Molecular phylogenetic and morphometric evaluation of *Calothrix* sp. N42 and *Scytonema* sp. N11

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

Hossein Fahimi: Assistant Prof., Department of Molecular and Cellular Sciences, Faculty of Advanced Sciences & Technology, Pharmaceutical Sciences Branch, Islamic Azad University (IAUPS), Tehran, Iran

Neda Ordodari: MSc Student, Pharmaceutical Sciences Research Center, Pharmaceutical Sciences Branch, Islamic Azad University (IAUPS), Tehran, Iran

Abstract

The taxonomy of cyanobacteria has been substantially modified in the last few decades, particularly after application of modern ultra structural and molecular methods. The present research, therefore, aimed to investigate, through a polyphasic approach, the differences in morphological and genotypic features of two cyanobacteria strains isolated from different agricultural and freshwaters areas in Kermanshah province (W Iran). In fact, identification and taxonomy of both *Calothrix* sp. N42 and *Scytonema* sp. N11 was problematic and confusing. Challenges arose when the two strains seemed to be *Calothrix*, because of early unbranched filaments, narrow cells and ending by heterocysts. Along with the progression of growth, branching arise. Therefore, molecular analysis was made and morphometric and genetic (16S rRNA) data were used to characterize the strains in liquid suspension cultures and solid media under photoautotrophic conditions. The results of sequencing the 16S rRNA fragment and phylogenetic comparison strongly indicated that, two *strains* fall into different phylogenetic clusters. They were named as *Calothrix* sp. N42 and *Scytonema* sp. N11 and have been registered under DDBJ, accession number KY548062 and KY548065, respectively.

Keywords: *Calothrix* sp. N42, *Nostocales*, phylogenetic approaches, *Scytonema* sp. N11, taxonomy

ارزیابی فیلوژنی مولکولی و مورفومتریک *Calothrix* sp. N42 و *Scytonema* sp. N11

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

حسین فهیمی: استادیار گروه آموزشی علوم مولکولی و سلولی، دانشکده علوم و فناوری‌های نوین، واحد علوم دارویی دانشگاه آزاد اسلامی، تهران، ایران

ندا اردودری: دانشجوی کارشناسی ارشد علوم مولکولی و سلولی، مرکز تحقیقات علوم دارویی، واحد علوم دارویی دانشگاه آزاد اسلامی، تهران، ایران

خلاصه

مطالعه گونه‌های سیانوباکتریایی، به خصوص اعضای راسته *Nostocales*، به دلیل برخورداری از قابلیت تثبیت نیتروژن اتمسفری و در نتیجه نقش آن‌ها در باروری خاک‌های کشاورزی در سراسر جهان، در جوامع علمی از اهمیت بسیاری برخوردار است. با این حال، به رغم اهمیت اکولوژیکی، تاکسونومی سیانوباکتری‌ها هنوز دارای پیچیدگی‌های بسیار است چرا که مطالعات تاکسونومیک سیانوباکتری‌ها، به دلیل ویژگی‌های مورفولوژیکی و فیزیولوژیکی، در شرایط مختلف بسیار متنوع بوده و این مسئله موجب بروز اختلالات زیادی در سیستم‌های طبقه‌بندی شده است. تاکسونومی سیانوباکتری‌ها، در دهه‌های اخیر، بویژه بعد از کاربرد روش‌های مولکولی و فراساختاری، به طور قابل توجهی تغییر کرده است. هدف از مطالعه حاضر این است که از طریق مطالعه پلی‌فازیک، تفاوت ویژگی‌های مورفولوژیک و ژنوتیپیک دو سویه سیانوباکتری جداسازی شده از زمین‌های زراعی و اکوسیستم‌های آب شیرین در استان کرمانشاه، مورد بررسی قرار گیرد. در حقیقت، شناسایی و تاکسونومی *Calothrix* sp. N42 و *Scytonema* sp. N11 جدا شده از ایستگاه‌های مطالعاتی مختلف واقع در استان کرمانشاه، دارای پیچیدگی‌های بسیاری بود، چرا که دو سویه در شناسایی‌های اولیه مورفولوژیک، به علت فیلامنت‌های غیرمنشعب، سلول‌های باریک و حضور هتروسیست در انتهای ریسه، *Calothrix* به نظر می‌رسیدند، اما با رشد بیشتر، ریسه‌ها منشعب شدند. بنابراین، آنالیزهای مولکولی و مورفومتریک با استفاده از اطلاعات ژنتیکی حاصل از16S rRNA برای شناسایی سویه‌ها، در کشت‌های مایع و جامد در شرایط فتواتوتروفیک انجام گرفت. نتایج حاصل از مقایسه توالی قطعات 16S rRNA و فیلوژنتیک نشان داد که دو سویه، در دو کلاستر فیلوژنتیکی متفاوت قرار می‌گیرند، لذا با نام‌های *Calothrix* sp. N42 و *Scytonema* sp. N11 در DDBJ به ترتیب با کدهای دسترسی KY548062 و KY548065 ثبت شدند.

واژه‌های کلیدی: ارزیابی‌های فیلوژنتیک، تاکسونومی، *Calothrix* sp. N42، *Nostocales*، *Scytonema* sp. N11

Introduction

Cyanobacteria are among the most successful microorganism on earth and were most likely present since 2600 million years ago (Hedges *et al.* 2001). Cyanobacteria are Gram-negative bacteria, which are the only bacteria capable of performing oxygenic photosynthesis. They were first regarded as algae, but by finding the typical bacterial cell structure finally, led to their placement into prokaryotic kingdom (Stanier *et al.* 1978). They are adaptive to wide range of habitats due to their unique metabolic flexibility with high dispersion capacity, enormous size of their populations and low probability of extinction (Fenchel 2003).

Taxonomically, cyanobacteria are grouped into unicellular forms that divide by binary fission (Order *Chroococcales*, or Bergey's subsection I) or multiple fission (Order *Pleurocapsales*, or Bergey's subsection II); and filamentous forms that are non-heterocystous (Order *Oscillatoriales*, or Bergey's subsection III) or differentiate heterocysts in non-branching (Order *Nostocales*, or Bergey's subsection IV) or even branching filaments (Order *Stigonematales*, or Bergey's subsection V) (Castenholz 2001). A sixth cyanobacterial order, *Gloeobacterales*, was also proposed by Cavalier-Smith (2002) to accommodate the genus *Gloeobacter* which was formerly included in order *Chroococcales*. Classically, the distinguishing of different species of cyanobacteria has relied essentially upon identifying morphological and developmental characteristics by light microscopy (Rippka 1988, Castenholz *et al.* 1992). Morphologically, similar strains differ greatly at molecular level and vice versa. Such changes become evident in field isolates maintained under artificial culture conditions. In some cases, it is not too difficult to identify cyanobacterial isolates up to the genus level, particularly where morphological characteristics are significantly different from most other genera, e.g. *Calothrix*. In the recent years, molecular genetic techniques have been developed to supplement or supplant conventional methods.

According to the traditional classification system, *Calothrix* belongs to order *Nostocales* and family *Rivulariaceae*, and *Scytonema* belongs to order *Nostocales* and family *Scytonemataceae*. The main characteristics of family *Scytonemataceae* are double due to its false branching which often begins at one or two heterocytes. However, all the members of family *Nostocaceae* have non-branching filaments. These strains are filamentous oxygenic phototrophic cyanobacteria colonizing in both terrestrial and aquatic habitats and due to their nitrogen fixing ability; some are important entity in agricultural zones (Dodds *et al.* 1995, Gao & Ai 2004). Both the studied genera, represent similar morphology in culture, hence led the authors in their incorrect identifications! Early trichomes are unbranched and mostly narrow and tapered towards the end in both strains and according to the valid keys it is identified as *Calothrix*. While, along with the progression of growth, the genus *Scytonema* began to branching in the part of the trichome near the heterocyst, which generates confusing on identification. Therefore, the objective of the present study was to characterize two cyanobacterial strains for morphological and molecular analysis using polymerase chain reaction in order to characterize and deduce phylogenetic relationships.

The present study, therefore, deals with the polyphasic characterization of two strains of heterocytous cyanobacteria, which their identification was very problematic and confusing.

Materials and Methods

1. Morphological studies

- Isolation of strains and culture conditions

In 2015, soil and fresh water samples were collected from different agricultural and freshwaters areas in Kermanshah province, Iran (Table 1). According to the pedological map of aforesaid province, soil types of different textures from agricultural and freshwaters areas were selected. Soil samples were collected from the surface of the soils up to five cm deep by using sterilized

spatula after removing debris from the surface, and for the fresh water samples, small plastic bottles were used (Rangaswamy 1966). Three replicates of each site were collected and transferred to the lab while they were kept in ice. Then, samples were transferred to sterile Petri dishes with adequate quantities of liquid and solid media BG11 medium (nitrate free) for culturing the soil and aquatic strains, respectively (Rippka *et al*. 1979). The pH

was adjusted to 7.1 after sterilization and the Petri dishes were incubated in a culture chamber at 28 °C, and were provided with continuous artificial illumination of approximately 1500–2000 lux for two weeks (Kaushik *et al*. 2009). Three small fragments of growing colonies were then placed equidistantly onto an agar surface for purification.

Table 1. Isolation source of the isolated strains

Name of the strain	Isolation source (locality)	Latitude	Longitude
Scytonema sp. N11	Fresh waters (Sirvan river)	34° 25′ 04″	47° 00′ 59″
Calothrix sp. N42	Agricultural soils (*Crataegus*)	34° 24′ 32″	47° 00′ 17″

- Phenotypic characterization of the cyanobacterial isolates

Detailed morphological analyses of these strains were carried out at the time of isolation in order to avoid difficulties in identification bright-field microscopy and by phase-contrast illumination of 10-day-old cultures using a Leica DM750 microscope. The following parameters were selected to describe the morphology of heterocytous cyanobacteria: Morphology of vegetative cells (including terminal cell), heterocytes, akinetes, presence or absence of terminal heterocytes; and the shape of the filament as well as its aggregation in colonies (Singh *et al*. 2011). The species were identified according to Desikachary (1959). However, identification and taxonomy of two strains of heterocytous cyanobacteria which were the most frequently observed species in that areas, was problematic and confusing. Therefore, they were selected for morphological and genetical analyses. During a 30 days experiment, morphological characteristics of the cultures were examined using an optic microscope equipped with a camera system.

2. Molecular studies

- Genomic DNA extraction

Genomic DNA was extracted utilising the E.Z.N.A.® SP Plant DNA kit (Omega Bio-tek). The

micro-tubes containing 100 mg wet cells were supplemented with 300 mg of two differently-sized glass beads (acid-washed, 180 µm and 425–600 µm, Sigma-Aldrich) as well as lysis buffer and RNase solution, both provided by the kit. In order to ensure proper disruption of the cells, tubes were homogenised three times for 20 s at a speed of 6.5 ms^{-1} with a FastPrep instrument (Savant Instruments). 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.).

- 16S rDNA gene amplification and sequencing

Two oligonucleotide primers comprised of one forward primer (359F, 5'-GGG GAA TYT TCC GCA ATG GG-3') and a reverse primer (781Ra, 5'-GAC TAC TGG GGT ATC TAA TCC CAT T-3') (Nübel *et al*. 1997), were used for partial amplification of 16S rRNA gene. One PCR reaction was comprised of $1 \times$ PCR Buffer solution (DyNAzyme^{TM} PCR buffer, Finnzymes), 0.5 µm forward primer, 0.5 µm reverse primer and 0.5 U *Taq* polymerase (DyNAzyme^{TM} II DNA polymerase, Finnzymes) as well as 1 µL template DNA and sterile water in a total volume of 20 µL. The template DNA concentration of the three strains in the reaction accounted for approximately 140 ng. Sterile water instead of template DNA was used as a negative control.

The amplification reactions were conducted in a thermocycler (iCycler, Bio-Rad) with the following program: Initial denaturation at 94 °C for 3 min, 30 cycles comprised of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and annealing at 72 °C for 30 s, as well as a final annealing phase at 72 °C for 5 min. PCR products were checked by electrophoresis 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^{TM} 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 (/HinfIII+ x/HaeIII, Finnzymes). The products were purified using the Geneclean® Turbo kit (Qbiogene, MP Biomedicals) and were quantified with a NanoDrop^{TM} ND-1000 spectrophotometer (Thermo Scientific). Sequencing has been done by Bioneer Company (S Korea).

- Phylogenetic analysis and construction of tree

BLAST N searches (http://www.ncbi.nlm.-nih.gov/BLAST) of the partial 16S rRNA gene of two strains and BLAST X search 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 GenBank 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 (Jones-Taylor-Thornton model) analyses were constructed by the MEGA version 5.1 (Tamura *et al.* 2007). The robustness of the tree was estimated by bootstrap percentages using 1000 replications. The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

All positions containing gaps and missing data were eliminated. *Synechocystis* sp. PCC 6714 (Accession No. AB041937.1) and *Synechococcus* elongates (Accession No. NR_074309.1) was used as the out group. Complete deletion handling of gaps and confidence levels were calculated via bootstrapping using a re-sampling number of 1,000. Reference sequences were obtained from GenBank (NCBI).

- Nucleotide accession numbers

The sequences of the 16S rRNA genes of the strains have been deposited in the NCBI GenBank and respective accession numbers were obtained.

Results

1. Morphological descriptions of the studied cyanobacterial strains

The morphological features of the *Calothrix* sp. N42 (CCC 42) and *Scytonema* sp. N11 (CCC 11), is summarized in Tables 2 & 3, respectively.

The confusing point of identification was that, early trichomes of *Scytonema* sp. N11 are unbranched and mostly narrow and tapered towards the end (a–f). However, along with the progression of growth, the trichomes are of the same diameter throughout and with cylindrical cells and filaments, began to make double false branching which generates confusing on identification. A distinctive feature of the genus *Scytonema* sp. N11 is that, its mature trichomes exhibit a low degree of tapering, whereas those of the genus *Calothrix* have a distinct basal-apical polarity and display a high degree of tapering. Moreover, there was no obvious colony on agar plates for both strains. *Calothrix* sp. N42 and and *Scytonema* sp. N11 can easily colonize and develop a wide range of macroscopic colonies that have different shapes, sizes and textures on agar plates. The colonies were light green to dark green in mature *Calothrix* sp. N42 colonies and light brown to dark brown in *Scytonema* sp. N11 (Figs 1–3).

2. Phylogenetic study

To infer the phylogenetic relationship (Fig. 4), a total of 44 and 21 16S rRNA gene sequences for *Calothrix* sp. N42 and *Scytonema* sp. N11, respectively, were selected including the strains under study. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Subsequent phylogenetic analyses gave a strong pattern of distinct clustering. For example, *Scytonema* sp. N11 was grouped to other strain of *Scytonema* (Accession Numbers: KT222810.1, KU058658.1, KU6689001.1, KT935473.1,

KT222810.1, KJ65537.1, HF911525.1, JN56281.1, NR 112180.1, AM709637.1, and AF132781.1), and *Calothrix* sp. N42 was clustered separately to other strain of *Calothrix* (Accession Numbers: AB694935.1, X99213.1, KM019959.1, AM230701.1, and KU668908.1).

Two studied strains were registered under DDBJ as *Calothrix* sp. N42 KY548062 and *Scytonema* sp. N11 KY548065, and deposited in Cyanobacteria Culture Collection (CCC) of Science and Research Branch, Islamic Azad University, Tehran, Iran.

Table 2. The morphological characters of the *Calothrix* sp. N42 (CCC 42)

Isolated	Trichomes	Vegetative cells	Heterocytes	Sheaths	Hormogonia	Akinetes	Ending of the filaments
Calothrix sp. N42 (CCC 42)	Mature trichomes tapers from base and enlarges during filaments development.	Vegetative cells disc-shaped, cylindrical cells quadratic, shorter or longer than broad, 3–6 µm long and 2–6 µm broad.	Heterocyst single, basal and spherical, 3–4 µm broad (a–j), sometimes there is immediate intercalary heterocyte near the basal heterocyte (a, e, g, h and f).	Sheaths always present, sometimes disintegration of the sheath was found (g).	Reproduction was done by motile hormogonia, which divide from the trichome, necridic cells which liberate from the sheath and Akinetes. Hormogonia giving rise to young filaments with terminal heterocyst at only one end of the trichome.	Akinetes slightly conical, more or less cylindrical, 8–11 × 20–35 µm, greenish Mostly are in basal parts (g).	Ending by the hair-like apical part composed from narrow, long, hyaline cells (f).

Table 3. The morphological characters of the **Scytonema** sp. N11 (CCC 11)

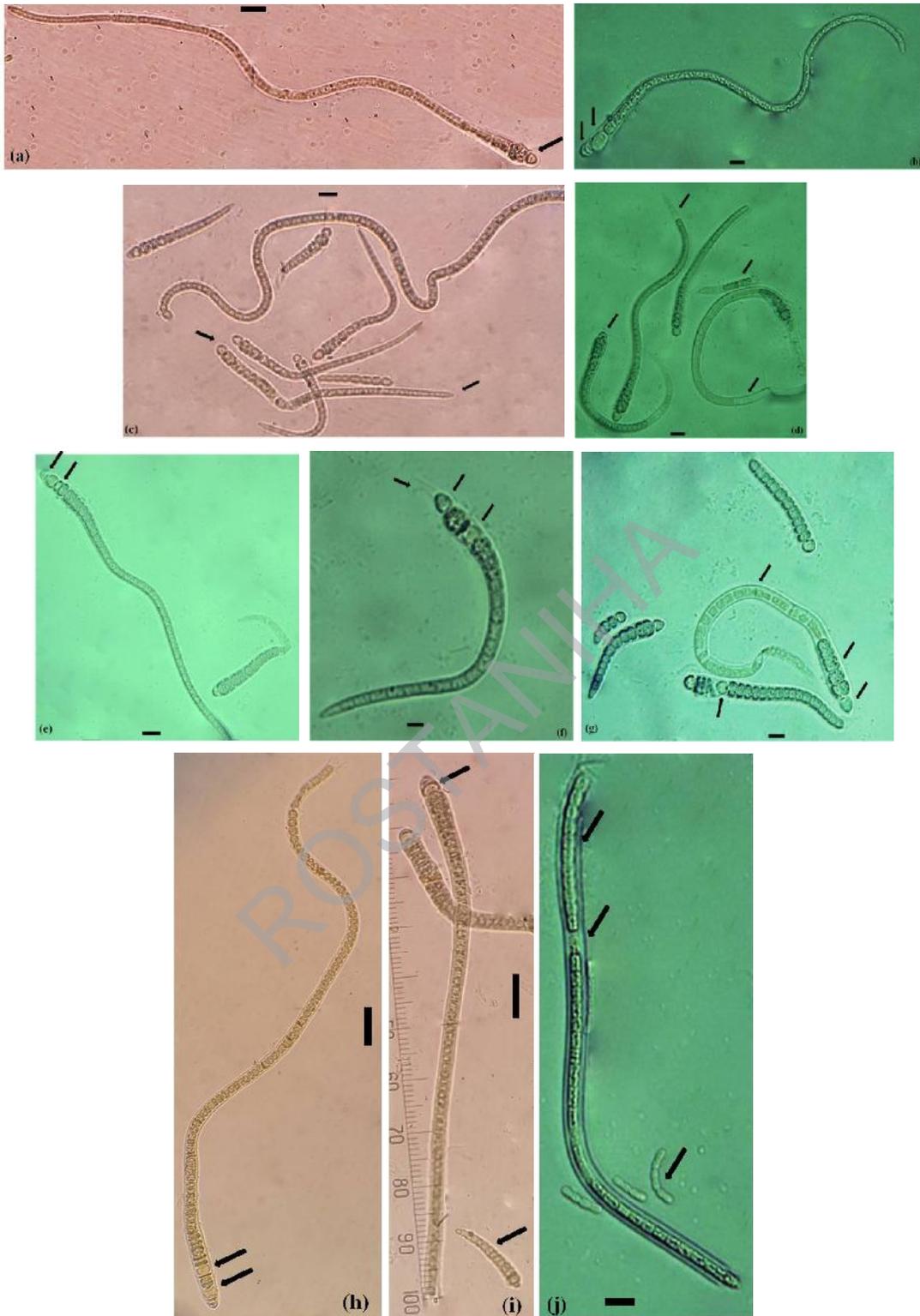| Isolated	Trichomes	Vegetative cells	Heterocytes	Sheaths	Hormogonia	Akinetes
Scytonema sp. N11 (CCC 11)	Filamentous branched, with one (n) or two lateral branches (m, o, p & q). Branching initiates after trichome dissociation by help of necridic cells between two heterocytes (g) or one heterocytes (j & h), both branches grow parallel aside (m, o, p & q). The filaments make sometimes typical loop-like lateral formations before branching, in which tops of the trichomes later divide (o & p). Usually, double false branching was found in this genus, this kind of false branching often begins at one or two heterocytes. In false branching, there is an appearance of a branch, but the cells in one branch are not actually continuous with those of the other.	Cells cylindrical, 4–6 µm long and 2–4 µm broad.	Solitary and rounded heterocytes At first, heterocytes were appeared in the middle parts of trichomes sometimes with elongated, cylindrical cells and the second, branching happened and the region of the false branches is correlated with the position of heterocysts. The other new and strange characteristics in *Scytonema* sp. N11is that in addition to intercalary heterocysts, there is hemispherical heterocyte at the end of filaments, that it is not usual in *Scytonema* sp. (o).	The ends of trichomes that develop from hormogonia grow through the old sheath of the parent filament, either singly or in pairs and then secrete a sheath of their own. Trichomes surrounded by a very distinct sheath, mostly narrow and tapered towards the end, usually yellow-brown (colored by scytonemin) in some parts.	Reproduction by hormogonia, which develop at the ends of branches and liberate from sheaths. Hormogonia germinate at both ends and form filaments (o).	Two or more akinetes (many in old cultures).

Fig. 1. Photomicrograph of *Calothrix* sp. N42, illustrating the proceeding of different representative developmental stages (Bars = 10 µm).
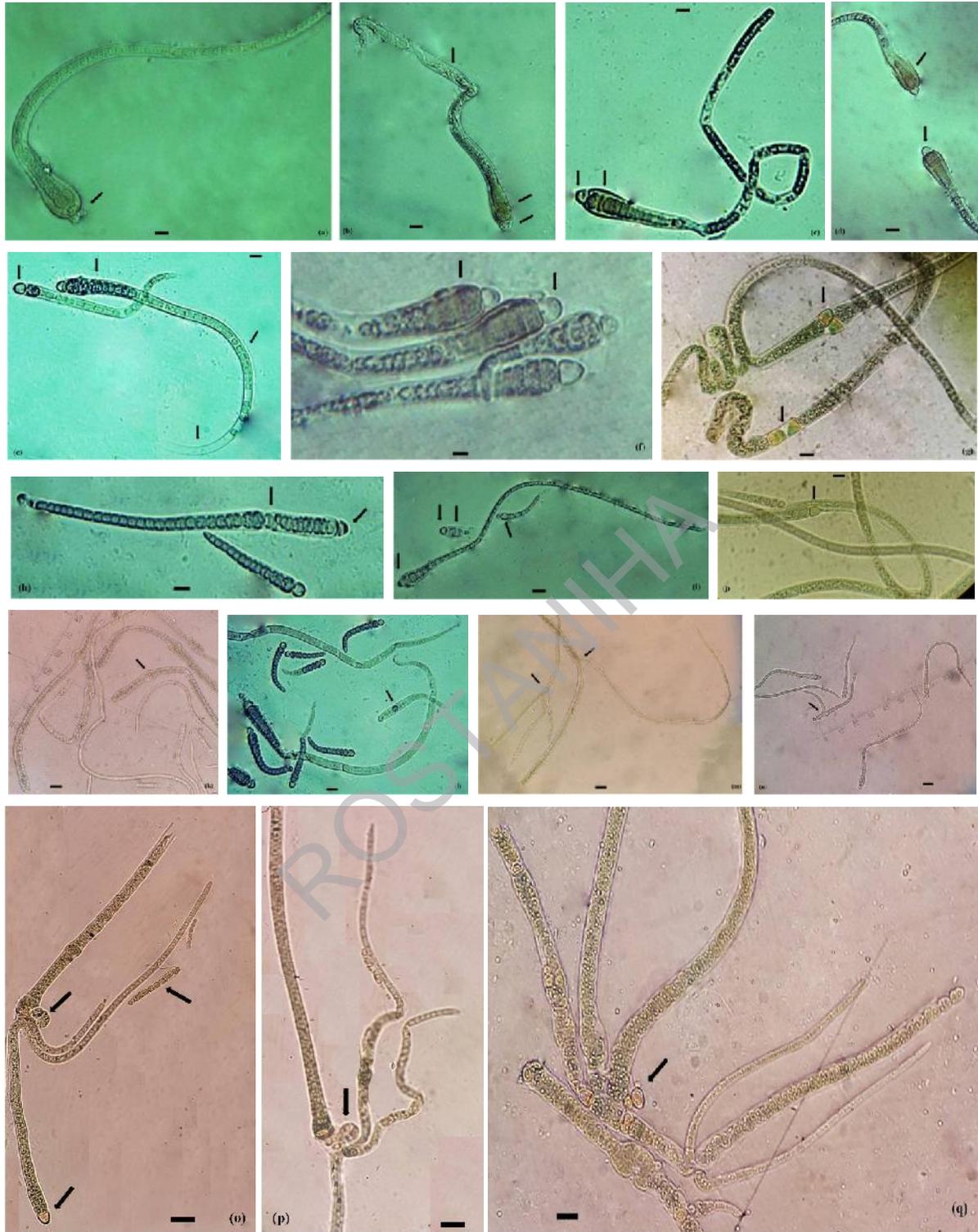
Fig. 2. Photomicrograph of *Scytonema* sp. N11, illustrating the proceeding of different representative developmental stages (Bars = 10 µm).

Fig. 3. Microphotographs of *Calothrix* sp. N42 (left side) and *Scytonema* sp. N11 (right side) colonies on agar solidified BG11 plates.

Fig. 4. Consensus bootstrap tree on the basis of maximum likelihood distances of 672 bp and 1343 bp long full-length 16S rRNA genes sequences of *Scytonema* sp. N11, *Calothrix* sp. N42 and sequences that were taken from the GenBank. Numbers near nodes indicate bootstrap values over 50% for ML analyses (Bar = 0.03 change per sequence position).

Discussion

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 & Anagnostidis 1989). These problems of traditional morphological classification, together with the lack of molecular data, pose serious hindrances for taxonomy and systematic of cyanobacteria (Komárek 2010). Moreover, some strains may lose some important features such as form of colony during long-term laboratory cultivation, which complicates identification (Singh *et al*. 2011). Komárek & Anagnostidis (1989) have estimated that, more than 50% of the strains in culture collections are misidentified. Therefore, isolates should be studied by combined morphological and genetic approaches.

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 (Thajamanbi *et al*. 2016).

The *Rivulariaceae* and *Scytonemataceae* are considered amongst the most morphologically complex cyanobacteria with tapered and branching trichomes (Rai *et al*. 1978, Bohuslav 2007, Korelusová 2008, Komárek *et al*. 2013), apart from short phases of hormogonium formation that has a terminal heterocyst in both strains, although in *Scytonema* sp. N11 intercalary heterocyst is also present and branching is largely localized to a region near the heterocyst.

The results of this study showed that, two strains of *Calothrix* sp. N42 and *Scytonema* sp. N11 are intensely varied in various conditions, and produce morphologically different developmental stages and morphological states. Such incorrect strain designations lead to many errors in experimental investigations. Therefore, the very precise studies of the variability of culture material combined with the perfect knowledge of the natural population and molecular analysis are important for the correct taxonomic classification of cyanophytes. Therefore, the revision of determination keys on the basis of such studies is necessary.

In addition, the separate and strong clustering of two strains in two separate clustering, strengthens our observation beyond doubt that, both strains of *Calothrix* sp. N42 and *Scytonema* sp. N11 are belonging to different families. This study emphasizes that, the polyphasic microbial identification (using both morphological and molecular data) is indeed very important for the taxonomic study of an organism. Genetic approach along with detail morphological study in different developmental stage of an organism is essential for proper identification of a species. Moreover, intensive investigations on different cultures of cyanobacteria not only for the morphological features but also for their physiological characteristics were suggested for evolving a better system of classification by designating reference cultures.

To the best of the author's knowledge, this work is the first research work performed in Iran that describes two species of *Calothrix* sp. N42 and *Scytonema* sp. N11 using the phenotypic, genetic and phylogenetic approach.

References

Bohuslav, U. 2007. Morphological characterization of three subaerial *Calothrix* species (*Nostocales*, Cyanobacteria), Fottea 7: 33–38.

Castenholz, R.W. 2001. General Characteristics of the Cyanobacteria. Pp. 474–487. *In*: Boone, D.R. & Castenholz, R.W. (eds). Bergey's Manual of Systematic Bacteriology, 2nd. edition, Vol. 1. New York: Springer.

Castenholz, R.W., Manhart, L.R., Mccourt, R.M., Wood, A.M. & Leatham, T. 1992. Species usage, concept, and evolution in the cyanobacteria (blue-green algae). Journal of Phycology 28: 737–745.

Cavalier-Smith, T. 2002. The neomuran origin of archaebacteria, the negibacterial root of the universal tree and bacterial megaclassification. International Journal of Systematic and Evolutionary Microbiology 52: 7–76.

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

Dodds, W.K., Gudder, D.A. & Mollenhauer, D. 1995. The ecology of *Nostoc*. Journal of Phycology 31: 2–18.

Gao, K. & Ai, H. 2004. Relationship of growth and photosynthesis with colony size in an edible cyanobacterium, ge-xian-mi *Nostoc* (*Cyanophyceae*). Journal of Phycology 40: 523–526.

Hedges, S.B., Chen, H., Kumar, S., Wang, D.Y., Thompson, A.S. & Watanabe, H. 2001. A genomic timescale for the origin of eukaryotes. BMC Evolutionary Biology 1: 4.

Fenchel, T. 2003. Biogeography for bacteria. Science 301: 925–926.

Kaushik, P., Chauhan, P., Chauhan, G. & Goyal, P. 2009. Evaluation of *Nostoc* commune for potential antibacterial activity and UV-HPLC analysis of methanol extract. The Internet Journal of Microbiology ISSN: 1937–8289, 1.

Komárek, J. 2006. Cyanobacterial taxonomy: current problems and prospects for the integration of traditional and molecular approaches. Algae 21: 349–375.

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

Komárek, J. & Anagnostidis, K. 1989. Modern approach to the classification system of Cyanophytes, 4-*Nostocales*. Archiv für Hydrobiologie 82: 247–345.

Komárek, J., Sant´Anna, C.L., Bohunická, M., Mareš, J., Hentschke, G.S, Rigonato, J. & Fiore, M. 2013. Phenotype diversity and phylogeny of selected *Scytonema*-species (Cyanoprokaryota) from SE Brazil. Fottea 13(2): 173–200.

Korelusová, J. 2008. Phylogeny of Heterocystous Cyanobacteria (*Nostocales* and *Stigonematales*). Thesis, University of South Bohemia, Ceské Budejovice.

Nayak, S., Prasanna, R., Prasanna, B.M. & Sahoo, D.B. 2007. Analyzing diversity among Indian isolates of *Anabaena* (*Nostocales*, Cyanophyta) using morphological, physiological and biochemical characters. World Journal of Microbiology and Biotechnology 23: 1575–1584.

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

Rai, A., Pandey, K.D. & Kashyap, A.K. 1978. Heterocyst differentiation and polarity in *Calothrix*. New Phytologist 81(3): 647–651.

Rangaswamy, G. 1996. Agricultural Microbiology. Asia Publishing House, Bombay.

Rippka, R. 1988. Recognition and identification of cyanobacteria. Methods *in* Enzymology 167: 3–67.

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

Singh, S., Dhara, D.W. & Guptab, R.K. 2011. Morphological and molecular characterization of *Calothrix* isolates obtained from diverse environments in India. Microbiology 80(3): 411–419.

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. 1978. Proposal to place nomenclature of cyanobacteria (blue-green algae) under rules of international code of nomenclature

of bacteria. International Journal of Systematic and Evolutionary Microbiology 28: 335–336.

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.

Thajamanbi, M., Rout, J. & Thajuddin, N. 2016. Isolation and characterization of two cyanobacterial strains *Calothrix* sp. and *Microchaete* sp. from rice fields of Karimganj district, Assam, north east India. Current World Environment 11: 399.