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Full Length Research Paper

Phylogenetic and morphological evaluation of two species of *Nostoc* (Nostocales, Cyanobacteria) in certain physiological conditions

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Studies of cyanobacterial species are important to the global scientific community, mainly, the order, Nostocales fixes atmospheric nitrogen, thus, contributing to the fertility of agricultural soils worldwide, while others behave as nuisance microorganisms in aquatic ecosystems due to their involvement in toxic bloom events. However, in spite of their ecological importance and environmental concerns, their identification and taxonomy are still problematic and doubtful, often being based on current morphological and physiological studies, which generate confusing classification systems and usually vary under different conditions. In fact, the taxonomy of cyanobacteria has been substantially modified in the last few decades, particularly, after the application of modern ultrastructural and molecular methods. Therefore, the present research aimed to investigate through a polyphasic approach, the differences in morphological and genotypic features of two cyanobacteria strains isolated from paddy fields of Iran, belonging to the family Nostocaceae (subsection IV. I). In the present study, morphometric and genetic (16S rRNA) data were used to characterize the strains in liquid suspension cultures and solid media under photoautotrophic conditions. The heterocytous cyanobacteria form a monophyletic group according to 16S rRNA gene sequence data. Phylogenetic analysis of 16S rRNA indicated that strain FSN_E and ASN_M are parts of the *Nostoc* cluster. The result was verified with morphological observations. These cyanobacteria are named as *Nostoc* sp. FSN_E and *Nostoc* sp. ASN_M.

Key words: Cyanobacteria, *Nostoc* sp. FSN_E, *Nostoc* sp. ASN_M, hormogonia, phylogenetic analyses, morphological evaluation, 16S rRNA.

INTRODUCTION

Cyanobacteria of the genus *Nostoc* is cosmopolitan in a wide range of terrestrial and aquatic ecosystems (Katoh et al*.,* 2003). Although, there are vast paddy fields in Iran, fewer studies have been carried out toward ecology and taxonomy of heterocystous cyanobacteria of these ecosystems. Due to an entirely almost complete lack of information concerning which parts of the morphology if any of cyanobacteria are constant and which is not, no satisfactory classification of cyanobacteria exists (Rajaniemi et al., 2005). However, there are two basic approaches to classify cyanobacteria (Komárek, 2006):

1. The bacteriological taxonomy which has typically

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concentrated using genetic sequences. Sequence analysis of genes encoding the small- subunit of ribosomal RNA (16S rRNA) is currently the most promising approach to the phylogenetic classification of cyanobacteria. Furthermore, the comparative analysis of 16S rRNA gene sequences provides a new means to investigate the discrepancy between strain collections and natural communities (Komárek, 2009).

2. The Botanical taxonomy has classically focused on the morphological features such as morphology of the filament, vegetative cells, heterocysts and akinetes (Komarek and Anagnostidis, 1989) (Anand, 1988). The form of the colony, shape of terminal cells, presence of the sheath and gas vesicles, as well as life cycle, are additional features used for the identification of some genera (Rajaniemi et al., 2005). At the present, *Nostoc* is classified in order of *Nostocales*, family Nostocaceae by traditional classification (Komarek and Anagnostidis., 1989) and subsection IV.I by bacteriological classification (Rippka et al., 2001). Phylogenetic studies of cyanobacteria have demonstrated that genetic relationships sometimes conflict with the morphological classification (Galhano et al*.,* 2011). The comparison of morphological and genetic data is hindered by the lack of cultures of several cyanobacterial morphospecies and inadequate morphological data of sequenced strains.

Moreover, some strains may lose some important features such as form of colony during long-term laboratory cultivation, which complicates identification (Rajaniemi et al., 2005). Komarek and Anagnostidis (1989) have estimated that more than 50% of the strains in culture collections are misidentified. Therefore, new isolates should be studied by combined morphological and genetic approaches.

The present work focused on the combined genetic and phenotypic relationships of the two *Nostoc* species. Detailed morphological analyses of these strains were carried out at the time of isolation in order to avoid difficulties in identification. The phylogeny of the strains was investigated by sequencing 16S rRNA.

MATERIALS AND METHODS

In 2010, samples of soils were collected from five paddy fields in Golestan province of Iran and were grown in $BG11_0$ (Rippka *et al.,* 1979) liquid culture in a growth chamber at 28°C with (8 to 12 µmol m^{-2} s^{-1}) light intensity (Kaushik, 1987). Morphological observations were examined by bright-field microscopy and by phase-contrast illumination of 10 day-old cultures using a Leica DM750 microscope. The species were identified according to Anand (1989); Desikhachary (1959) and Komarek and Anagnostidis (1989). Two strains of heterocytous cyanobacteria which were the most frequent species of paddy fields were selected on the basis of initial morphological characteristics and released polysaccharide (RPS) production for evaluation of Phylogenetic and morphologic.

Cultivation media

For morphological studies, the strains were cultivated in $BG11_0$, nitrogen-free culture medium: [K_2HPO_4, 0.4 gL^{-1}; $MgSO_4.7H_2O$, 0.75 gL^{-1}; $CaCl_2.2H_2O$, 0.36 gL^{-1}; citric acid, 0.06 gL^{-1}; iron (III) ammonium citrate, 0.06 gL^{-1}; Na_2-EDTA, 0.01 gL^{-1}; Na_2CO_3, 0.2 gL^{-1}, 1 mL; trace elements solution, (H_3BO_3, 61 mgL^{-1}; $MnSO_4$. H_2O, 169 mgL^{-1}; $ZnSO_4.7H_2O$, 287 mgL^{-1}; $CuSO_4.5H_2O$, 2.5 mgL^{-1}; $(NH_4)6Mo_7O_{24}.4H_2O$, 12.5 mgL^{-1}) pH 7.2] (Rippka et al., 1979).

For DNA extraction, the strains were grown in a 4-fold dilution of the nitrogen-free liquid medium AA (Allen and Arnon, 1955). This medium was made by mixing solution A (6.25 ml L^{-1}) with solution B (3.10 ml L^{-1}). Stock solution A was made by combining at the proportion 1:1: 1:1 (v/v), the following stock solutions (mM): $MgSO_4.7H_2O$, 162.3; $CaCl_2.2H_2O$, 81.6; NaCl, 684.5 and microelements. The micro-elements stock solution was composed of (mM): H_3BO_3, 9.25; $MnCl_2.4H_2O$, 1.82; $ZnSO_4.7H_2O$, 0.15; $Na_2MoO_4.2H_2O$, 0.25; $CuSO_4.5H_2O$, 0.06; $CoCl_2.6H_2O$, 0.03; NH_4VO_3, 0.04; and Fe EDTA solution, 160 ml L^{-1} (5.2 g KOH was dissolved in 186 ml distilled water and 20.4 g $Na_2EDTA2H_2O$ was added to it; and in another solution 13.7 g $FeSO_47H_2O$ was dissolved in 364 ml distilled water; afterwards, these two solutions were mixed and bubbling filtered air through the solution until the color changes). Stock solution B contained 245 mM $K_2HPO_4.3H_2O$.

After autoclaving and cooling, the pH of medium was about 7.4. Erlenmeyer flasks (250 ml) containing 100 ml of the appropriate medium (AA/4 or $BG11_0$) were inoculated axenically with 1 ml of 10 25 day-old cultures and incubated under continuous illumination provided by an array of cool white fluorescent tubes giving a mean photon flux density of 3 klx at the flask surface at 30°C (Kashic, 1987).

For growth in solid media, *ca*. 0.1-ml inoculum was inoculated onto each agar-solidified BG11 plate (Liu and Chen, 2003). Prior to inoculation, the culture was microscopically examined to make sure that hormogonium of *Nostoc* strains were the dominant form, accounting for more than 95% of the total cell number. For differentiation of hormogonia, plates were placed in the dark for 2 days, and hormogonia were found during the dark period (Katoh et al., 2003). All treatments were performed in triplicate. Cultures were routinely checked for purity by both microscopic examination and by plating on nutrient agar and examining for bacterial colonies. During a 30 days experiment, morphological characteristics of the cultures were examined using an optic microscope equipped with a camera system.

Morphological study

The following parameters were selected to describe the morphology of the studied strains: length and width of vegetative cells, heterocytes and akinetes; morphology of terminal cell; distance between heterocystes and distance between a heterocyste and the nearest akinete (counted as the number of cells); presence or absence of terminal heterocystes; and shape of filament and its aggregation in colonies (Rajaniemi et al., 2005).

DNA isolation

Filamentous *Nostoc* cells showed aggregated colonies, therefore, their culture flasks were subjected to sonic shock for 10 min. This treatment allowed cell separation and filament breakage. An aliquot of cultured cells (1.5 to 5 ml) were harvested in mid to late exponential phase (10 to 25 days) by centrifugation (12 000 g for 5 min at 25°C) in a sterile 1.5 ml microcentrifuge tube. Cyanobacterial cells that still presented aggregated colonies were dispersed by repeated pipetting with a 1.0 ml micropipette prior to the final centrifugation (Fiore et al., 2000).

Genomic DNA Purification Kit (Bio flux corporation, Tokyo, Japan) was used for DNA extraction and purification using cetyltrimethyl ammonium bromide (CTAB) to reduce contaminations

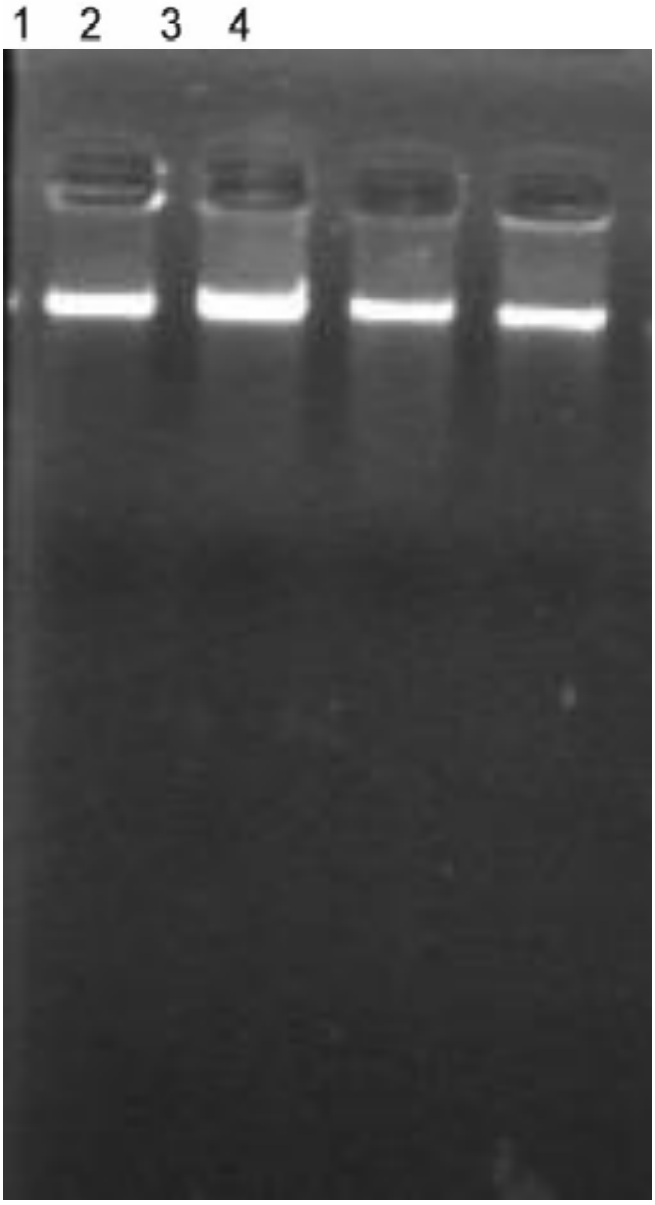


Figure 1. Electrophoresis of extracted cyanobacterial DNA in 1.0% (w/v) agarose gel. Lanes 1 and 2 are *Nostoc* sp. FSN_E and Lanes 3 and 4 are *Nostoc* sp. ASN_M.

by polysaccharides. Finally, supernatant containing the DNA was transferred to a sterile 0.5 ml microcentrifuge tube. DNA samples were electrophoresed in a 1% (w/v) agarose gel using Tris–acetate /EDTA electrophoresis buffer (TAE; 40 mM Tris–acetate and 1 mM EDTA, pH 8.0). The gel was photographed under UV light (Figure 1). The DNA extraction was repeated at least thrice for each species to ensure that the method was reproducible. DNA was resuspended in TE buffer and stored at −20°C (WU et al., 2000). DNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc).

Polymerase chain reaction (PCR) amplifications

The two oligonucleotide primers were used for amplification of cyanobacterial 16S rRNA gene (approximately 420 bp). The set was comprised of one forward primer (359F, 5'-GGGGAATYTTCCGCAATGGG-3') and reverse primers (781Ra, 5'-GACTACTGGGGTATCTAATCCCATT-3') (Nubel et al., 1997). PCRs were performed by Primus advanced Thermal Cycler (MWG, Germany) with 50 µl reaction mixtures each containing 1.5 mM $MgCl_2$, 0.2 mM of dNTP, 1 × PCR buffer, 20 pmol of each primer, 1.25 U of *Taq* DNA polymerase and 1 µg template DNA. The PCR programme for the primer set targeting the *16S rRNA* gene fragment had an initial denaturation step at 94°C for 5 min, followed by 30 cycles consisting of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C, with a final extension step at 72°C for 10 min. Negative and positive control reactions were carried out simultaneously. PCR products were checked by electro-phoresis on 1% agarose gels (SeaPlaque® GTG®, Cambrex Corporation) at 100 V, followed by 0.10 µg mL^{-1} EtBr (Ethidium bromide, Bio-Rad) staining.

PCR products were visualised in the gel by UV light utilising the Molecular Imager® Gel Doc™ XR system (Bio-Rad). A digital gel image was obtained utilising the QUANTITY ONE® 1-D V 4.6.7 analysis software. The size of the products was estimated by comparison to marker DNA (λ/HinfIII + φx/HaeIII, Finnzymes) (Figure 2). Products were purified using the Geneclean® Turbo kit (Qbiogene, MP Biomedicals) and their quantity was measured with a Nanadrop™ ND-1000 spectrophotometer (Thermo Scientific).

Sequencing

Sequencing was subsequently carried out by the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Life Technologies) with 10 µl reaction mixtures each containing 2 µl of sequencing buffer, 1 µl of big dye, 1 µM of primer and 42 ng of PCR product. Reactions for forward and reverse primer were prepared separately.

Cycle sequencing reactions were conducted in a thermocycler (iCycler, Bio-Rad) followed by 25 cycles consisting of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C. Extension products were purified by precipitation: 40 µl saline solution (0.125 M NaCl) as well as ethanol (Etax-A, 96.1%, Alko) were added to the extension reaction products to yield a final volume of 175 µl. The mixture was vortexed and subsequently centrifuged at 13000 g for 10 min at 4°C. The resulting pellet was washed with 5 volumes of 70% (v/v) ethanol (Alko) and subsequently centrifuged for 5 min at 13000 g at 4°C. The pellet was dried at 37°C for several minutes and resuspended in 12 µl of formamide (Hidi™, Applied Biosystems, Life Technologies). Product DNA was denatured at 95°C for 2 min in a thermocycler (iCycler, Bio-Rad) and subsequently analysed with an ABI PRISM® 310 Genetic Analyser (Applied Biosystems, Life Technologies). The runtime for each reaction was 45 min with a running voltage of 15 kV at a temperature of 50°C and the polymer used was POP-6™ (Applied Biosystems, Life Technologies).

RESULTS

Morphological characteristics of the studied cyanobacterial strains

Nostoc sp. FSN_E

This strain has a gelatinous mass, adhering to under surface, and is brownish or dark-colored. Cells are similar in form, cylindrical (3.5 to 4 µ broad and 7 to 11 µ in length), brownish or olive. Heterocysts are somewhat spherical or oblong (5 to 6.5 µ wide and 6 to 12.5 µ long). The spores are ellipsoidal to oblong (5 to 5.5 µ broad and

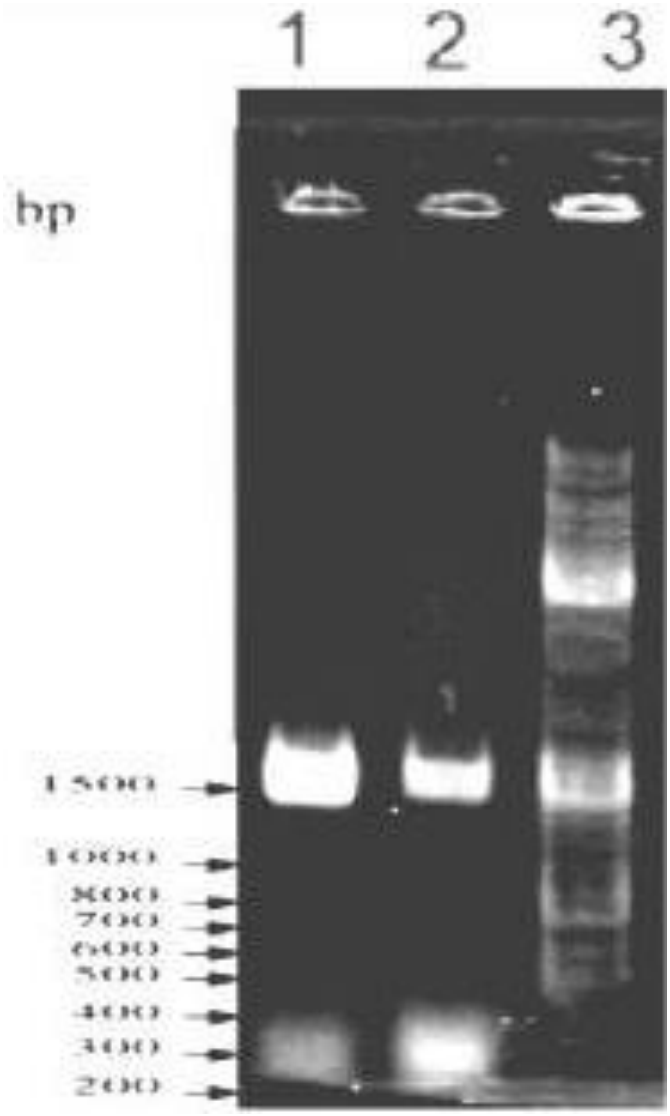


Figure 2. Amplification of *Nostoc* sp. 16S rRNA gene fragments (1.4 kb) using the extracted DNA. Lane 1, *Nostoc* sp. FSN_E, Lane 2, *Nostoc* sp. ASN_M, Lane 3, 100 bp DNA ladder.

10 to 12 µ long).

Nostoc sp. ASN_M

This strain has a gelatinous thallus, adhering to under surface, with dull olive. Cells are spherical or slightly longer than broad (4 to 5 µ broad and 5.5 to 7 µ long) with olive. Heterocysts are somewhat globose (4.5 to 7 µ broad and 4 to 8.5 µ long). Spores are oblong, with many chains (5 to 6 µ broad and 6.5 to 11 µ long).

Colony morphology on agar plates $BG11_{0\ medium}$

Nostoc sp. FSN_E and *Nostoc* sp. ASN_M can easily colonize and develop a wide range of macroscopic colonies that have different colors, shapes, sizes and textures on agar plates (Figures 3A, B and C and 4A, B and C). The size of the spherical colonies (or pearls) in both species ranged from 0.5 to 5 mm, with a majority of 0.5 to 3 mm in *Nostoc* sp. ASN_M. Along with the progression of growth on the plates, there was a color change from light brown (Figure 3A and B) to dark brown (Figure 3C) in *Nostoc* sp. FSN_E and light green (Figure 4A and B) to dark green in *Nostoc* sp. ASN_M (Figure 4C). Microscopic observation of these ball-like colonies revealed that early colonies were completely packed with long filaments, each with more than 20 vegetative cells and 2 to 5 heterocysts in *Nostoc* sp. FSN_E and nearly 10 to 15 vegetative cells and 2 to 3 heterocysts in *Nostoc* sp. ASN_M, while the mature colonies in both of species (Figures 3C and 4C) consisted of relatively short filaments and amorphous substances that were assumed to be exopolysaccharides (Liu and Chen, 2003).

Cell morphology in liquid suspension culture

A distinct life cycle can be observed in the form of the various colony appearances on the culture media. At the onset of incubation, hormogonia tended to prevail (Figure 5) with different lengths and nearly 7 to 15 cells within each hormogonium. Hormogonial cells are typically smaller and more elongated than the square cells of vegetative filaments (compare Figures 5 and 6) with tapered end cells (Campbell et al., 1989). These characteristic end cells eventually differentiate into heterocysts as the filaments return to the vegetative growth state (Figures 5 and 6). After a short period of growth (1 to 2 days), two changes took place, namely, the formation of proheterocysts and heterocysts. Then, cell differentiation into akinetes commenced, indicating that cells grew in suboptimal conditions, such as the depletion of nutrients and light attenuation due to the increased viscosity of the culture. The akinetes were either randomly distributed in the absence of heterocysts or, in the presence of heterocysts, they were located close to their neighboring terminal or to intercalary heterocysts. Degenerate heterocysts also appeared as empty cells, which eventually disintegrated or became detached from the filaments resulting in filament fragmentation. Individual akinetes were released by the senescent colonies to await optimal conditions for germination. Under optimal conditions, akinetes germinated to several hormogonia. At the peak of the growth curves, the colony turned deep green in color due to the concomitant growth of the vegetative cells (Figures 7 and 8).

In the *Nostoc* sp. ASN_M, cell division mainly occurred in the longitudinal plane, leading to the gradual disappearance of the filamentous character. In addition, cells increased in size and tended to be granulated. In fact, they formed spiral aggregates (Figure 9). The whole

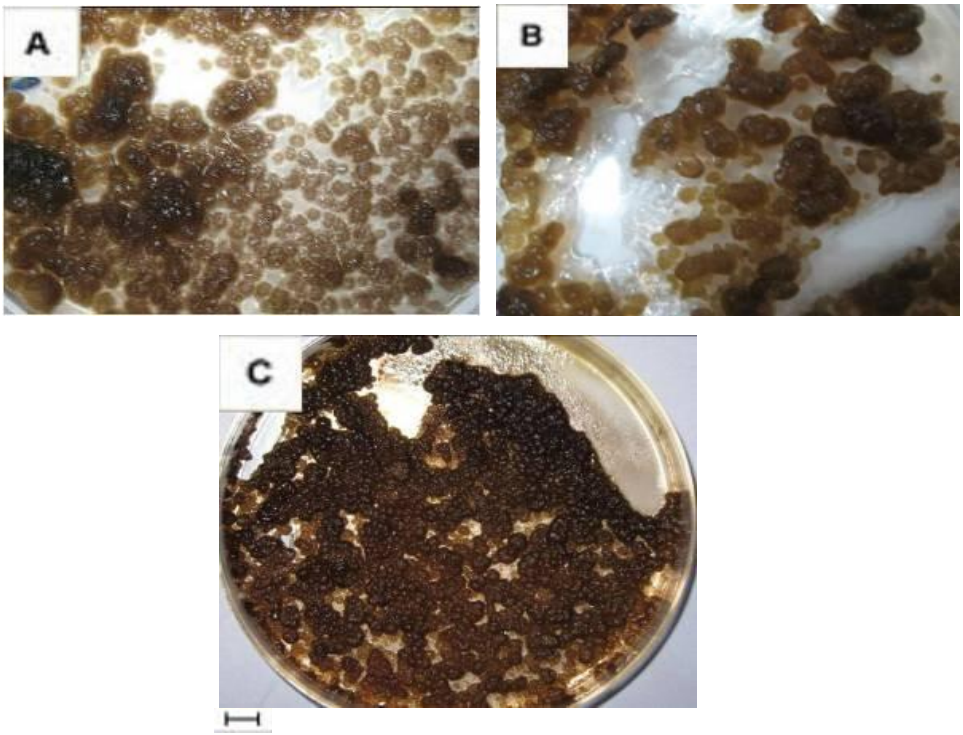


Figure 3. Microphotographs of *Nostoc* sp. FSN_E spherical colonies on agar solidified BG11 plates; A–**C** arranged in chronological order; bar = 10 mm.

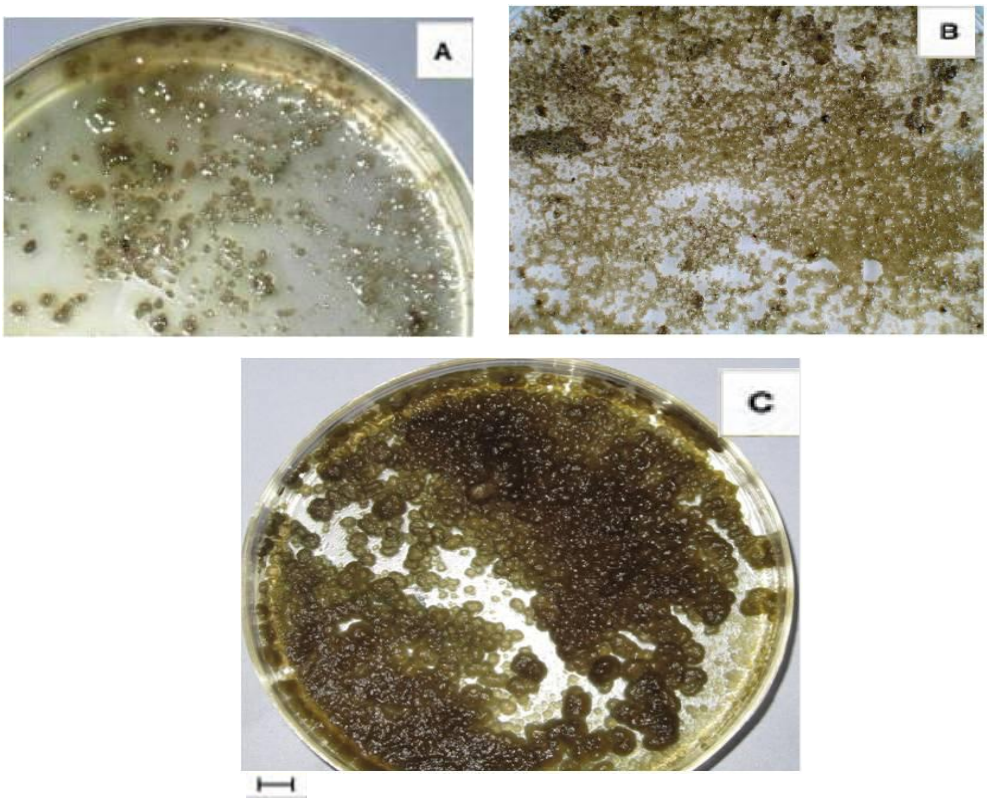


Figure 4. Microphotographs of *Nostoc* sp. ASN_M spherical colonies on agar solidified BG11 plates; A–**C** arranged in chronological order; bar = 10 mm.

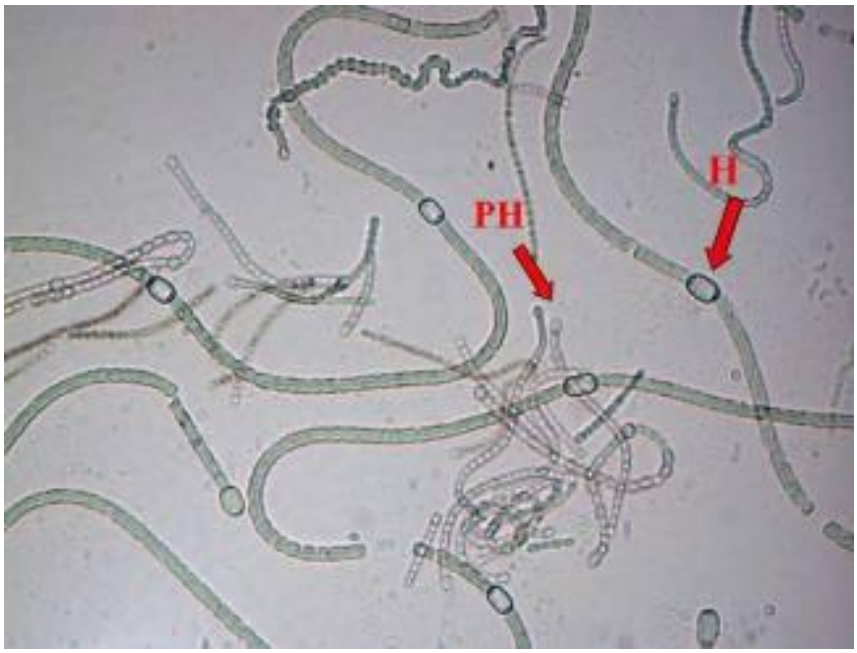


Figure 5. Photomicrograph of *Nostoc* sp. ASN_M, illustrating the proceeding of some representative developmental stages. Cells show cell differentiation young hormogonia with the rare proheterocyst (PH) and vegetative cell developmental alternatives with intercalary heterocysts (H) (400X).

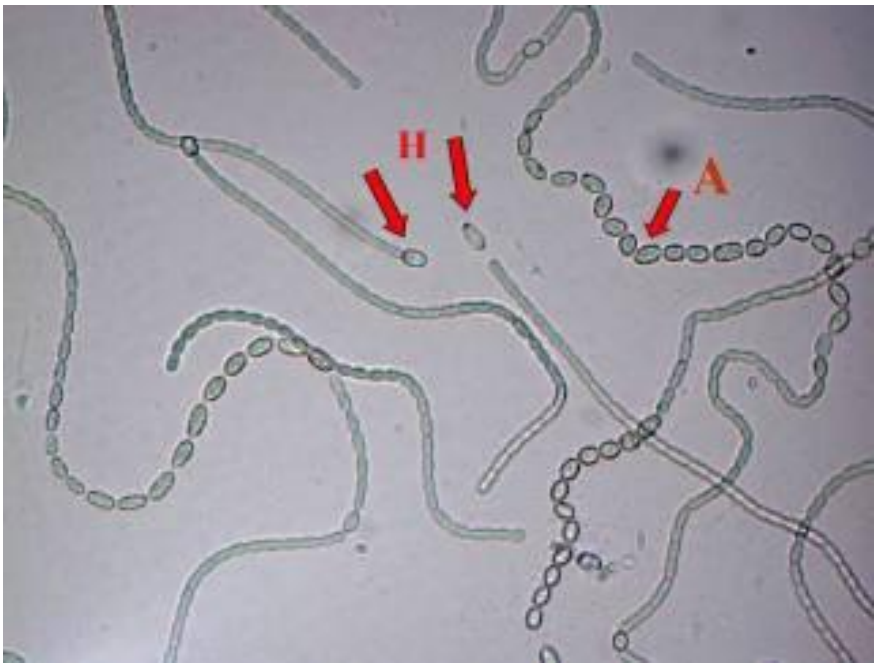


Figure 6. Photomicrograph of *Nostoc* sp. FSN_E, cell differentiation of vegetative cell developmental alternatives with terminal heterocysts (H) and string of akinetes (A) (400X).

colony appeared to be contorted and separated into several compartments, each containing 5 to 10 cells. In contrast to *Nostoc* sp. FSN_E, in *Nostoc* sp. ASN_M four different developmental stages in the life span can be identified namely, hormogonia, filament, seriate colony, and aseriate colony.

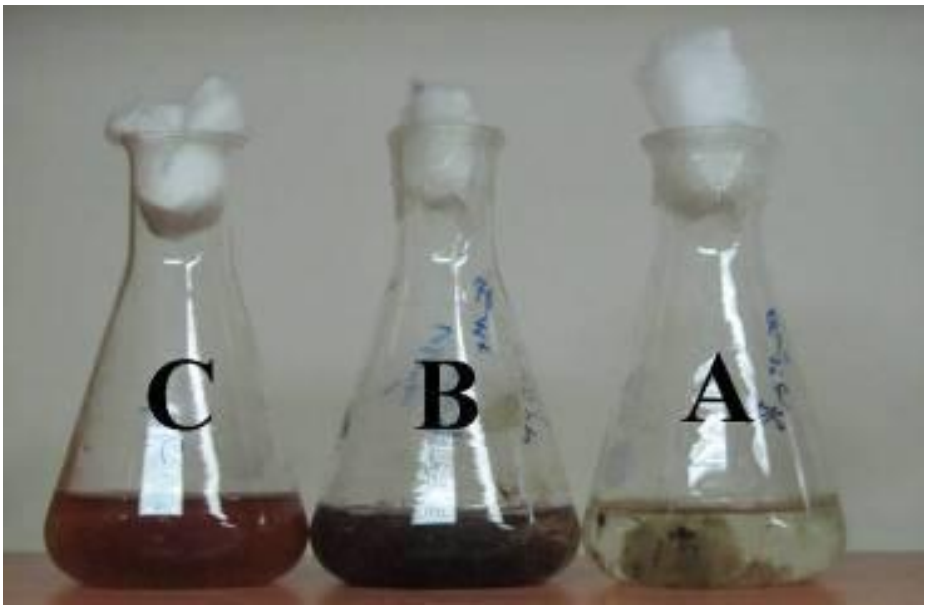


Figure 7. Cultures of *Nostoc* sp. FSN_E (A–C) arranged in chronological order.

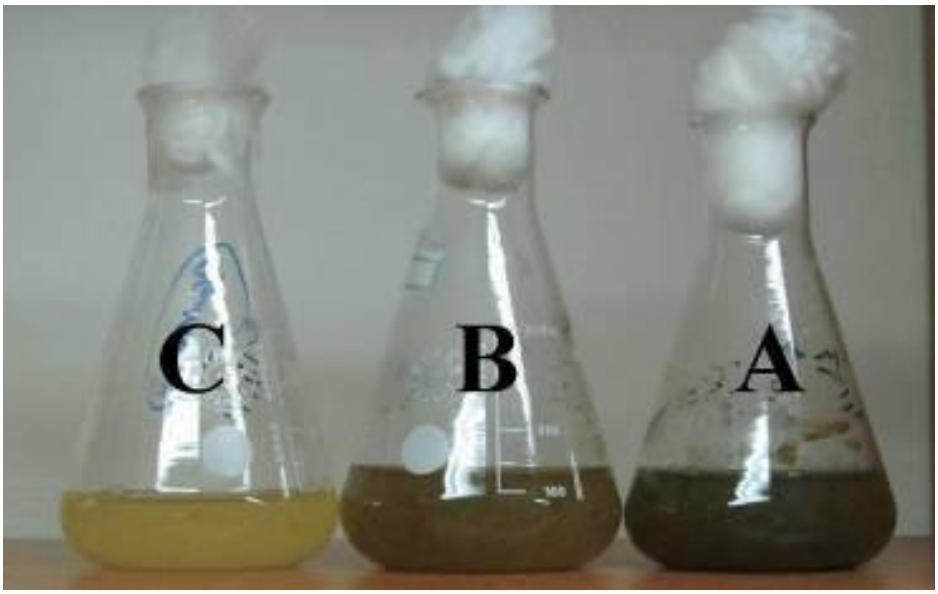


Figure 8. Cultures of *Nostoc* sp. ASN_M (A–C) arranged in chronological order.

Colonies on both agar plates and in liquid medium is pigmented, with coloration ranging from dark green to yellowish green in *Nostoc* sp. ASN_M and reddish brown in *Nostoc* sp. FSN_E. This was probably related to the secretion of some hydrophilic photosynthetic accessory pigments such as scytonemin (Liu and Chen, 2003).

16S rRNA phylogenetic analysis

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.

Phylogenetic trees using the neighbour-joining method and maximum Likelihood analyses was constructed using MEGA5 software package (version 5.0) using the Kimura

Figure 9. Small aseriate (ball-like) colonies with akinetes and heterocysts, encapsulated in a gelatinous sheath.

two-parameter model with complete deletion gap handling. The robustness of the tree was estimated by bootstrap percentages using 1000 replications. Gloeobacter violaceus VP3-01 (accession no. FR798924.1) was used as the outgroup (Schopf, 2000) and (Svenning et al., 2005).

Nucleotide sequence accession number

The nucleotide sequences of the 16S rRNA for isolated *Nostoc* sp. FSN_E and *Nostoc* sp. ASN_M have been registered under DDBJ accession number JF795278 and JF272482, respectively.

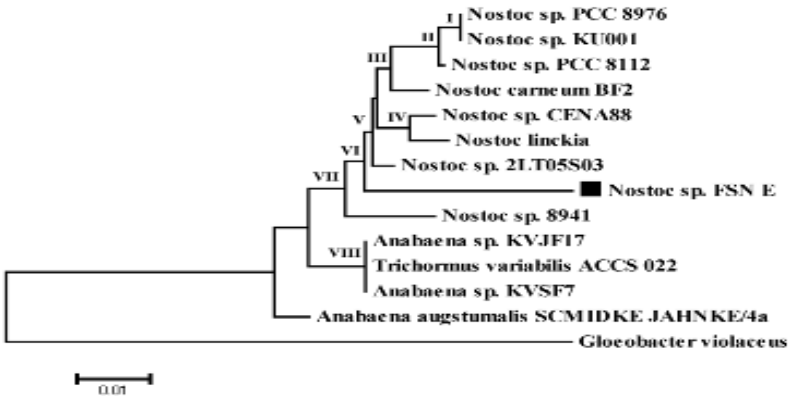
DNA sequencing and phylogenetic analysis

To determine the genus of strain *Nostoc*, the 16S rRNA region was sequenced. The 16S rRNA sequence of this strain was homologous to that of *Nostoc*. The identity of the sequence of strain *Nostoc* sp. FSN_E with *Nostoc* sp. 2LT05S03, *Nostoc* sp. CENA88, *Nostoc* linckia 129, *Nostoc* sp. PCC 8112, *Nostoc* sp. PCC 8976 and *Nostoc* sp. KU001 were 96, 96, 96, 95 and 95%, respectively. The identity of the sequence of strain *Nostoc* sp. ASN_M with *Nostoc calcicola* 99, *Nostoc* sp. PCC 8112, *Nostoc* sp. PCC 8976, *Nostoc* sp. KU001, *Nostoc elgonense* TH3S05, *Nostoc* sp. 2LT05S03, *Nostoc linckia* 129, *Nostoc carneum* BF2 and *Nostoc* sp. CENA88 was 95, 94, 94, 94, 93, 93, 93, 93 and 93%, respectively. On the basis of 16S rRNA gene-sequence analyses, the isolates are identified as *Nostoc* sp (Figures 10, 11 and 12).

DISCUSSION

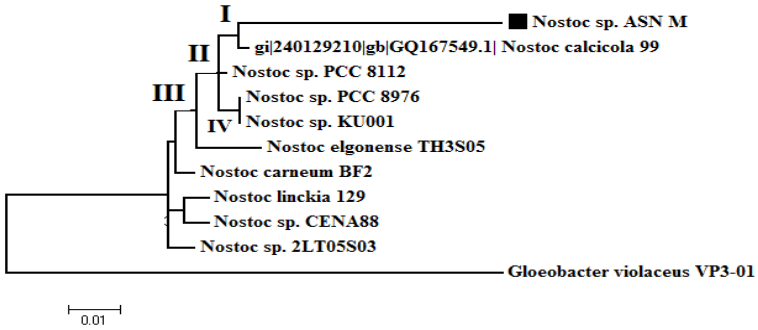
The isolation and purification of cyanobacteria are difficult and the procedures are time-consuming. Methods should be deliberately chosen to take into account the physiological characteristics of the isolated cyanobacterium. The motile hormogonia of *Nostoc* species made the isolation easier, that is, some hormogonia on the plate moved far from their original colonies and settled where no other bacteria or fungi existed (Rajaniemi et al., 2005). Based on these methods, we could isolate and purify *Nostoc* sp. FSN_E and *Nostoc* sp. ASN_M.

In the course of cultivation, it was found that dark and poor- nutrient conditions induced the cells to differentiate into hormogonia and that irradiation inhibited the differentiation. This dark-dependent formation of hormogonia is not observed in *Nostoc muscorum* A and *Calothrix* sp. PCC 7601, where red light is required for cells to differentiate into hormogonia and green light reverses the effect. The differentiation into hormogonia under dark and poor-nutrient conditions seems to be a strategy to spread widely on nutrient – poor soils. In the daytime, the colonies photosynthesize and increase in cell number, while



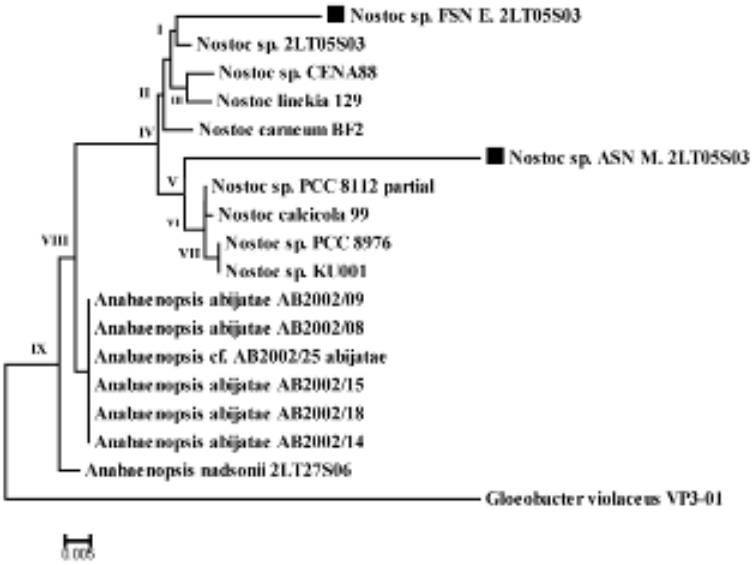
Clades	NJ	ML
I	97	99
II	98	98
III	56	64
IV	71	60
V	72	81
VI	82	87
VII	88	96
VIII	100	99

Figure 10. Distance tree of cyanobacteria constructed on the basis of almost complete 16S rRNA sequences (more than 130 nucleotides). The phylogenetic tree was constructed using the NJ algorithm as implemented within CLUSTAL W. The root of the tree was determined using the 16S rRNA of *Gloeobacter violaceus* VP3-01 (accession no. FR798924.1) as the out-group. The studied *Nostoc* sp. FSN_E is shown in full square. The significant bootstrap percentages obtained from 1,000 replicates using neighbour-joining (NJ) and maximum likelihood (ML) are indicated in the table. The scale bar indicates 0.02 substitutions per nucleotides.



Clades	NJ	ML
I	73	88
II	84	61
III	56	55
IV	97	96

Figure 11. Distance tree of cyanobacteria constructed on the basis of almost complete 16S rRNA sequences (more than 130 nucleotides). The phylogenetic tree was constructed using the NJ algorithm as implemented within CLUSTAL W. the root of the tree was determined using the 16S rRNA of *Gloeobacter violaceus* VP3-01 (accession no.FR798924.1) as the out group. The studied *Nostoc* sp. ASN_M is shown in full square. The significant bootstrap percentages obtained from 1,000 replicates using neighbour-joining (NJ) and maximum likelihood (ML) are indicated in the table. The scale bar indicates 0.02 substitutions per nucleotides.



Clades	NJ	ML
I	57	52
II	54	41
III	64	60
IV	99	99
V	77	83
VI	89	55
VII	92	96
VIII	73	94
IX	98	90

Figure 12. Co-existence of *Nostoc* sp. FSN_E and *Nostoc* sp. ASN_M in Distance tree constructed on the basis of almost complete 16S rRNA sequences (more than 130 nucleotides). The phylogenetic tree was constructed using the NJ algorithm as implemented within CLUSTAL W. the root of the tree was determined using the 16S rRNA of *Gloeobacter violaceus* VP3-01 (accession no.FR798924.1) as the out group. The studied *Nostoc* sp. FSN_E and *Nostoc* sp. ASN_M are shown in full square. The significant bootstrap percentages obtained from 1,000 replicates using neighbour-joining (NJ), maximum likelihood (ML) are indicated in the table. The scale bar indicates 0.02 substitutions per nucleotides.

during the night they produced motile hormogonia and expand their territories (Katoh et al., 2003). The classification of cyanobacteria has routinely relied on morphological characteristics which are not always trustworthy, as they may show variation depending on culturing and environmental conditions (Nayak et al., 2007), and lead to misidentifications (Komárek and Anagnostidis, 1989). These problems of traditional morphological classification, together with the lack of molecular data, posed serious hindrances for taxonomy and systematics of cyanobacteria (Komárek, 2010).

Besides morphological examination, it is currently accepted that characterization and taxonomy of cyanobacteria must combine multidisciplinary approaches (Komárek, 2010). This so-called polyphasic methodology (including phenotypic, chemotaxonomic and genotypic data) has been increasingly followed by many cyanobacteriologists worldwide, for example, Rajaniemi et al. (2005) and Nayak et al. (2007). Among the molecular methods, the analysis of the 16S rRNA gene sequences has proved to be a useful tool for exploring phylogenetic relationships among cyanobacteria (Gugger et al., 2002; Rajaniemi et al., 2005).

In the present study, morphological characteristics of the studied strains (belonging to the family Nostocaceae) revealed remarkable morphological differences between

them and were congruent with recent studies but also with traditional references. The 16S rRNA indicates that strains of *Nostoc* sp. FSN_E and *Nostoc* sp. ASN_M is part of the *Nostoc* cluster. Moreover, we showed that the life cycle of *Nostoc* sp. FSN_E is similar to those of *Nostoc* sp. ASN_M, in which the forms of hormogonia filaments but not in spiral aggregates (aseriate colonies). At the stage of hormogonia and filaments, *Nostoc* sp. FSN_E appears to be have more heterocystous cells than that of *Nostoc* sp. ASN_M and the formation of intercalary heterocysts is very vigorous in *Nostoc* sp. ASN_M while in *Nostoc* sp. FSN_E, the intercalary heterocysts are relatively rare (Figures 3 and 4).

Conclusion

A polyphasic methodology, which integrates phenotypic, chemotaxonomic and genotypic data, is undoubtedly becoming imperative for a better characterization of cyanobacterial strains. In the present study, results from morphologic and genetic criteria showed congruency.

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