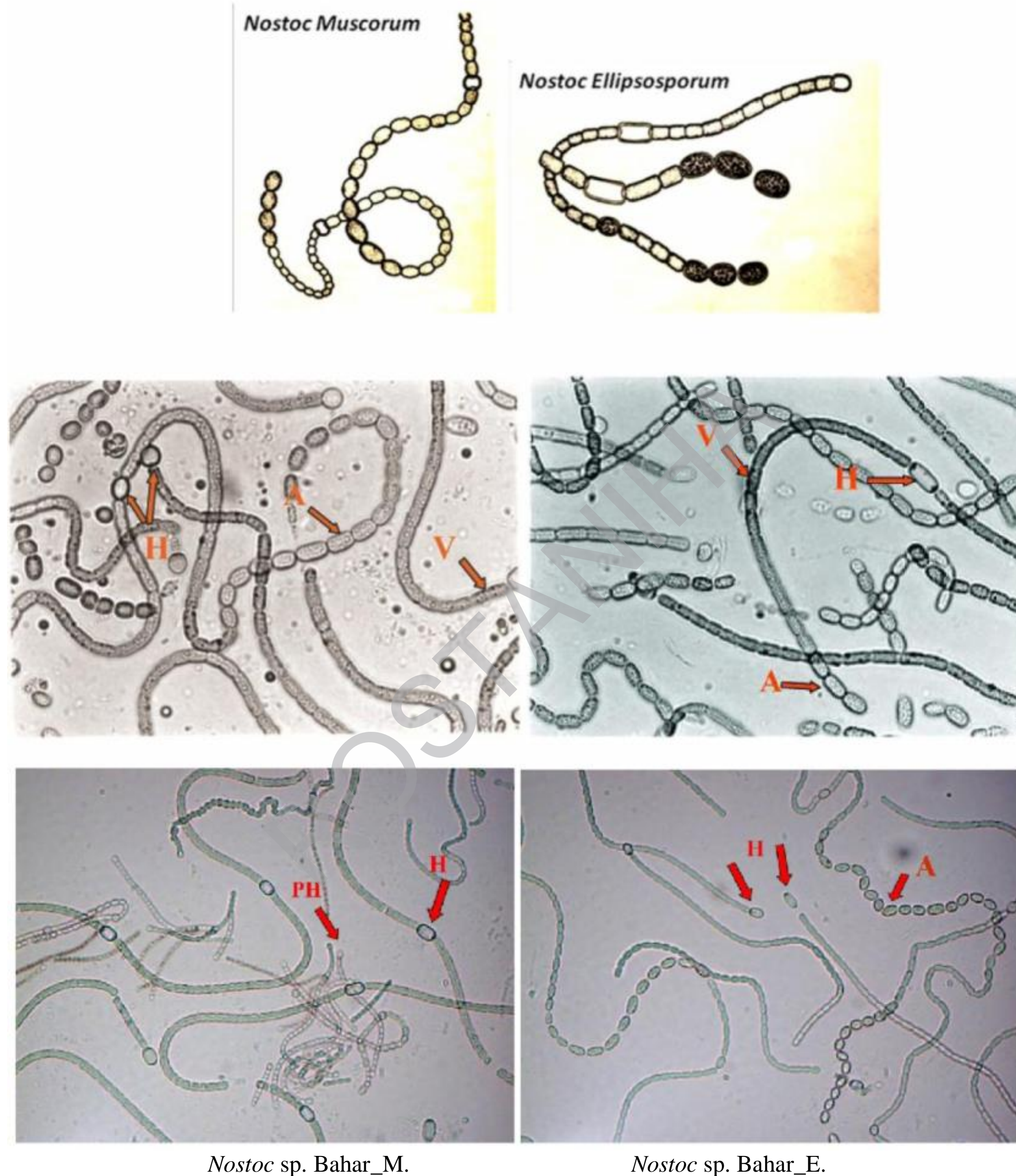
- Morphological characteristics of two *Nostoc* strains

Based on the description of the morphology provided by Desikachary (1959) two strains were identified as *Nostoc ellipsosporum* and *N. muscorum.* Moreover, the identification was checked according to Komarek (2010) and the name of *N. muscorum* has been changed to *Desmonostoc muscorum.*

The strain had been identified as *Nostoc ellipsosporum* had these characters: Mass gelatinous, brownish or dark-colored. Cells cylindrical, 3.5–4 µm broad, 7–11 µm in length, brownish or olive. Heterocysts somewhat spherical or oblong, 5–6.5 µm wide, 6–12.5 µm long. Spores ellipsoidal to oblong, 5–5.5 µm broad, 10–12 µm long and the strain had been identified as *N. muscorum*, had these characters: Thallus gelatinous, dull olive. Cells spherical or slightly longer than broad, 4–5 µm broad, 5.5–7 µm long., olive. Heterocysts

somewhat globose 4.5–7 µm broad, 4–8.5 µm long. Spores oblong, many in chain, 5–6 µm broad and 6.5–11 µm long.

Two *Nostoc* strains can easily colonize and develop a wide range of macroscopic colonies that have different colors, shapes, sizes and textures on agar plates. The size of the spherical colonies (or pearls) in both species ranged from 0.5 to 5 mm, with a majority of 0.5–3 mm in *N. muscorum*. Along with the progression of growth on the plates, there was a color change from light brown to dark brown in *N. ellipsosporum* and light green to dark green in *N. muscorum* (Figs 1–2).



Nostoc sp. Bahar_M. *Nostoc* sp. Bahar_E.

Fig. 1. Morphological comparison of two *Nostoc* strains identified based on the description of the morphology provided by Desikachary (1959) (top figures) and Photomicrograph of two *Nostoc* strains in paddy fields of Iran illustrating the proceeding of some representative developmental stages. Cells show differentiation in young hormogonia vegetative cell (V), Akinetes (A), developmental alternatives with intercalary heterocysts (H) (400X) (below figures). As illustrated, length and width of vegetative cells, heterocytes and akinetes, shape of filaments and their aggregation in colonies, congruent with traditional keys.

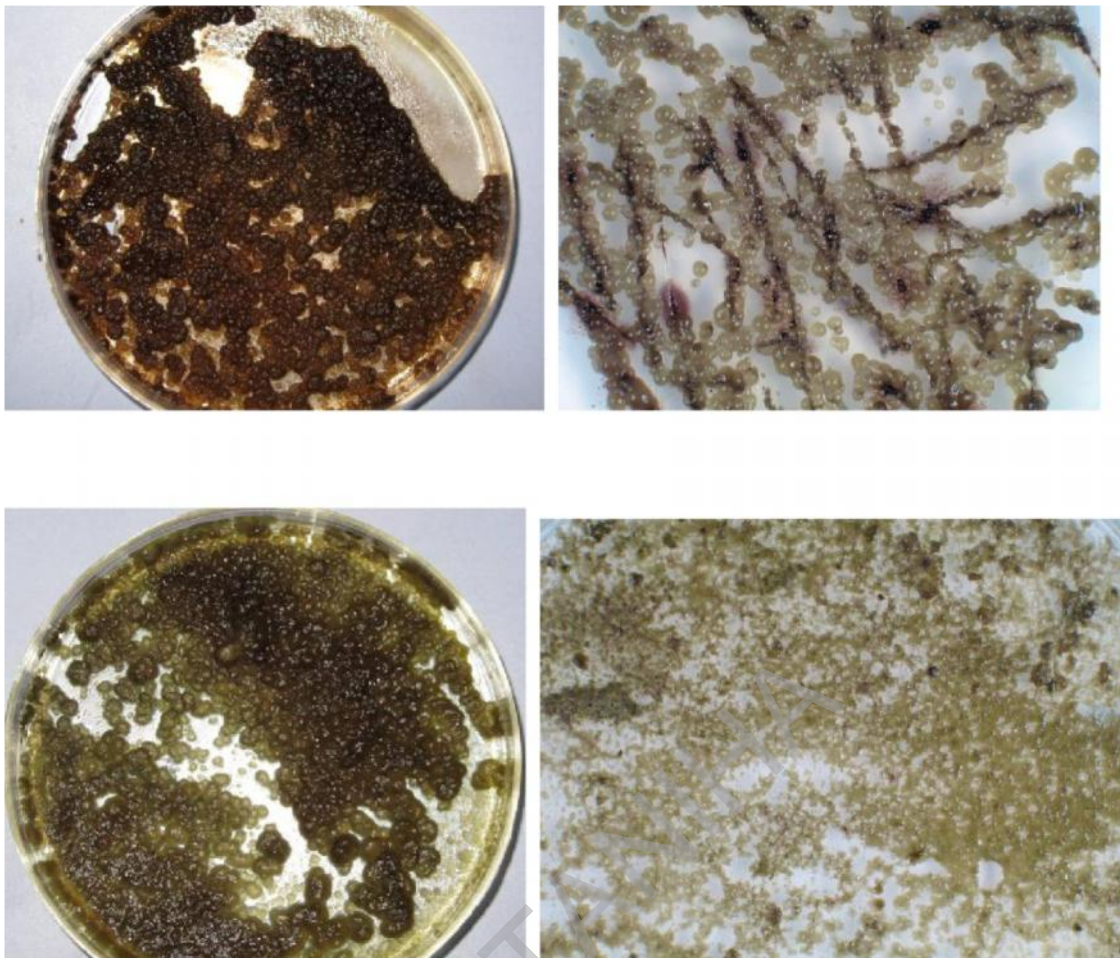


Fig. 2. Microphotographs of *Nostoc* sp. Bahar_E (top figures) and *Nostoc* sp. Bahar_M (below figures) spherical colonies on agar solidified BG11 plates. Colonies and size of cells of two *Nostoc* species significantly are different in color during their life and it became easy to re-identify them.

ROSTANIHA

- Phylogenetic analysis of the 16S rRNA and ITS genes

An 1815 bp portion of the 16S rRNA gene was successfully amplified from the PCR amplicon obtained from two *Nostoc* strains. The results of cloning showed that, two strains being 99 percent similar in their sequences and there were only five different nucleotides. In addition, results of the degree of relatedness between organisms showed that, all DNA-DNA reassociation values between two strains of *Nostoc* exceeded 85%.

The 16S rRNA gene sequences obtained in this study and reference sequences retrieved from GeneBank were first aligned with CLUSTAL W with the default settings and then manually edited in BioEdit version 7.0.0. A BLAST search was used to detect similar sequences deposited in the GenBank™ database of NCBI (http://www.ncbi.nlm.nih.gov/). The positions with gaps and undetermined and ambiguous sequences were removed for subsequent phylogenetic analyses. The phylogenetic tree which was constructed using the maximum likelihood method is shown in Fig. 3.

The complete 16S rRNA gene sequence of two sequences has been registered in the Data Bank of Japan (DDBJ) under the accession numbers: KT763390 and KT763391. Two *Nostoc* species named as *Nostoc* sp. Bahar_E and *Nostoc* sp. Bahar_M.

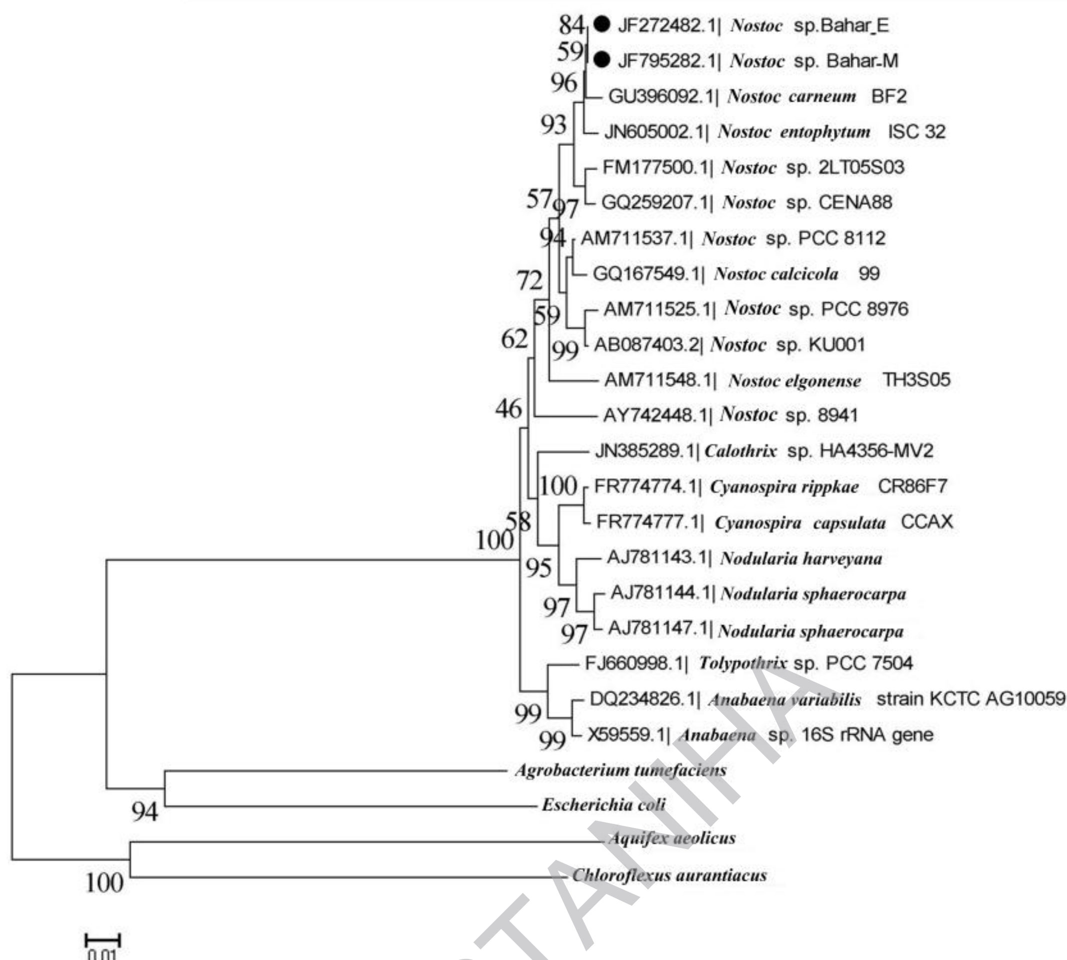


Fig. 3. Consensus bootstrap tree on the basis of maximum likelihood distances of 1815 bp long full-length 16S rRNA genes sequences and sequences that were taken from the GenBank. The studied strains are shown in full circle. Numbers near nodes indicate bootstrap values over 50% for ML analyses (Bar = 0.01 change per sequence position).

ROSTANIHA

Discussion

In this study, result of 16S sequence comparisons, showed 100 percent similarity, when they are aligned. However, the internal transcribed spacer (ITS) sequence separating the 16S and 23S rRNA genes is highly polymorphic and also more variable than the 16S rRNA gene sequence, so it provides a template for discriminating closely related bacterial and cyanobacterial strains (Barry *et al*. 1991, Jensen *et al*. 1993, Neilan *et al*. 1997b). For example *Pseudanabaena* sp. PCC7409 and *Limnothrix redekei* strain Mef 6705 had the ITS sequence similarity of 88% while the similarity of the 16S rRNA gene sequences was 99.7% (Wilmotte 1994).

Moreover, the ITS region of bacteria is variable both in length and in the nucleotide sequence (Boyer *et al*. 2001). For instance, in cyanobacteria, the lengths of the ITS reported, vary from 262 bp of *Anabaena crassa* strain PH215 to 771 bp of *Synechococcus* sp. (Suzuki *et al*. 2001). Since we did not have any estimation about the lengths of the ITS of two *Nostoc* strains and also the result of sequencing showed that they were homologues together again, so cloning was used for sequencing the full-length 16S rRNA genes. The lengths of the 16S rDNAs of the present two strains were identical, 1815 bp and two strains had high sequence similarity, exceeding 99 ± 5%. Thus, the two strains were found to be closely related to each another and can be the same genospecies because of the DNA relatedness of 85%. The motile hormogonia were used for isolation, so we were sure that two *Nostoc* species were pure. However, in the *Nostoc* cluster of the maximum likelihood tree based on 16S

rDNA sequences (Fig. 3), there is no clear division, which agrees with the current species classification.

However, two *Nostoc* species revealed remarkable morphological differences and it was not congruent with traditional references. Therefore, the weak point of the traditional morphological classification system, which is on the morphological transformation of cells conflicted with phylogenetic studies of two *Nostoc* species. Nevertheless, Palinska *et al*. (1996) showed that the great morphological diversity observed in nature and (partially) in culture does not necessarily reflect genetic diversity and mentioned the likelihood that more ecophenic and or phenotypic forms have been described rather than genotypic species. As a result, these problems of traditional morphological classification, together with the lack of molecular data, pose serious hindrances for taxonomy and systematic of cyanobacteria (Komárek 2010). Therefore, besides morphological examination, it is currently accepted that characterization and taxonomy of cyanobacteria must combine multidisciplinary or polyphasic approaches (Komárek 2010).

Morphological characteristics can give important information about the organism of interest; however, it is necessary to bear in mind that there are organisms, which cannot be classified solely by morphological characteristics. The current taxonomy of cyanobacteria depends too much upon morphological characteristics and must be reviewed by means of bacteriological methods as well as traditional botanical methods.

Molecular investigations, especially those based on 16S rDNA and DNA-DNA hybridization would bring unification or division to some species, genera and even taxa of higher rank. There remain many cyanobacteria to be reconsidered regarding their taxonomy. The genus *Nostoc* is an example; we studied two strains that can be distinguished phenotypically but, do not exhibit enough genotypic difference that they can be correctly assigned to two species based on current criteria. Moreover, genetic distances between the two *Nostoc* species in the maximum likelihood tree (Fig. 3) seem too short for them to be classified in a single species. What then is to be concluded about these taxa? This is an important issue because 16S rRNA sequence characterization is being widely used in evolutionary, taxonomic and ecological studies, functional consistency, both variable and conserved regions and large size and thus, rather high information content-characteristics are needed for a good phylogenetic marker gene (Woese 1987, Ludwig & Klenk 2001) not only to define taxa, but also to characterize which taxa are present. In addition, the 16S rRNA gene sequences are relatively easy to align and a large database has accumulated (currently over 6000 cyanobacterial sequences), allowing comparisons between strains (Ludwig & Klenk 2001).

In the present task, two organisms with effectively identical 16S rRNA sequences (i.e. less than five differences) might be assumed to belong to the same species. In terms of extremely closely related strains, the key issue is the relationship between 16S rRNA sequence similarity and DNA-DNA hybridization results. It is apparent from this data set that when the level of 16S rRNA identity is 99% or more, DNA-DNA hybridization may document the existence of species identity. Clearly, at these levels of sequence similarity the accuracy of the 16S rRNA determination is subject to inherent errors and small differences cannot necessarily be regarded as significant. What is observed then is that effective identity of 16S rRNA sequences does not guarantee species identity as determined by the DNA-DNA hybridization criterion. In contrast, we have not found any instances in which strains that are well resolved by 16S rRNA methods are indistinguishable on the basis of DNA-DNA hybridization results. In a trivial sense, these results simply verify what is well known, that 16S rRNA sequences are more appropriate for determining inter- and intrageneric relationships. Nevertheless, it would be extremely helpful if these two criteria were interfaced in a consistent way. The alternative of claiming that 16S rRNA data have no relevance to species definition is also not appropriate. It is very apparent that if two strains have effectively identical 16S rRNA sequences and then they are much more closely related than typical distinct species. In fact, if the 16S rRNA sequences are 100% identical, the strains almost certainly will be shown to be

ROSTANIHA

the same species by the DNA-DNA hybridization criterion as well. In the absence of definitive DNA-DNA data, strains with effectively identical 16S rRNA sequences are best regarded as belonging to the same “rRNA species complex” or “rRNA superspecies”, but could never convincingly establish that the remaining genetic diversity was consistent with the existence of only one species.

This interpretation has already been recognized and implemented by Collins and coworkers 1989). In recent studies of *Lactobacillus lactis*, *Streptococcus parasanguis* and *Mycobacterium intracellulare* strains, less than 10 sequence differences were encountered in each case. Since the strains had been assigned to their respective species on the basis of other criteria, the authors attributed the differences to possible interspecies diversity rather than create a new species. In contrast, studies of *Bacillus anthracis*, *B. cereus*, *B. Rnycoides*, and *B. thuringiensis* also revealed less than 10 differences among the various strains examined, but in this case, the authors did not propose reassignment of the strains to a single species.

As with the 16S rDNA similarities, the genomic DNA homologies correspond with the current species classification. It is considered that the relative binding percentage should be higher than 70% within a bacterial species (Wayne *et al*. 1987).

Currently, prokaryotic species definition relies on DNA-DNA relatedness, which is measured as the relative binding ratio (RBR) and/or the difference in the thermal denaturation midpoint (T_m) between DNAs from two organisms (heteroduplex DNA’s) (Rosselló-Mora & Amann 2001). In RBR and T_m determination, denatured DNAs of two organisms are mixed, allowed to re-associate and form hybrid molecules in controlled experimental conditions (Vandamme *et al*. 1996). The more similar are the DNAs of two organisms, the more hybridization occurs. The RBR and T_m are determined by comparing the results from the heteroduplex DNA to the results obtained from homoduplex DNAs (Rosselló-Mora & Amann 2001).

According to this criterion, the genomes of two *Nostoc* strains have to share above 70% RBR or less than 5° C T_m to be considered members of the same species (Wayne *et al.* 1987). However, this view was recently revised by Stackebrandt & Ebers (2006) who recommended a 16S rRNA similarity value of (above) “98.7–99% as the point at which DNA-DNA association experiments should be mandatory…” (Stackebrandt & Ebers 2006).

Within the cyanobacteria, the case of the morphologically different but phylogenetically closely related and inseparable two *Nostoc* strains are an excellent illustration of the difficulties that can be encountered in species demarcation and classification.

ROSTANIHA

Acknowledgement

This study was designed and performed in University of Helsinki, Department of Biology, Science and Research Branch. The authors would like to thank to Dr. Prashant Singh of the Department of Biotechnology, Curator of the Cyanobacterial Culture Collection at MCC, Pune, India for his helpful discussion and also wish to thank, Lyudmila Saari for helping to isolate the axenic *Nostoc* sp. Bahar_E and *Nostoc* sp. Bahar_M strains.

References

Barry, T., Colleran, G., Glennon, M., Dunican, L. & Gannon, K. 1991. The 16S-23S ribosomal spacer region as a target for DNA probes to identify eubacteria. PCR Methods and Applications 1: 51–56.

Boyer, S.L., Flechtner, V.R. & Johansen, J.R. 2001. Is the 16S-23S rRNA internal transcribed spacer Region a good tool for use in molecular systematics & population genetics? A case study in cyanobacteria. Molecular Biology and Evolution 6: 1057–1069.

Castenholz, R.W. & Norris, T.B. 2005. Revisionary concepts of species in the cyanobacteria and

their applications. Algological Studies 117: 53–69.

Cohan, F.M. 2002. What are bacterial species? Annual Review of Microbiology 56: 457–487.

Collins, M.D., Ash, C., Farrow, J.A.E., Wallblanks, S. & Williams, A.M. 1989. 16S rRNA sequence analysis of lactococci and related taxa. Description of *Vagococcus fluvialis* gen. nov., sp. nov. Journal of Applied Microbiology 67: 453–460.

Dembitsky, V.M. & ezanka, T. 2005. Metabolites produced by nitrogen-fixing *Nostoc* species. Folia Microbiologica 5: 363–391.

Desikachary, T.V. 1959. Cyanophyta. Indian Council of Agricultural Research, New Delhi, 1–686.

Edwards, U., Rogall, T., Blocker, H., Emde, M. & Bottger, E.C. 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Research 17: 7843–7853.

Fox, G.E., Wisotzkey, J.D. & Jurtshuk, P. 1992. How Close Is Close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. International Journal of Systematic Bacteriology 42: 166–170.

Gupta, R.S. 1998. Protein phylogenies and signature sequences: a reappraisal of evolutionary relationships among archaebacteria, eubacteria and eukaryotes. Microbiology and Molecular Biology Reviews 62: 1435–1491.

Gurtler, V. & Stanisich, V.A. 1996. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. Microbiology 142: 3–16.

Jensen, M.A., Webster, J.A. & Strauss, N. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Applied and Environmental Microbiology 59: 945–952.

Kaushik, B.D. 1987. Laboratory methods for blue-green algae. Associated Publishing Co., New Delhi: 17–63.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16: 111–120.

Komárek, J. 2003. Problem of the taxonomic category “species” in cyanobacteria. Algological Studies 109: 281–297.

Komarek, J. 2010. Modern taxonomic revision of planktic nostocacean cyanobacteria: a short review of genera. Hydrobiologia 639: 231–243.

Konstantinidis, K.T. & Tiedje, J.M. 2005. Towards a genome-based taxonomy for prokaryotes. Journal of Bacteriology 187: 6258–6264.

Lepere, C., Wilmotte, A. & Meyer, B. 2000. Molecular diversity of *Microcystis* strains (*Cyanophyceae*, *Chroococcales*) based on 16S rDNA sequences. Systematics and Geography of Plants 70: 275–283.

Ludwig, W. & Klenk, H.P. 2001. Overview: a phylogenetic backbone and taxonomic framework for procaryotic systematics. *In*: Bergey’s Manual of Systematic Bacteriology, pp. 49–65.

Mayr, E. 1942. Systematics and the Origin of Species from the Viewpoint of a Zoologist. New York, Columbia University Press: 1–372.

Neilan, B.A., Stuart, J.L., Goodman, A.E., Cox, P.T. & Hawkins, P.R. 1997. Specific amplification and restriction polymorphisms of the cyanobacterial rRNA operon spacer region. Systematic and Applied Microbiology 20: 612–621.

Neilan, B.A., Jacob, S.D., Dot, T.D., Blackall, L., Hawkins, P.R., Cox, P.T. & Goodman, A.E. 1997. rRNA sequences and evolutionary relationships among toxic and nontoxic

ROSTANIHA

cyanobacteria of the genus *Microcystis*. International Journal of Systematic Bacteriology 47: 693–697.

Nelissen, B., De Baere, R., Wilmotte, A. & De Wachter, R. 1996. Phylogenetic relationships of nonaxenic filamentous cyanobacterial strains based on 16S rRNA sequence analysis. Journal of Molecular Evolution 42: 194–200.

Nubel, U., Garcia-Pichel, F. & Muyzer, G. 1997. PCR primers to amplify 16S rRNA genes from cyanobacteria. Applied and Environmental Microbiology 63: 3327–3332.

Oren, A. 2004. A proposal for further integration of the cyanobacteria under the bacteriological code. International Journal of Systematic and Evolutionary Microbiology 54: 1895–1902.

Ossello-Mora, R. & Amann, R. 2001. The species concept for prokaryotes. FEMS Microbiology Reviews 25: 39–67.

Palinska, K.A., Liesack, W., Rhiel, E. & Krumbein, W.E. 1996. Phenotype variability of identical genotypes: the need for a combined approach in cyanobacterial taxonomy demonstrated on Merismopedia-like isolates. Archives of Microbiology 166: 224–233.

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). International Journal of Systematic and Evolutionary Microbiology 55: 11–26.

Rippka, R., Deruelles, J.J.B., Waterbury, M. & Stanier, R.Y. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. Journal of General Microbiology 111: 1–61.

Sambrook, J. 2001. Molecular Cloning: A Laboratory Manual (3rd ed.). Cold Spring Harbor Laboratory Press. ISBN 978-0-87969-577-4.

Stackebrandt, E. & Ebers, J. 2006. Taxonomic parameters revisited: tarnished gold standards. Microbiology Today 33: 152–155.

Stackebrandt, E. & Goebel, B.M. 1994. Taxonomic note: a place for DNA-DNA re-association and 16S rRNA sequence analysis in the present species definition in bacteriology. International Journal of Systematic Bacteriology 44: 846–849.

Stanier, R.Y., Sistrom, W.R., Hansen, T.A., Whitton, B.A., Castenholz, R.W., Pfennig, N., Gorlenko, V.N., Kondratieva, E.N., Eimhjellen, K.E., Whittenbury, R., Gherna, R.L. & Trüper, H.G. 1978. Proposal to place the nomenclature of the cyanobacteria (blue-green algae) under the rules of the International Code of Nomenclature of Bacteria. International Journal of Systematic Bacteriology 28: 335–336.

Suzukim, M.T., Beja, O., Taylor L.T. & Delong, E.F. 2001. Phylogenetic analysis of ribosomal RNA operons from uncultivated coastal marine bacterioplankton. Environmental Microbiology 3: 323–331.

Tamura K., Dudley, J., Nei, M. & Kumar, S. 2007. MEGA 4: molecular evolutionary genetics analysis (MEGA) software, version 4.0. Molecular Biology and Evolution 8: 1596–1599.

Vandamme, P., Pot, B., Gillis, M., Vos, P.de., Kersters, K. & Swings, J. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiological Reviews 60: 407–438.

Vetrovsky, T. & Baldrian, P. 2013. The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. PLos One 8: 57–923.

Ward, D.M. 1998. A natural species concept for prokaryotes. Current Opinion in Microbiology 1: 271–277.

ROSTANIHA

Wayne, L.G., Brenner, D.J., Colwell, P.A.D., Grimont, O., Kandler, A., Krichevsky, M.I., Moore, L.H., Moore, W.E.C. & Murray, R.G.E. 1987. International Committee on Systematic Bacteriology. Report of the ad-hoc committee on reconciliation of approaches to bacterial systematic. International Journal of Systematic Bacteriology 37: 463–464.

Wilmotte, A. 1994. Molecular evolution and taxonomy of the cyanobacteria. The Molecular Biology of Cyanobacteria. *In*: D. Bryant (ed.). Dordrecht. The Netherlands, Kluwer Academic Publishers: 1–25.

Woese, C.R. 1987. Bacterial evolution. Microbiological Reviews 51: 221–227.

ROSTANIHA