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MOLECULAR AND PHYLOGENETIC CHARACTERIZATION OF TWO NOSTOC STRAINS ISOLATED FROM ALAGOL AND AJIGOL SOILS (GOLESTAN PROVINCE), IRAN

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Abstract
Accurate identification and phylogenetic characterization of cyanobacterial strains are essential for understanding their diversity and ecological roles. In this study, *Nostoc* strains isolated from lake-adjacent soils of Alagol and Ajigol (Golestan province) were analyzed by amplifying functional, structural, and palindromic genes. Considering the content of this article, it is important to acknowledge the problems and inefficiencies of relying solely on morphological traits for the accurate identification of cyanobacteria. Morphological features alone often fail to distinguish closely related strains due to their variability and overlap. Therefore, new molecular techniques and polyphasic studies are justified to achieve more reliable identification. The sampling results revealed two strains belonging to the Nostocaceae family. The two strains were designated as *Nostoc* sp. 1981 and *Nostoc* sp. 1982. The phylogenetic tree constructed using the 16S rRNA gene correlated with the morphological characteristics of the studied strains, placing both in a separate clade within Nostocaceae with strong bootstrap support of 83.2%. Comparative analysis of the ITS regions revealed differences in length and in the secondary structure of the D1-D1 and BOX B helices compared to other strains. Amplification of palindromic sequences also distinguished the two strains. Phylogenetic analysis using the *rpo*C1 gene demonstrated its suitability as a marker for discriminating closely related strains. This study combines fingerprinting techniques and gene analysis to effectively differentiate closely related *Nostoc* strains, providing new molecular and phylogenetic insights.

Keywords: Cyanobacteria; molecular phylogeny; palindromic genes, *rpo*C1; structural genes

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شناسایی مولکولی و فیلوژنتیکی دو سویه *Nostoc* جدا شده از خاک‌های اطراف دریاچه های آلاگل و آجی‌گل (استان گلستان) ایران

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چکیده: شناسایی دقیق و تعیین جایگاه فیلوژنتیکی سویه‌های سیانو باکتریایی برای درک تنوع و نقش‌های اکولوژیکی آنها بسیار حیاتی است. در این مطالعه، سویه‌های *Nostoc* که از خاک‌های حاشیه تالاب‌های آلاگل و آجی گل جدا شده بودند، با تکثیر ژن‌های عملکردی، ساختاری و نشانگرهای به شدت تکراری مورد بررسی قرار گرفتند. با توجه به ناکارآمدی تکیه بر صفات ریخت‌شناسی به دلیل تغییرپذیری آنها برای شناسایی دقیق سیانوباکتریها و تمایز سویه‌های نزدیک به هم، استفاده از روش‌های مولکولی و مطالعات پلی فازیک برای شناسایی قابل اعتمادتر ضروری است. نتایج حاصل از نمونه‌برداری، دو سویه متعلق به تیره Nostocaceae را نشان داد. دو سویه به نام‌های *Nostoc* sp. 1982 و *Nostoc* sp. 1981 نامگذاری گردیدند. نتیجه حاصل از ساخت درخت فیلوژنتیک با استفاده از توالی نوکلئوتیدی ناحیه ژنی ۱۶S rRNA، با شناسایی مورفولوژیک دو سویه مورد مطالعه همبستگی داشت. به طوری‌که هر دو سویه با حمایت بالای بوت استرپ ۸۳/۲ درصد با سایر جنس‌های *Nostoc* در یک کلاد جداگانه قرار گرفتند. نتایج حاصل از آنالیز مقایسه طول مناطق ITS نشان داد که طول منطقه و همچنین ساختار مارپیچ های D1-D1 و BOX B با سایر سویه ها متفاوت است. نتایج به دست آمده از تکثیر توالی‌های پالیندرومیک نیز منجر به تفکیک دو سویه شد. حاصل از رسم درخت بر اساس ژن *rpo*C1 نشان داد، که استفاده از ژن *rpo*C1 می تواند معیار مناسبی برای تفیک دو سویه نزدیک به هم تلقی گردد. این مطالعه تکنیک‌های انگشت نگاری و تحلیل ژنی را ترکیب می‌کند تا سویه‌های نزدیک *Nostoc* را به‌طور مؤثر متمایز کند و بینش‌های جدیدی درباره ویژگی‌های مولکولی و فیلوژنتیکی ارائه دهد.

INTRODUCTION

Cyanobacteria are a group of Gram-negative, photosynthetic prokaryotes and represent one of the most widespread and ecologically significant microbial groups on earth. Evolutionary assessments of cyanobacteria indicate notable similarities between extant forms and their fossilized counterparts. Their presence in freshwater blooms, marine ecosystems, rice paddies, limestone formations, saline soils, deserts, polar environments, and symbiotic relationships highlights their genetic adaptability and evolutionary resilience across diverse ecological niches. Collectively, these findings underscore that cyanobacteria comprise a highly diverse clade of prokaryotes (Sánchez-Baracaldo & al., 2022).

The phylogeny of filamentous heterocystous cyanobacteria, which exhibit considerable morphological and physiological diversity, has long posed challenges for taxonomists. The variation in morphology has often led to misidentification, and it is now widely recognized that morphological and physiological similarities among different cyanobacterial strains do not necessarily reflect genetic relatedness. In one study, researchers reported that over 50% of cyanobacterial strains in culture collections had been misidentified (Valerio & al. 2009). This issue has drawn the attention of many scientists worldwide and has driven the development of more reliable methods for the taxonomic evaluation and biodiversity assessment of cyanobacteria (Strunecký & al., 2023).

In this context, DNA-based analytical methods-particularly PCR techniques have enabled extensive evaluations of genetic variation at the nucleotide level, profoundly influencing nearly all fields of modern biology. Advances in molecular biology and bioinformatics have made it possible to explore an organism's genome in search of unique sequences (Nowruzi & Fahimi, 2022). These distinct sequences can be employed to differentiate a specific group of microorganisms from their closely related counterparts. Various types of repetitive DNA sequences are present in prokaryotic genomes; due to their widespread occurrence and conserved nature, they play a significant role in DNA fingerprinting techniques and have been introduced as alternative approaches for the identification of species and strains (Strunecký & al., 2023). Rep-PCR techniques, utilizing molecular markers such as highly repetitive palindromic sequences (HIP), Short Tandemly Repeated Repetitive (STRR), and Enterobacterial Repetitive Intergenic Consensus (ERIC), have been applied in the molecular phylogeny of cyanobacteria. These techniques use the repetitive oligonucleotide sequences present in bacterial strains to distinguish closely related members of the same genus and have been successful in differentiating members of multiple bacterial genera

(Strunecký & al., 2023). Despite the production of a wide range of secondary metabolites by cyanobacteria, their molecular phylogenetic and evolutionary genetics have been less

explored, leading to challenges in identifying superior strains for bioactive compound production. Given the importance of molecular and evolutionary genetic studies in the context of cyanobacterial strains, and considering that limited research has been conducted in Iran regarding the use of HIP, STRR, and ERIC in the molecular phylogeny of these organisms. This study aims to investigate the molecular and phylogenetic study of heterocystous cyanobacteria isolated from the soils surrounding Alagol and Ajigol lakes. This region has been selected as an ideal environment for examining the diversity of heterocystous cyanobacterial taxa due to its dense and diverse cyanobacterial cover during the spring and summer. This study, which is being conducted for the first time on cyanobacterial strains isolated from the soils surrounding Alagol and Ajigol lakes, intends to amplify highly repetitive genes and represents one of the first investigations in Iran. It will not only lead to the construction of dendrograms based on molecular similarity and phylogenetic proximity of the strains, but also provide new evidence for understanding the phylogenetic relationships between strains. The use of genetic sequences has always been a reliable method for confirming and identifying microbial flora, as genetic sequences, unlike morphological traits, are never subject to change and consistently serve as a stable characteristic in the classification and identification of microbes.

MATERIALS AND METHODS

Collection, Culturing, Purification, and Phenotypic Analyses

Five different areas were selected for sampling, which were relatively large for the area, and the goal was to cover the entire area. Soil samples were randomly collected from the surface to a depth of 5 cm from the peripheral areas of Alagol and Ajigol lakes in Golestan Province. These lakes serve as permanent habitats for significant populations of rainbow trout. The collected soil samples were cultured in Z8 liquid medium (Kotai, 1972) and incubated in a growth chamber at temperatures ranging from 28 to 30°C, with a light intensity of 40 to 60 µmol photons per square meter per second (Nowruzi & Zakerfirouzabad, 2024).

To isolate and purify cyanobacteria, it was necessary to culture them on a solidified, selective medium. For this purpose, 10 grams of agar were added to each liter of Z8 liquid medium. After the medium solidified, a portion of each colony, differentiated by color, was transferred with a loop onto the solid medium in a zigzag pattern under sterile conditions in a laminar flow hood (Nowruzi & al., 2024b). To ensure purity, sub-culturing was carried out three to five times, ensuring that the cultures were completely pure. The incubation period ranged from one to four weeks. To confirm the purity of the strains, inoculation was performed on R2A LAB163 (R2A) medium, and after growth, the presence or absence of colonies around each inoculation point was checked (Sarchizian & Ardelean, 2010). The following parameters were selected to describe the morphology of cyanobacteria: morphology of vegetative cells (including terminal cells), heterocytes, akinetes; presence or absence of terminal heterocytes; and the shape of the filament and its aggregation in colonies according to Komárek, (2016).

Dry type material and voucher specimens were deposited into the ALBORZ herbarium, Cyanobacteria Culture Collection (CCC) at Science and Research Branch of the Islamic Azad University (Tehran, Iran (sweetgum.nybg.org/science/ih/herbarium_details.php ?irn=253911).

Identification Based on 16S rRNA Sequencing

For molecular identification of the genus, DNA extraction was performed manually using the phenol-chloroform method (Liu & al., 2022). To assess DNA quality, each qualitative analysis was conducted using gel electrophoresis, and quantitative analysis was performed with a NanoDrop spectrophotometer (Gaget & al. 2017). 16S rRNA gene was amplified utilizing a cyanobacteria-specific primer set (Nübel & al. 1997). Forward primer (359F, 5'-GGGGAATYTTCCGCAATGGG-3') and reverse primers (781Ra, 5'-GACTACTGGGGTATCTAATCCCATT-3'). One PCR reaction was comprised of 1 × Buffer solution, 0.5 µM forward primer, 0.25 µM of each reverse primer, and 0.5 U Taq polymerase, as well as sterile water and 1 µl of template DNA in a total volume of 20 µl. 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. The reactions were allowed to cool at 4°C. Sequencing of the amplified 16S rRNA gene segment was performed by cycle sequencing at Gene Iran Company.

Phylogenetic Tree Construction

For constructing the phylogenetic tree, *Gloeobacter violaceus* PCC 9601 was used as the root. A nucleotide BLAST search was performed to find similar sequences (27 strains) of the 16S rRNA gene in the GenBank database at NCBI.

Table 1: 16S rRNA gene sequences obtained in this study, along with other similar sequences retrieved from GenBank at NCBI. The new sequences of species were considered by asterisk.

Strains	References	Isolation source	Accession number
Gloeobacter violaceus PCC 9601	https://www.ncbi.nlm.nih.gov/nuccore/KC004020	wet calcareous rock, near Kastanienbaum, Switzerland	KC004020
Nostoc sp. 1981 *	https://www.ncbi.nlm.nih.gov/nuccore/OP279618	Soils of Alagol and Ajigol (Golestan Province)	OP279618
Nostoc sp. 1982 *	https://www.ncbi.nlm.nih.gov/nuccore/OP039567	Soils of Alagol and Ajigol (Golestan Province)	OP039567
Cylindrospermum moravicum strain	https://www.ncbi.nlm.nih.gov/nuccore/NR_125684	cave sediment	NR_125684
Cylindrospermum licheniforme CCALA	https://www.ncbi.nlm.nih.gov/nuccore/KF052610	Prairie soil. USA: Pyramid State Recreation Area, Illinois.	KF052610
Cylindrospermum licheniforme strain	https://www.ncbi.nlm.nih.gov/nuccore/NR_125686	prairie soil. USA: Pyramid State Recreation Area, Illinois.	NR_125686
Cylindrospermum pellucidum strain	https://www.ncbi.nlm.nih.gov/nuccore/NR_125683	Soil, Czech Republic: Dlouha Ves near Vodnany, South Bohemia.	NR_125683
Cylindrospermum marchicum strain	https://www.ncbi.nlm.nih.gov/nuccore/NR_125689	Seep wall, USA: Big Horn Seep, Grand Staircase-Escalante National	NR_125689
Cylindrospermum badium CCALA 1000	https://www.ncbi.nlm.nih.gov/nuccore/KF142524	Reclaimed coal mine soil, USA: Pyramid State Recreation Area, Illinois.	KF142524
Cylindrospermum badium strain CCALA	https://www.ncbi.nlm.nih.gov/nuccore/NR_125688	Reclaimed coal mine soil, USA: Pyramid State Recreation Area, Illinois.	NR_125688
Nostoc sp. CCAP 1453 35	https://www.ncbi.nlm.nih.gov/nuccore/HE974997	United Kingdom: England, Cambridgeshire	HE974997
Nostoc sp. AH-12	https://www.ncbi.nlm.nih.gov/nuccore/KC699844	Pakistan	KC699844
Nostoc. sp. FM177500 1	https://www.ncbi.nlm.nih.gov/nuccore/298103756	Lake Trasimeno	2LT05S03
Nostoc sp. CENA88	https://www.ncbi.nlm.nih.gov/nuccore/GQ259207	Brazil	GQ259207
Nostoc sp. MGL001	https://www.ncbi.nlm.nih.gov/nuccore/KX721474	water sample	KX721474
Nostoc sp. CENA543	https://www.ncbi.nlm.nih.gov/nuccore/KX458489	Alkaline-saline Lake, Salina 67 Mil, municipality of Aquidauana,	KX458489
Nostoc sp. CENA544	https://www.ncbi.nlm.nih.gov/nuccore/KX458490	Alkaline-saline Lake, Salina 67 Mil, municipality of Aquidauana,	KX458490
Nostoc sp. CENA536	https://www.ncbi.nlm.nih.gov/nuccore/KX458487	Alkaline-saline Lake, Salina Verde, municipality of Aquidauana,	KX458487
Nostoc sp. CENA548	https://www.ncbi.nlm.nih.gov/nuccore/KX458492	Alkaline-saline Lake, Salina Verde, municipality of Aquidauana,	KX458492
Nostoc sp. CENA535	https://www.ncbi.nlm.nih.gov/nuccore/KX458486	Alkaline-saline Lake, Salina Verde, municipality of Aquidauana,	KX458486
Nostoc sp. CENA511	https://www.ncbi.nlm.nih.gov/nuccore/KX458482	Alkaline-saline Lake, Salina Verde, municipality of Aquidauana,	KX458482
Nostoc sp. CENA524	https://www.ncbi.nlm.nih.gov/nuccore/KX458485	Alkaline-saline Lake, Salina Verde, municipality of Aquidauana,	KX458485
Nostoc sp. CENA514	https://www.ncbi.nlm.nih.gov/nuccore/KX458484	Alkaline-saline Lake, Salina Verde, municipality of Aquidauana,	KX458484
Nostoc entophytum	https://www.ncbi.nlm.nih.gov/nuccore/JN605002	soil, Golestan, Iran	JN605002
Nostoc sp.CENA175	https://www.ncbi.nlm.nih.gov/nuccore/KC695867	Soil, Brazil: Bertioga.	KC695867
Nostoc carneum BF2	https://www.ncbi.nlm.nih.gov/nuccore/GU396092	rice rhizosphere, India: Shivri, Lucknow, Uttar Pradesh.	GU396092
Cylindrospermum siamensis SAG 11 82	https://www.ncbi.nlm.nih.gov/nuccore/KM019950	River, Goettingen 37073, Germany	KM019950
Nostoc sp. Esp20	https://www.ncbi.nlm.nih.gov/nuccore/FJ661022	Soil, Mexico City, DF 04510, Mexico	FJ661022
Calothrix desertica PCC 7102	https://www.ncbi.nlm.nih.gov/nuccore/AF132779	Fresh water, Bloomington, IN 47405, USA	AF132779

Multiple sequence alignment of the 16S rRNA gene sequences obtained in this study, along with other similar sequences retrieved from GenBank, was carried out using the MAFFT program (Ver. 7). After selecting the best model, the phylogenetic trees were constructed using the IQ-TREE server, and the analysis was performed. The resulting tree was edited using the Fig Tree program.

Secondary Structure Analysis of the ITS Region

To visualize the secondary structure of the ITS region, the Mfold web server was used. The secondary structures for D1-D1' and Box-B were constructed, and the number of nucleotides, their distances, and the number of loops in each structure were analyzed (Nowruzi & al. 2023).

Amplification of Palindromic Sequences

The primers used for the ERIC sequences were (ERIC1A 5'-ATGTAAGCTCCTGGGGATTCAC-3') and (ERIC1B, 5'-AAGTAAGTGACTGGGGTGAGCG-3'). The PCR program consisted of an initial cycle at 95°C for 7 minutes, followed by 30 cycles (94°C for 1 minute, 52°C for 1 minute, 65°C for 8 minutes), and one cycle at 65°C for 16 minutes with a final incubation at 4°C for 30 minutes (Nowruzi & Fahimi, 2022). For amplification of the HIP sequences, the primers HIP-TG; 5'-GCGATCGCTG-3', HIP-GC; 5'-GCGATCGCGC-3', and HIP-CA; 5'-GCGATCGCCA-3' were used. The PCR program included an initial cycle at 95°C for 5 minutes, followed by 30 cycles (95°C for 30 seconds, 30°C for 30 seconds, 72°C for 60 seconds), and one cycle at 72°C for 5 minutes. For the amplification of the STRR1a primer (5'-CCARTCCCCARTCCCC-3'), the PCR program consisted of an initial cycle at 95°C for 6 minutes, followed by 30 cycles (94°C for 1 minute, 56°C for 1 minute, 65°C for 5 minutes), and one cycle at 65°C for 16 minutes with a final incubation at 4°C for 30 minutes (Nowruzi & Fahimi, 2022).

The PCR profiles of ERIC, HIP, and STRR primers were loaded onto agarose gels. The presence or absence of distinct and amplified bands in each DNA fingerprinting pattern generated by the ERIC, HIP, and STRR profiles was converted into binary data. This information was then used to construct a composite dendrogram (Nowruzi & Fahimi, 2022).

Amplification of the Functional Gene *rpo*C1

For the amplification of the functional gene *rpo*C1, all reactions were performed with 12.5 µL master mix, 1 µL forward primer (F), 1 µL reverse primer (R), 2 µL template DNA, and 8.5 µL deionized distilled water (DDW). The primers used for *rpo*C1 sequencing were (rpoF: 5'-TGGGGHGAAAGNACAYTNCCTAA-3') and (rpoR: 5'-GCAAANCGTCCNCCATCYAAYTGBA-3').

The PCR program included an initial denaturation step at 95°C for 15 minutes, followed by 40 cycles consisting of three stages: denaturation at 95°C for 30 seconds, primer annealing at 51°C for 30 seconds, and DNA extension at 72°C for 45 seconds. Finally, a final extension step was performed at 72°C for 10 minutes (Nowruzi & Shalygin, 2021).

RESULTS

Morphological Observations

The results of morphological analysis of cyanobacteria isolated from soil samples collected around Alagol and Ajigol lakes indicated the presence of purified strains belonging to the genus *Nostoc*, which is classified within the family Nostocaceae (Fig. 1). In fact, two strains examined in this study were the dominant cyanobacterial strains consistently found across all sampled locations. Although other cyanobacterial strains were also isolated and successfully cultured from the soil samples, these were neither as abundant nor as widespread as the two dominant strains. Due to the relatively low frequency of these other strains and considering the high costs associated with molecular analyses, only the two predominant strains were selected for further detailed molecular and phylogenetic investigations. A comprehensive list of all isolated strains is included in Table 2, but the focus was deliberately placed on the main strains representing the majority of the cyanobacterial community in the sampled soils.

Phylogenetic Analysis Results

The best-fit model determined for tree construction using the IQ-TREE server was TVM+F+I+G4. The numbers next to each branch node indicate bootstrap support values derived from 1000 replicates. Based on the constructed phylogenetic tree, each branch indicates the evolutionary relationships between taxa in terms of ancestry. Branch length reflects the degree of divergence from a common ancestor, essentially representing the number of nucleotide changes that have occurred along the branch. The phylogenetic tree constructed using the 16S rRNA gene showed concordance with the morphological identification of the two studied strains. Both strains were grouped in a separate clade with other *Nostoc* species, supported by a high bootstrap value of 83.2%. The two strains were designated as *Nostoc* sp. 1981 and *Nostoc* sp. 1982. According to the phylogenetic tree, the two strains were located together within the same clade, suggesting the necessity of additional analyses using ITS sequences, functional gene sequences, and DNA fingerprinting to differentiate between the two strains (Fig. 2).

Table 2: A complete list of all isolated strains collected around Alagol and Ajigol lakes.

Sampling site 1	Sampling site 2	Sampling site 3	Sampling site 4	Sampling site 5
Fischerella ambiqua	*Trichormus khannae*	*Nostoc punctifoeme*	*Nodularia harveyana*	*Calothrix stagnalis*
Nostoc muscorum	*Nostoc microscopicum*	*Nostoc muscorum*	*Nostoc spongiforme*	*Nostoc spongiforme*
Nodularia harveyana	*Nostoc muscorum*	*Nostoc ellipsosporum*	*Nostoc ellipsosporum*	*Nostoc muscorum*
Nostoc spongiforme	*Nostoc ellipsosporum*	*Anabaena oscillarioides*	*Nostoc muscorum*	
Stigonema minutum	*Nodularia harveyana*	*Nodularia harveyana*		
Aphanizomenon flosaquae	*Anabaena torulosa*	*Stigonema minutum*		
Scytonema ocellatum	*Fischerella ambiqua*	*Scytonema ocellatum*		
Anabaena variablilis	*Stigonema minutum*	*Fischerella ambiqua*		
Nostoc ellipsosporum	*Nostoc commune*	*Nostoc spongiforme*		
Trichormus naviculoides		*Nostoc commune*		
Cylindrospermum majus				
Anabaena circinalis				
Nostoc punctiforme				

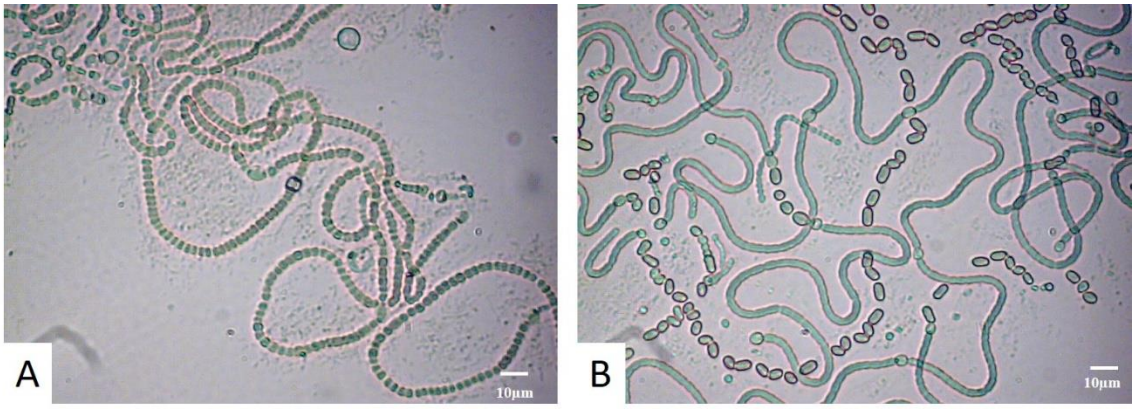

Fig. 1. A: *Nostoc* sp. 1981, the presence of spherical heterocysts in the middle of the filament and rounded heterocysts at the terminal ends is a characteristic feature of this strain. Additionally, the presence of bead-like akinetes arranged in succession is another defining trait of this genus. B: *Nostoc* sp. 1982, the presence of cylindrical heterocysts located both centrally and terminally along the filament, as well as vegetative cells with a length shorter than their width, are distinguishing characteristics of this strain.

Secondary structure analysis of ITS

The analysis of the ITS region was carried out to identify the D1-D1' helix, BOX B, and BOX A structural domains. The results of comparing the lengths of the ITS regions of the studied strains with other phylogenetically related strains (as identified in the phylogenetic tree) indicated that the length of the D1-D1' helix and BOX B regions differed from those of the related strains. Furthermore, the secondary structure modeling of the D1-D1' helix and BOX B yielded distinct results (Figs. 3 and 4). The two studied strains were similar in all examined characteristics, except for a difference in the number of loops within the D1-D1' helix. Therefore, further analysis using functional gene sequences and DNA fingerprinting remains necessary to distinguish between these two strains (Table 3).

Results obtained from the amplification of palindromic sequences

The electrophoresis gels resulting from the amplification of primers HIP-TG, HIP-GC, HIP-AT, HIP-CA, STRR1a, and ERIC1 for the two studied strains showed different results, which could help differentiate the two strains. The results regarding the presence or absence of palindromic primer bands in the two studied strains are presented in Table 4.

Table 3. Comparison of the ITS region lengths of the studied strains with other phylogenetically related strains identified in the phylogenetic tree.

	D1–D1 helix			Box-B helix		
Strains	**Total number of nucleotides**	**Number of loops**	**Base pairs in the stem**	**Total number of nucleotides**	**Number of loops**	**Base pairs in the stem**
Nostoc sp. 1981	99	7	4	29	2	6
Nostoc sp. 1982	99	8	4	29	2	6
Nostoc carneum BF2	70	6	4	27	2	6
Nostoc entophytum	99	8	4	25	2	5

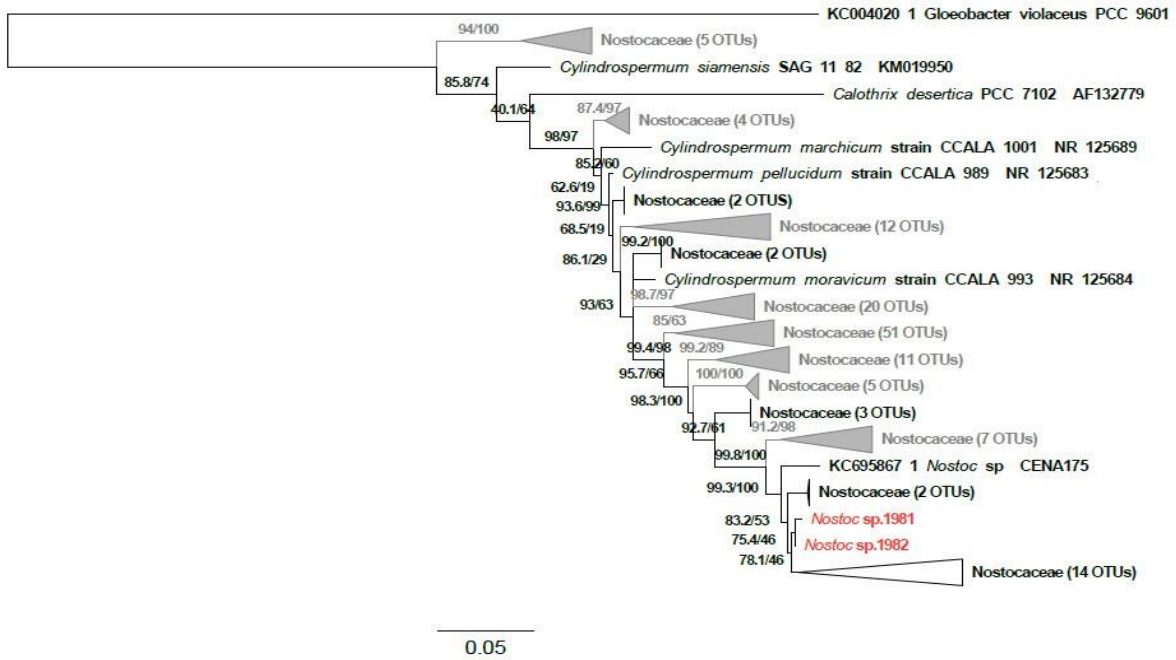

Fig. 2. Maximum likelihood phylogenetic tree constructed based on the 16S rRNA gene. The resulting scale bar of 0.05 represents the number of substitutions per nucleotide position. The best-fit evolutionary model selected for the 16S rRNA gene was TVM+F+I+G4. The numbers next to each branch node indicate bootstrap support values derived from 1000 replicates. Numbers near nodes indicate standard bootstrap support (%)/ultrafast bootstrap support (%) for ML analyses.

Results of *rpo*C1 gene amplification

The results of identifying and amplifying the functional gene *rpo*C1 in both studied strains yielded notable findings. The phylogenetic tree constructed based on the *rpo*C1 gene showed that the studied strains were placed in two distinct branches with strong bootstrap support of 100%. This indicates that the *rpo*C1 gene can serve as an effective marker for distinguishing between closely related strains (Fig. 5).

DISCUSSION

The rpoC1 gene is considered a better marker than 16S rRNA for distinguishing closely related genera and species due to its higher variability. However, the limited database coverage for functional gene sequences can reduce identification accuracy and lead to less precise results. (Singh & al. 2015). In 2018, Kabirnejad & al. used a polyphasic approach to identify heterocystous cyanobacteria from rice paddies in Mazandaran, Iran, combining morphology with 16S rRNA and functional gene sequences including tufA, rbcL, psbA, and rpoC1. They found that rpoC1 provided high resolution at the genus level, complementing and confirming 16S rRNA phylogeny.

Table 4. Presence and absence of palindromic primer bands in the two studied strains

	***Nostoc* sp. 1981**						***Nostoc* sp. 1982**					
	HIP-AT	HIP-CA	HIP-GC	HIP-TG	ERIC1A	STRR	HIP-AT	HIP-CA	HIP-GC	HIP-TG	ERIC1A	STRR
200-300					1						1	
300-400				1	1	1				1	1	1
400- 500		1	2		1	1		1	2		1	1
500- 650		1		1	1	1	2	1		1	1	1
650- 850	1	1	2	2	1	2	2				1	2
850-1000					1	1	2				1	
1000-1650	2				1	1	2				1	
1650-2000	2				1		2				1	
1900-2000	1						1					
2000-5000					1						1	
5000-12000	1	1	1	1	2	1	1	1	1	1	2	1
Total	7	4	5	5	11	8	12	3	3	3	11	6

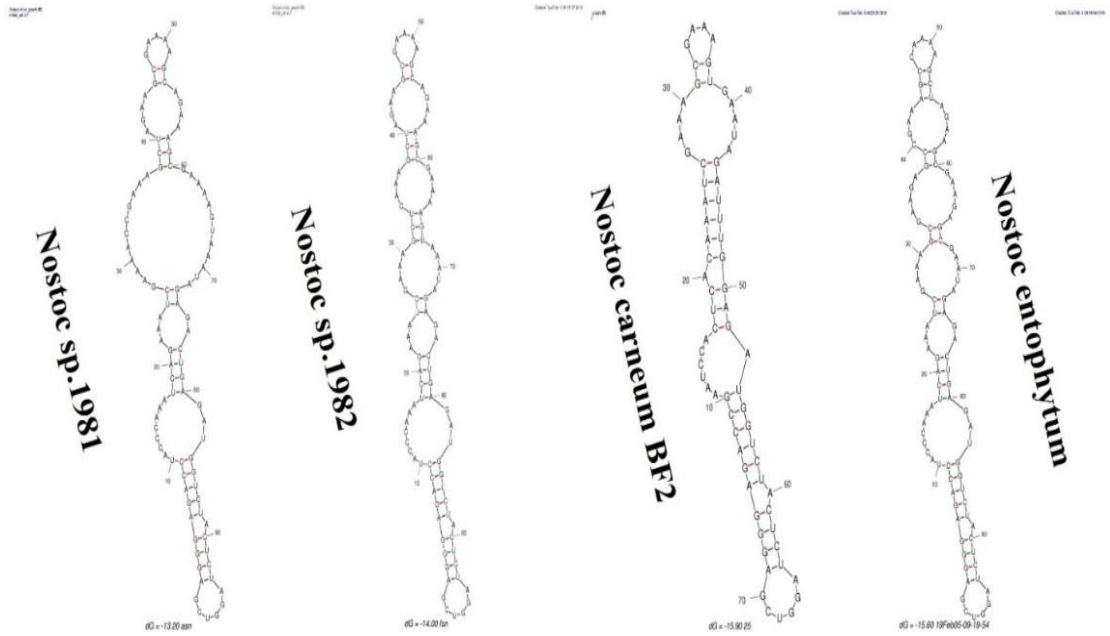

Fig. 3. D1-D1 helix structure of the two studied strains, along with GenBank strains.

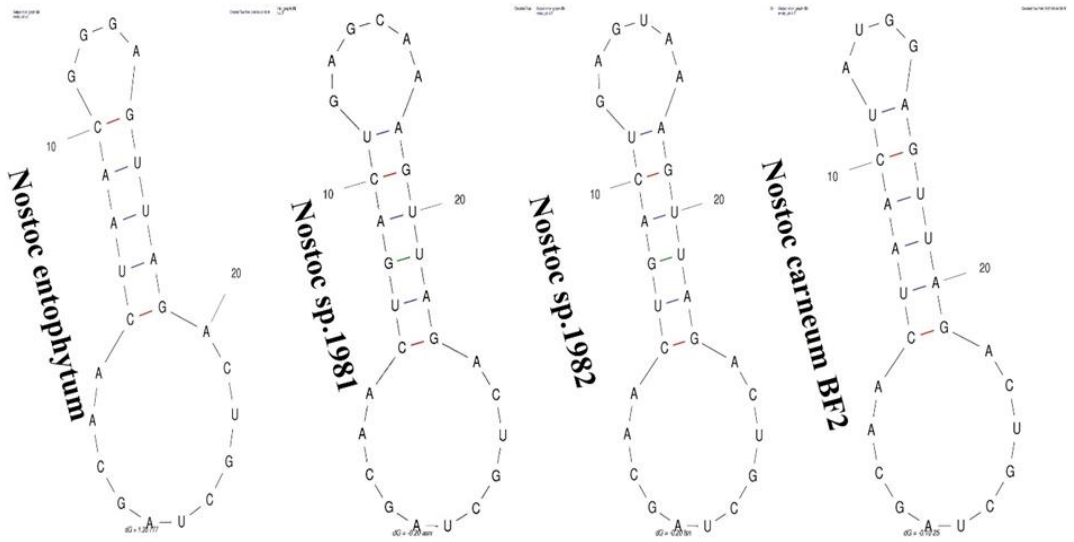

Fig. 4. BOX B helix structure of the two studied strains, along with GenBank strains.

In 2020, Kabirnejad & al. described new species of Aliinostoc, Desikacharya, and Desmonostoc using a polyphasic approach on five Nostoc-like cyanobacterial strains isolated from Mazandaran Province, Iran. The phylogenetic analysis of the rpoC1 gene grouped *Neowestiellopsis persica* A1387 with related strains, demonstrating high sequence similarity. The rpoC1 gene has greater discriminatory power at the species level than 16S rRNA and has been successfully used before (Nowruzi & al., 2024a). Integrating molecular data with bioinformatics and biostatistics is vital for advancing the understanding of evolutionary patterns. Mathematical modeling of DNA sequences using computational tools allows evaluation of evolutionary rates and reflects environmental and geographical variations. In this study, PCR-based molecular marker analysis was combined with biostatistics and bioinformatics, with secondary structure modeling via mfold, revealing that identical nucleotide sequences can yield different structures and loop configurations. In 2021, Rivandi & al. performed morphological and phylogenetic analyses on a toxic cyanobacterial strain from Lavāsān Lake using 16S rRNA and ITS markers. Similarly, the present study found differences between the two strains in the number of loops in the D1–D1′ region.

In 2017, Nowruzi & al. used a polyphasic approach to study two cyanobacterial strains from agricultural and freshwater habitats in Kermanshah Province, Iran. Molecular analysis, specifically 16S rRNA gene sequencing, revealed that the strains belonged to different phylogenetic clades, leading to their identification as *Calothrix* sp. N42 and *Scytonema* sp. N11.

The ITS sequence is significantly more variable than the 16S rRNA gene sequence and is widely used to differentiate various species within a genus. Bohunická & al. (2015) utilized the ITS sequence along with Box-B and V3 helices to distinguish four species within the genus *Roholtiella*. In the present study, as well, variable regions within the ITS were employed for the differentiation and identification of strains.

In 2021, Nowruzi & Shalygin identified a new cyanobacterial strain, *Dulcicalothrix alborzica*, from agricultural fields in Kermanshah Province using genetic markers rbcL, rpoC1, and 16S–23S ITS. This taxonomic placement was further confirmed by rbcL and rpoC1 markers, while secondary structure analysis of the 16S–23S ITS region revealed a unique structure distinguishing the *alborzica* strain from the other *Dulcicalothrix* species.

In 2009, Valério & al. used M13 PCR fingerprinting and ERIC-PCR to identify nine toxic *Cylindrospermopsis raciborskii* strains from freshwater sources in Portugal. Unlike the present study's analysis of two unidentified cyanobacterial samples based on PCR banding patterns, Valério & al. emphasized genomic fingerprinting to assess strain-level differentiation.

In 2016, Liaimer & al. used the STRR sequence to fingerprint *Nostoc* strains from plants and soil in Northern Norway, revealing significant genetic diversity and identifying toxic compounds and bioactive metabolites, but no antimicrobial activity was found. Their study highlights the need for multiple molecular markers to better understand *Nostoc* diversity and bioactivity, beyond what morphology alone can show.

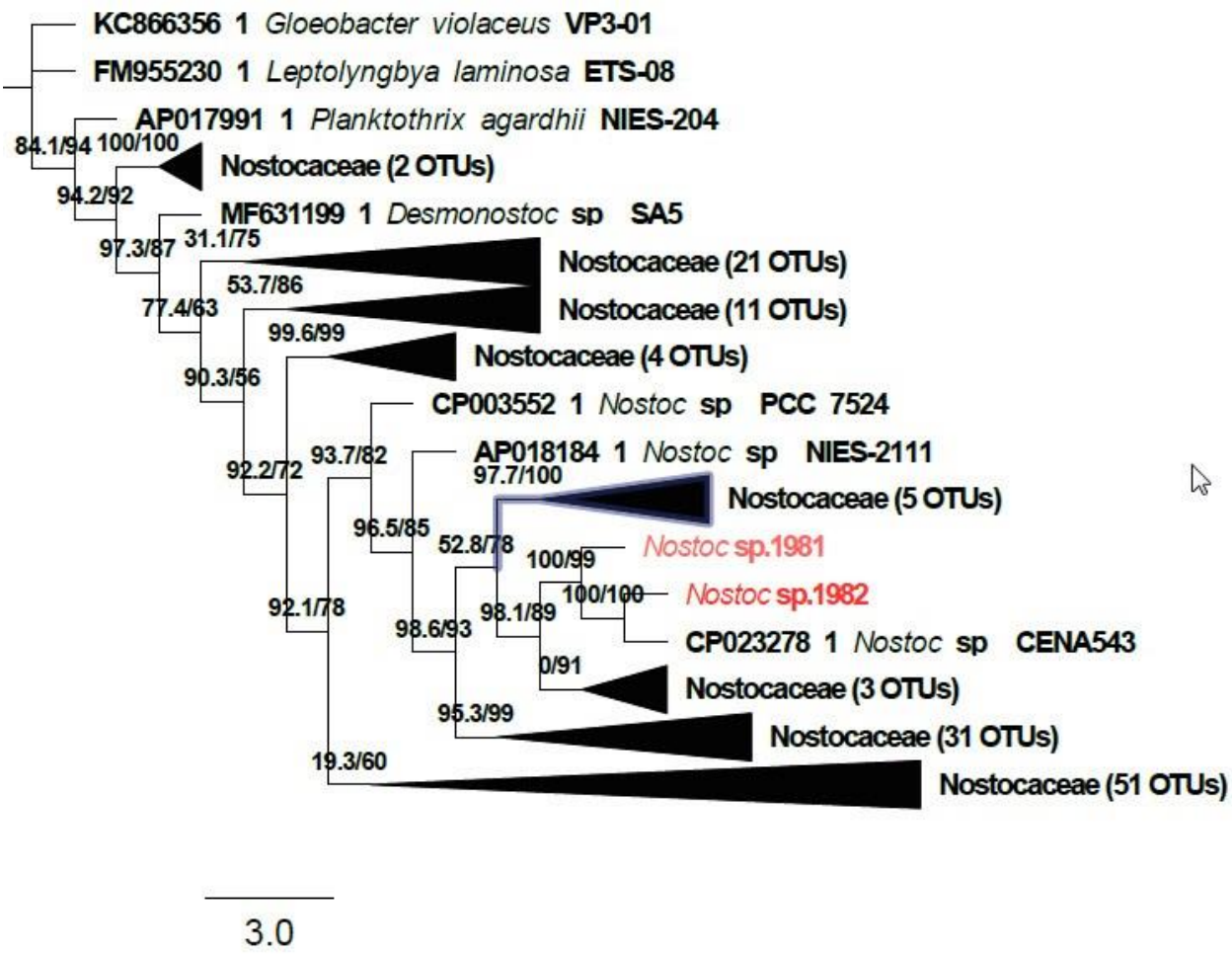

Fig. 5. Maximum likelihood phylogenetic tree based on the *rpo*C1 gene. The scale value of 10 indicates the number of mutations per nucleotide site. The best-fit model for the *rpo*C1 gene was determined to be TVMe + me + G4 + F. The numbers next to each branch node indicate bootstrap support values derived from 1000 replicates. Numbers near nodes indicate standard bootstrap support (%)/ultrafast bootstrap support (%) for ML analyses.

Although microscopic observations showed clear morphological differences between the two studied cyanobacterial strains, these traits alone are insufficient for accurate identification due to their variability and overlap among species. In this study, while the 16S rRNA gene phylogeny grouped both strains in a single clade, the rpoC1 gene analysis distinguished them into separate clades. This discrepancy underscores the limitations of relying solely on morphology or single-gene analysis and highlights the importance of using advanced molecular methods and comprehensive polyphasic approaches that integrate genetic, morphological, ecological, and biochemical data for more accurate and reliable cyanobacterial identification and diversity assessment.

REFERENCES

Allen, M.B. & Arnon, D.I. 1955: Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. -Plant Physiol. 30: 366. https://doi.org/10.1104/pp.30.4.366

Bohunická, M., Pietrasiak, N., Johansen, J.R., Gómez, E.B., Hauer, T., Gaysina, L.A. & Lukešová, A. 2015: Roholtiella, gen. nov. (Nostocales, Cyanobacteria)—a tapering and branching cyanobacterium of the family Nostocaceae. -Phytotaxa. 197: 84–103.

Gaget, V., Keulen, A., Lau, M., Monis, P. & Brookes, J. 2017: DNA extraction from benthic cyanobacteria: comparative assessment and optimization. -J. Appl. Microbiol. 122: 294-304.

https://doi.org/10.1111/jam.13332

Kabirnjad, S., Nematzadeh, G. A., Talebi, A. F., Saraf, A., Suradkar, A., Tabatabaei, M. & Singh, P. 2020: Description of novel species of *Aliinostoc*, *Desikacharya*, and *Desmonostoc* using a polyphasic approach. -Int. J. Syst. Evol. Microbiol. 70: 3413-3426. https://doi.org/10.1099/ijsem.0.004188

Kabirnejad, S., Nematzadeh, G.A., Talebi, A.F., Tabatabaei, M. & Singh, P. 2018: *Neowestiellopsis* gen. nov, a new genus of true branched cyanobacteria with the description of *Neowestiellopsis persica* sp. nov. and *Neowestiellopsis bilateralis* sp. nov., isolated from Iran. -Plant Syst. Evol. 304: 501-510. https://doi.org/10.1007/s00606-017-1488-6

Komárek, J. 2016: A polyphasic approach for the taxonomy of cyanobacteria: principles and applications. -Eur. J. Phycol. 51: 346-353. https://doi.org/10.1080/09670262.2016.1163738

Kotai, J. 1972. Instructions for the preparation of modified nutrient solution Z8 for algae. Norwegian Institute for Water Research, Oslo, 11(69): 5-11.

Liaimer, A., Jensen, J.B. & Dittmann, E. 2016: A Genetic and Chemical Perspective on Symbiotic Recruitment of Cyanobacteria of the Genus Nostoc into the Host Plant Blasia pusilla L. -Front. microbiol. 7: 161.169. https://doi.org/10.3389/fmicb.2016.01693.

Liu, A.W., Villar-briones, A., Luscombe, N.M. & Plessy, C. 2022. Automated phenol-chloroform extraction of high molecular weight genomic DNA for use in long-read single-molecule sequencing. *F1000Research*, 11**,** 240-261. https://doi.org/10.12688/f1000research.109251.1.

Nowruzi, B., Becerra-Absalón, I. & Metcalf, J.S. 2023: A novel microcystin-producing cyanobacterial species from the genus *Desmonostoc*, *Desmonostoc alborizicum* sp. nov., isolated from a water supply system of Iran. -Curr. Microbiol. 80: 49-61. https://doi.org/10.1007/s00284-022-03144-5

Nowruzi, B. & Fahimi, H. 2022: Fingerprinting and molecular phylogeny of some heterocystous cyanobacteria using 16S rRNA, ITS regions, and highly iterated palindromes as molecular markers. -Rostaniha. 23: 79-104.

Nowruzi, B., Fahimi, H. & Ordodari, N. 2017: Molecular phylogenetic and morphometric evaluation of *Calothrix* sp. N42 and *Scytonema* sp. N11. -Rostaniha. 18: 210-221.

Nowruzi, B., Ghazi, S., Norouzi, R. & Norouzi, R. 2024a: The impact of plasma-activated water on the process of nickel bioremediation by Neowestiellopsis persica A1387. -Ecotoxicol. Environ. Saf. 285: 117-128. https://doi.org/10.1016/j.ecoenv.2024.117101

Nowruzi, B., Hutarova, L., Vešelenyiova, D. & Metcalf, J.S. 2024b: Characterization of Neowestiellopsis persica A1387 (Hapalosiphonaceae) based on the cpc A, psb A, rpo C1, nif H and nif D gene sequences. -BMC Ecol. Evol. 24: 57-65. https://doi.org/10.1186/s12862-024-02244-z

Nowruzi, B., Khavari-nejad, R.-A., Nejadsattari, T., Sivonen, K. & Fewer, D. 2016: A proposal for the unification of two cyanobacterial strains of Nostoc as the same species. -Rostaniha. 17: 161-172.

Nowruzi, B. & Shalygin, S. 2021: Multiple phylogenies reveal a true taxonomic position of *Dulcicalothrix alborzica* sp. nov. (Nostocales, Cyanobacteria). -Fottea. 21: 235-246. https://doi.org/10.5507/fot.2021.008

Nowruzi, B. & Soares, F. 2021: *Alborzia kermanshahica* gen. nov., sp. nov. (Chroococcales, Cyanobacteria), isolated from paddy fields in Iran. -Int. J. Syst. Evol. Microbiol. 71-89. https://doi.org/10.1099/ijsem.0.004828

Nowruzi, B. & Zakerfirouzabad, M. 2024: Antifungal activity of *Neowestiellopsis persica* against *Rhizoctonia solani* in root and crown of Faba bean cultivated under modified BG-110 medium composition. -The Microbe. 4: 100-112.

Nübel, U., Garcia-Pichel, F., & Muyzer, G. 1997. PCR primers to amplify 16S rRNA genes from cyanobacteria. AFM, 63(8): 3327-3332. https://doi.org/10.1128/aem.63.8.3327-3332.1997

Rasouli-dogaheh, S., Komárek, J., Chatchawan, T. & Hauer, T. 2022: *Thainema* gen. nov.(Leptolyngbyaceae, Synechococcales): A new genus of simple trichal cyanobacteria isolated from a solar saltern environment in Thailand. -PLoS One. 17: 261-311. https://doi.org/10.1371/journal.pone.0261682

Rivandi, M., Nowruzi, B. & Fahimi, H. 2021: Molecular phylogenetic study of toxic cyanobacterium *Anabaena* sp. strain B3 isolated from Lavasan Lake, Tehran (Iran). -Rostaniha. 22: 120-133.

Ronquist, F., Teslenko, M., Van der mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., LIU, L., Suchard, M.A. & Huelsenbeck, J.P. 2012: MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. -Syst. Biol. 61: 539-542. https://doi.org/10.1093/sysbio/sys029

Sánchez-baracaldo, P., Bianchini, G., Wilson, J.D. & Knoll, A.H. 2022: Cyanobacteria and

biogeochemical cycles through Earth history. -Trends Microbiol. 30: 143-157. https://doi.org/10.1016/j.tim.2021.05.008

Sarchizian, I. & Ardelean, I. 2010: Axenic culture of a diazotrophic filamentous cyanobacterium isolated from mesothermal sulfurous springs (Obanul Mare-Mangalia). -Plant Biol. 55: 47-59.

Singh, P., Singh, S.S., Aboal, M. & Mishra, A. K. 2015: Decoding cyanobacterial phylogeny and molecular evolution using an evonumeric approach. -Protoplasma. 252: 519-535. https://doi.org/10.1007/s00709-014-0699-8

Strunecký, O., Ivanova, A.P. & Mareš, J. 2023: An updated classification of cyanobacterial orders and families based on phylogenomic and polyphasic analysis. -J. Phycol. 59: 12-51. https://doi.org/10.1111/jpy.13304

Valerio, E., Chambel, L., Paulino, S., Faria, N., Pereira, P., & Tenreiro, R. 2009: Molecular identification, typing, and traceability of cyanobacteria from freshwater reservoirs. -Microbiology, 155 (2): 642-656. https://doi.org/10.1099/mic.0.022848-0