MOLECULAR IDENTIFICATION OF TOXIC CYANOBACTERIA ISOLATED FROM TUSHAN LAKE, IRAN BASED ON *STRR*, *MCY*, *RPOC1*, AND *RBCL* GENES

BAHAREH NOWRUZI[1], HASSAN BEIRANVAND[2]

Abstract: Tushan Lake (Iran) is not only a popular public recreational site, especially for children during the warmer seasons, but due to its shallow depth, it has also become a place for swimming. Local reports of adverse effects following swimming in the lake – such as itching and skin blistering – among individuals exposed to the water in this area suggest the possible presence of cyanotoxins. Accordingly, this study aimed to screen for potentially toxigenic cyanobacterial strains in the Tushan Lake, Gorgan, by amplifying functional and structural genes along with palindromic sequences. Cluster analysis based on the amplification of the STRR gene in four cyanobacterial colonies revealed that colonies 1 and 3 shared 90% similarity. Colony 4, with 80% similarity, was grouped in the same clade with colonies 1 and 3. However, colony 2 was located in the most distant clade and exhibited the lowest similarity to the other samples. Therefore, all subsequent analyses – including the identification of pathogenic, structural and functional genes – were exclusively conducted on colony 2. Amplification results of *mcy* genes showed that only the *mcyG* gene was present in the strain under study. Phylogenetic tree construction based on the 16S rRNA gene demonstrated congruence with trees derived from *mcyG*, *rbcL* and *rpoC1* genes, placing the studied strain within the same clade as *Calothrix* sp. PCC_7715 with a bootstrap value of 71.8%. Analysis of the ITS region length and its structural motifs, including the D1-D1′ helix and BOX B region, in comparison with closely related strains from the phylogenetic tree, indicated distinct differences in both length and secondary structure. This study represents one of the first molecular phylogenetic investigations of toxic cyanobacteria in Tushan Lake, Gorgan.

Keywords: Cyanobacteria, Molecular Phylogeny, Structural Genes, 16S rRNA and ITS, Palindromic Sequences, STRR, *mcy*G, *rpo*C1, *rbc*L.

INTRODUCTION

Cyanobacteria are a group of photosynthetic prokaryotes that play a key role in the biological cycles of aquatic ecosystems. Some species of these microorganisms are capable of producing toxic secondary metabolites that can be

[1] Department of Biology, SR.C., Islamic Azad University, Tehran, Iran (*bahare77biol@gmail.com*)

[2] Department of Biology, SR.C., Islamic Azad University, Tehran, Iran

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dangerous for humans, animals, and other organisms residing in aquatic ecosystems (Moreira, Vasconcelos *et al.* 2022). These toxins, known as cyanotoxins, may have deleterious effects on public health and the environment, potentially causing issues such as water contamination, biodiversity loss and even mortality in aquatic organisms. Therefore, investigating these organisms and characterizing their genetic and phylogenetic traits is of critical importance (Semedo-Aguiar, Pereira-Leal *et al.* 2018, Kadiri, Isagba *et al.* 2020).

Blooms of pathogenic cyanobacteria have been observed not only in marine waters but also in freshwater systems, where they play a significant role in human and animal poisonings. Despite structural similarities among these blooms across different ecosystems, their toxic properties vary markedly depending on the producing species. Microcystin and nodularin production, as the most common toxins, is mainly reported by dominant species in freshwater blooms. Furthermore, these blooms are often accompanied by the concurrent presence of other cyanobacteria producing neurotoxins such as anatoxin-A and saxitoxin (Ash and Patterson 2022).

In recent years, PCR-based techniques have emerged as powerful tools in phylogenetic studies and genomic fingerprinting. These methods, which rely on DNA sequence polymorphisms and repetitive elements, enable more precise species differentiation and the investigation of evolutionary relationships. Consequently, such approaches have led to the development of new genetic classifications that, in some instances, may not align with traditional taxonomy or may even contradict it. The widespread use of molecular techniques in recent years for amplifying specific genomic regions to identify and infer phylogenetic relationships among cyanobacteria has increased considerably (Yuan and Yoon, 2021).

Short Tandemly Repeated Regions (STRR) are employed to generate strain-specific DNA profiles. These sequences are used both as primers in PCR reactions and as oligonucleotide probes and are highly effective even in non-axenic strains due to their strain-specific nature (Ali Anvar, Nowruzi *et al.* 2023).

Among housekeeping genes, the *rpoC1* gene, which encodes the gamma subunit of DNA-dependent RNA polymerase, is recognized as a valuable molecular marker in cyanobacterial phylogenetic studies. In these bacteria, the *rpoC1* and *rpoC2* genes are the functional equivalents of the *rpoC* gene in other bacteria and are transcribed separately (Yan, Feng *et al.* 2020). In cyanobacterial genomes, these genes are present in single copies, making the *rpoC1* gene a valuable molecular marker for identifying these microorganisms. The sequence of this gene can be readily and specifically amplified using PCR. Studies have shown that this gene has higher discriminatory power compared to the 16S rRNA gene and is capable of distinguishing closely related species, making it a suitable candidate for genus/species-level differentiation (Valério 2008, Nowruzi, Hutarova *et al.* 2024).

Another key gene in this context is *mcyG*, which plays a critical role in the biosynthetic pathway of microcystin, one of the most significant cyanotoxins. Microcystins are cyclic heptapeptides with a complex structure, predominantly produced by *Microcystis aeruginosa* (Zhou, Chen *et al.* 2021). Owing to their persistence in aquatic systems and toxic effects on humans and animals, these compounds have become a major environmental concern. Studies have demonstrated that inactivation of the *mcyG* gene results in the cessation of microcystin production, underscoring the biological importance of this gene in the regulation of toxicity (Yancey 2023, Wei, Hu *et al.* 2024).

In addition to the aforementioned genetic markers, functional genes such as *rbcL*, which encodes the large subunit of the key photosynthetic enzyme RuBisCO, are also considered powerful molecular tools for investigating the diversity and classification of phytoplankton, including cyanobacteria. Due to its appropriate evolutionary rate and widespread presence among photosynthetic organisms, *rbcL* sequencing offers high resolution for identification at the species and genus levels, thereby complementing data obtained from markers such as 16S rRNA. The use of this gene in studies of phytoplankton community structure across various aquatic ecosystems has demonstrated its efficacy as a robust molecular tool for ecological and taxonomic research on cyanobacteria (Zhang, Wang *et al.* 2024).

Tushan Lake in Gorgan is a shallow water body in northern Iran that, due to its specific environmental conditions and public usage – especially during warmer seasons – is highly susceptible to cyanobacterial proliferation and bloom formation. Local reports of symptoms such as itching, skin blistering, diarrhea, and vomiting among swimmers indicate the potential presence of cyanotoxins in these waters. This situation highlights the urgent need for a thorough investigation into the genetic and toxicological composition of the cyanobacterial community inhabiting this ecosystem.

This study aims to screen potentially toxic cyanobacterial strains from Tushan Lake, Gorgan, using molecular techniques based on the amplification of the STRR, *mcyG*, *rpoC1* and *rbcL* genes. The findings of this research may contribute to a better understanding of the genetic diversity and phylogenetic patterns of pathogenic cyanobacteria in this ecosystem and provide a foundation for more effective environmental monitoring and management strategies.

MATERIALS AND METHODS

SAMPLING AND CULTIVATION OF WATER SAMPLES

Water samples were collected from the surface to a depth of five centimeters at three distinct locations within Tushan Lake. The samples were immediately transported to the laboratory and inoculated into sterile Z8 medium. Petri dishes containing the

cyanobacterial inoculum were incubated in a growth chamber under continuous fluorescent light with an intensity of 40–60 µmol photons m^{-2} s^{-1} and a temperature of 28-30ºC for a period of 14 days (Kotai 1972, Nowruzi and Zakerfirouzabad 2024)

PREPARATION OF SOLID MEDIUM AND ISOLATION OF STRAINS

To isolate and purify heterocystous cyanobacteria, 10 grams of agar were added per liter of liquid Z8 medium. After the medium solidified, portions of cyanobacterial colonies exhibiting distinct pigmentation were streaked in a zigzag pattern on the surface. For more precise purification and elimination of microbial contaminants, the motile filament (hormogonium) identification method was employed. This physical trait of cyanobacteria – gliding motility across the surface of solid media – served as a biological criterion to distinguish them from other contaminants. To verify axenic culture status, samples were subsequently cultured in pre-prepared R2A medium (Nowruzi and Lorenzi 2024).

GENOMIC DNA EXTRACTION

Genomic DNA was extracted manually using the phenol–chloroform method (Baptista, Cunha *et al.* 2021) DNA concentration was measured using a NanoDrop spectrophotometer, and absorbance was recorded at wavelengths of 260 and 280 nm. The concentration was expressed in nanograms per microliter (ng/µL) (Gaget, Keulen *et al.* 2017). To assess DNA quality, the samples were electrophoresed on a 1% agarose gel (SeaPlaque® GTG®, Cambrex) prepared with 1X TAE buffer, diluted from a 50X TAE stock solution. The gel was visualized under UV light using a Gel Doc XR transilluminator (Bio-Rad), and DNA bands were analyzed with QUANTITY ONE® software version 4.6.7 (Green and Sambrook 2019, Baptista, Cunha *et al.* 2021).

STRR SEQUENCE ANALYSIS

The rep-PCR technique was employed to analyze the STRR1a genetic structure. The STRR1a primer with the sequence 5′-CCARTCCCCARTCCCC-3′ was used (Rasmussen and Svenning 1998). The PCR reaction was carried out in a final volume of 25 µL, comprising 12.5 µL of master mix, 0.5 µL of each primer, 2 µL of template DNA, and 9.5 µL PCR-grade water.

The STRR-PCR reactions began with an initial denaturation step at 95ºC for 6 minutes, followed by 30 cycles consisting of denaturation at 94ºC for 1 minute, annealing at 56ºC for 1 minute, and extension at 65ºC for 5 minutes. A final extension step was performed at 65ºC for 16 minutes (Singh, Kaushik *et al.* 2014).

Following PCR amplification, the PCR products were loaded onto an agarose gel along with an appropriate molecular marker. The band size ranges observed on the gel were carefully evaluated, and for each marker applied to different strains,

categorical divisions were established (*e.g.*, 300–400, 400–500 bp, etc.). The presence or absence of bands within each size range was recorded in a binary format (0/1). For the genomic fingerprinting analysis of highly repetitive palindromic sequences, the software Biodiversity Pro was utilized (Singh, Kaushik *et al.* 2014, Shokraei, Fahimi *et al.* 2019).

IDENTIFICATION OF PATHOGENIC CYANOBACTERIAL STRAINS BASED ON THE PRESENCE OF *MCY*, *RPOC1* AND *RBCL* GENES

Amplification of the *mcy* genes (*mcyG*, *mcyD* and *mcyE*), *rpoC1* and *rbcL* was performed using specific primers to identify pathogenic strains (Table 1). PCR reactions were conducted in a final volume of 25 µL, including 12.5 µL of master mix, 1 µL of each primer, 2 µL of template DNA, and 8.5 µL PCR-grade water.

PCR reactions were performed according to the thermal cycling program described by Nowruzi and Lorenzi (2021). For the *mcy* gene, an initial denaturation was carried out at 95ºC for 5 minutes, followed by 30 cycles consisting of denaturation at 95ºC for 30 seconds, annealing at 53ºC for 30 seconds, and extension at 72ºC for 1 minute. A final extension was performed at 72ºC for 5 minutes (Nowruzi, Bouaïcha *et al.* 2021).

The PCR protocol for the *rpoC1* gene began with an initial denaturation at 95ºC for 15 minutes. This was followed by 30 cycles comprising denaturation at 95ºC for 30 seconds, annealing at 51ºC for 30 seconds, and extension at 72ºC for 45 seconds. A final extension was conducted at 72ºC for 10 minutes (Rantala, Fewer *et al.* 2004). For the *rbcL* gene, the initial denaturation was performed at 94ºC for 5 minutes, followed by 30 cycles of denaturation at 92ºC for 1 minute, annealing at 55ºC for 1 minute, and extension at 72ºC for 2 minutes. A final extension step was carried out at 72ºC for 6 minutes (Singh, Fatma *et al.,* 2015).

Table 1

List of primers used for *mcy*, *rpoC1* and *rbcL* genes

Target gene/ sequence	**Sequence 5´ 3´**	**Reference**	**Amplicon size**
*mcy*GF *mcy*GR	GAAATTGGTGCGGGAACTGGAG TTTGAGCAACAATGATACTTTGCTG	(Fewer, Rouhiainen *et al.,* 2007)	247 bp
rpoC1	TGGGGHGAAAGNACAYTNCCTAA GCAAANCGTCCNCCATCYAAYTGBA	(Rantala, Fewer *et al.,* 2004)	723 bp
*rbcL*F *rbcL*R	GACTTCACCAAAGAYGACGAAAACAT GAACTCGAACTTRATYTCTTTCCA	(Singh, Fatma *et al.,* 2015)	950 bp
*mcy*DF *mcy*DR	GATCCGATTGAATTAGAAAG GTATTCCCCAAGATTGCC	(Rantala, Rajaniemi-Wacklin *et al.,* 2006)	818 bp
*mcy*EF *mcy*ER	GAAATTTGTGTAGAAGGTGC AATTCTAAAGCCCAAAGACG		812 bp

IDENTIFICATION BASED ON 16S RRNA AND ITS SEQUENCES

Following the identification of strains carrying pathogenicity-related genes, molecular identification was performed by amplifying the 16S rRNA and ITS gene regions. PCR reactions were carried out in 50 µL volumes and conducted in nine replicates (including three replicates per sample, one positive control, one negative control, and one contamination control). The reactions were performed using an iCycler thermal cycler (Bio-Rad) (Nowruzi and Shalygin, 2021).

The ITS region is considered a suitable marker for assessing intraspecific diversity among cyanobacteria, as its sequence exhibits considerably greater variability than the 16S rRNA gene (Nowruzi, Becerra-Absalón *et al.,* 2023).

The primers used for the PCR reactions are listed in Table 2. PCR comprised four cycles. The first cycle was the initial denaturation step at 94ºC for 5 minutes. The second cycle consisted of three steps: the first step was denaturation at 94ºC for 30 seconds; the second step was annealing, during which primers bind to the template DNA single strands, performed at 56ºC for 30 seconds; the third step was extension at 72ºC for 2 minutes. The third cycle, termed final extension, was conducted at 72ºC for 15 minutes. The fourth cycle, designated the final hold, was maintained at 4ºC.

Table 2

Primers used in the PCR reactions for ITS and 16S rRNA genes

27F1	(Neilan, Jacobs *et al.,* 1997) 5'- AGAGTTTGATCCTGGCTCAG (8-27)
23S30Ra	(Lepère, Wilmotte *et al.,* 2000) 5'- CTTCGCCTCTGTGTGCCTAGGT(30-52)
ITS16CF	(Iteman, Rippka *et al.,* 2000) CCATGGAAGYTGGTCAYG (1411-1428) (16S)
ITS23CR	(Iteman, Rippka *et al.,* 2000) CCTCTGTGTGCCTAGGTATCC (26-46) (23S)

SECONDARY STRUCTURE ANALYSIS OF ITS USING THE MFOLD PROGRAM

Secondary structure analysis of the ITS region was performed using the Mfold web server. The “RNA Folding Form” option was selected, and the target sequence was submitted. In the “STRUCTURE DRAW MODE” section, the “Untangle with loop fix” option was selected, followed by the “fold RNA” command. The secondary structures of the D1–D1′ and Box-B regions were visualized, and the number of nucleotides, their spacing, and the number of loops were analyzed (Nowruzi, Becerra-Absalón *et al.,* 2023).

PHYLOGENETIC TREE CONSTRUCTION

After confirming the accuracy and integrity of both forward and reverse sequences, pairwise alignment was conducted using the BioEdit program to construct

consensus sequences. Subsequently, BLASTN was used for the 16S rRNA and ITS genes, while BLASTX was employed for the *rpoC1* and *rbcL* genes. Following the retrieval and downloading of homologous sequences, multiple sequence alignment was performed using MAFFT version 7. For phylogenetic tree construction, appropriate evolutionary models were selected for each gene as follows: TVM+F+I+G4 for the 16S rRNA gene, TVMe+I+G4+F for *rpoC1*, HKY+G+F for *rbcL*, and TIM2+F+I+G4 for *mcyG*. Maximum Likelihood analysis was then conducted to construct the trees. Necessary adjustments and annotations were applied using the FigTree software. The robustness of phylogenetic relationships among taxa was assessed using bootstrap analysis with 1000 replicates (Nowruzi and Soares, 2021).

RESULTS

CULTURING RESULTS OF WATER SAMPLES IN Z8 MEDIUM

The various stages of cultivation, isolation, and purification of water samples collected from Tushan Lake in Gorgan are shown in Figures 1 and 2.

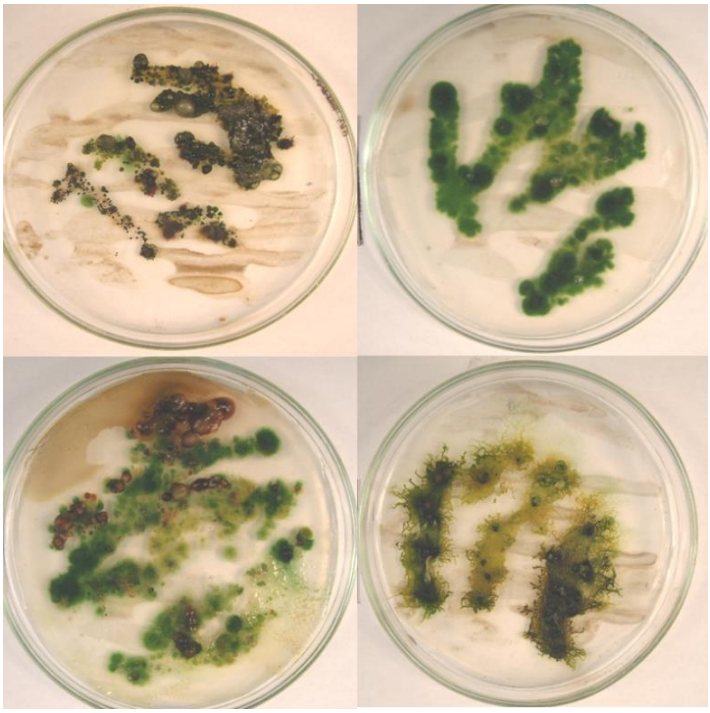

Figure 1. Cultivation of water samples on solid Z8 medium after two weeks. It should be noted that only a few Petri dishes are shown as representative samples.

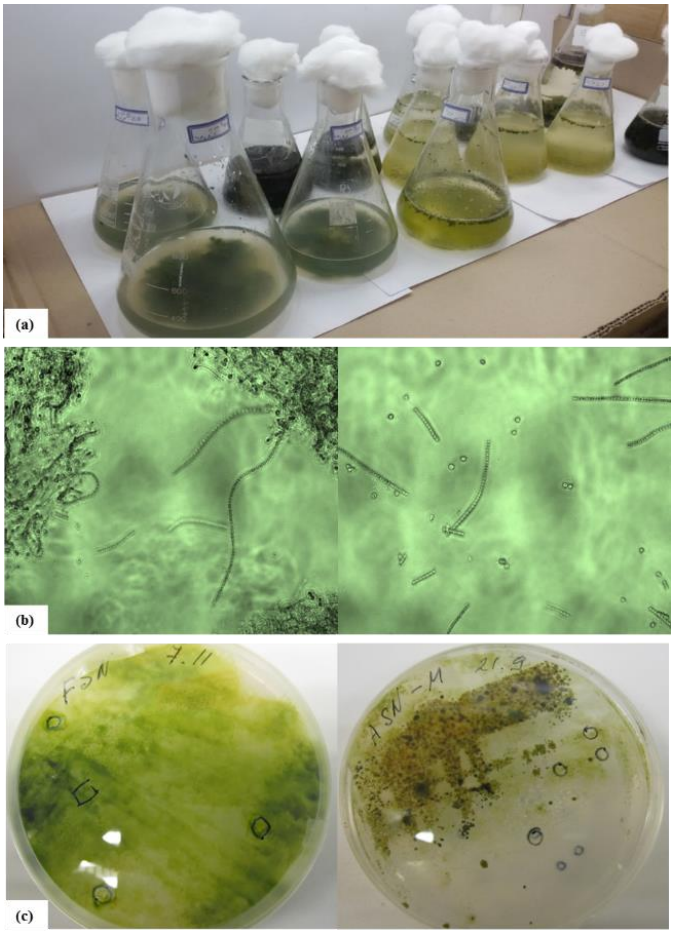

Figure 2. (a) Cultivation of purified colonies in liquid growth medium,
(b) purification process using hormogonia on agar medium, magnification 100×,
|(c) cultivation of purified samples on solid growth medium.

DNA CONCENTRATION DETERMINATION RESULTS

NanoDrop analysis indicated that the DNA purity of all samples fell within the standard range (Table 3).

Table 3

DNA concentration and purity measured using NanoDrop (ng/µL)

Colony	**Concentration (ng/µl)**	**A260 (AU)**	**A280 (AU)**	**A260/A280 Ratio**	**A260/A230 Ratio**
1	21.05	0.421	0.224	1.88	2

Table no.3 (continued)

2	141.81	2.836	1.508	1.88	2.09
3	46.29	0.92	0.49	1.89	2.2
4	258.31	5.166	2.739	1.89	2.14

RESULTS FROM STRR PRIMER DENDROGRAM

Electrophoresis gels resulting from the amplification of palindromic bands in four tested colonies are shown in Figure 3. Cluster analysis of the STRR results (Figure 4) revealed a 90% similarity between colonies 1 and 3. Colony 4 clustered with these two with 80% similarity, while colony 2 was placed in a separate, distant clade and exhibited the lowest similarity with the other samples. Additionally, the number of STRR-amplified bands in the four tested colonies was consistent with the cluster analysis results. Colonies 1 and 3 each exhibited 10 bands, colony 4 exhibited 9 bands, and colony 2 exhibited only 4 bands. Consequently, further analyses, including the identification of pathogenic genes and other structural and functional genes, were conducted exclusively on colony 2.

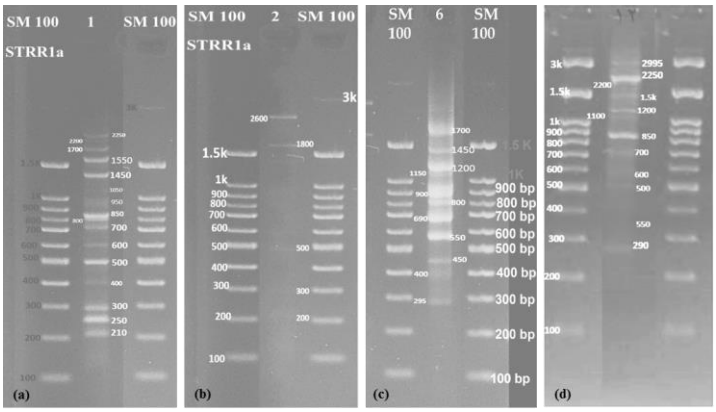

Figure 3. PCR electrophoresis gel using STRR primers. (a) Colony 1, (b) Colony 2, (c) Colony 3, (d) Colony 4. (Bands are in the size range of 100–3000 bp.)

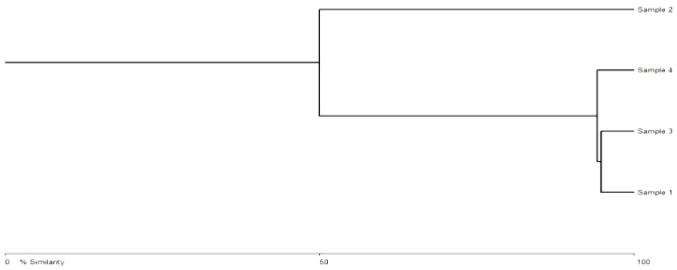

Figure 4. Dendrogram resulting from cluster analysis of the STRR gene in the four examined colonies.

IDENTIFICATION OF THE PATHOGENIC CYANOBACTERIAL STRAIN BASED ON THE PRESENCE OF THE *MCY* GENE

Amplification of the *mcyG*, *mcyD* and *mcyE* genes using their respective primers was performed to identify the pathogenic strain. Among the amplified genes, only the *mcyG* gene, with an approximate size of 247 bp, was detected. The results from the phylogenetic tree construction, based on BLAST analysis of shared sequences, were depicted in two distinct trees.

As shown in Figure 5, the detected gene in the studied strain belongs to the group of non-ribosomal peptide synthetase (NRPS) genes. This is because polyketide synthase (PKS) genes encoding microcystins are positioned in a separate cluster. Since the *mcyG* gene is a hybrid of both NRPS and PKS genes, and the amplified region comprises only 247 base pairs of this gene, its synthesis is thus attributed to non-ribosomal peptide synthetases.

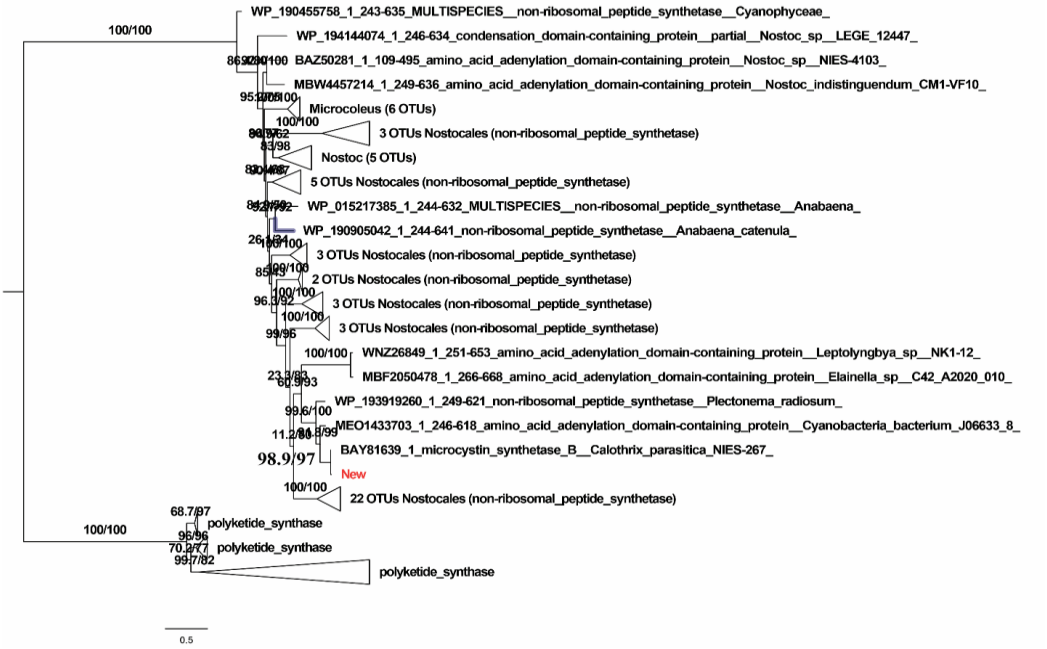

Figure 5. Phylogenetic trees based on the *mcy* gene, constructed using NRPS and PKS genes. The resulting scale of 0.05 indicates the number of nucleotide substitutions per site.

ELECTROPHORESIS RESULTS OF THE PCR PRODUCT OF THE 16S RRNA GENE

Following the identification of the pathogenic strain harboring the *mcyG* gene, molecular identification was carried out using amplification of the ITS and 16S rRNA genes. The results demonstrated bands of approximately 500 bp and

1500 bp for the ITS and 16S rRNA genes, respectively. Both the DNA band and the PCR product band exhibited high quality, which ensures successful sequencing of the amplified products.

The phylogenetic tree constructed using the 16S rRNA gene showed a correlation with the tree derived from the *mcyG* gene. The studied strain clustered with *Calothrix* sp. PCC_7715 (KM019959) within the same clade, exhibiting phylogenetic affinity with a bootstrap support of 85.7%. This result confirms the genus identity of the studied strain (Figure 6).

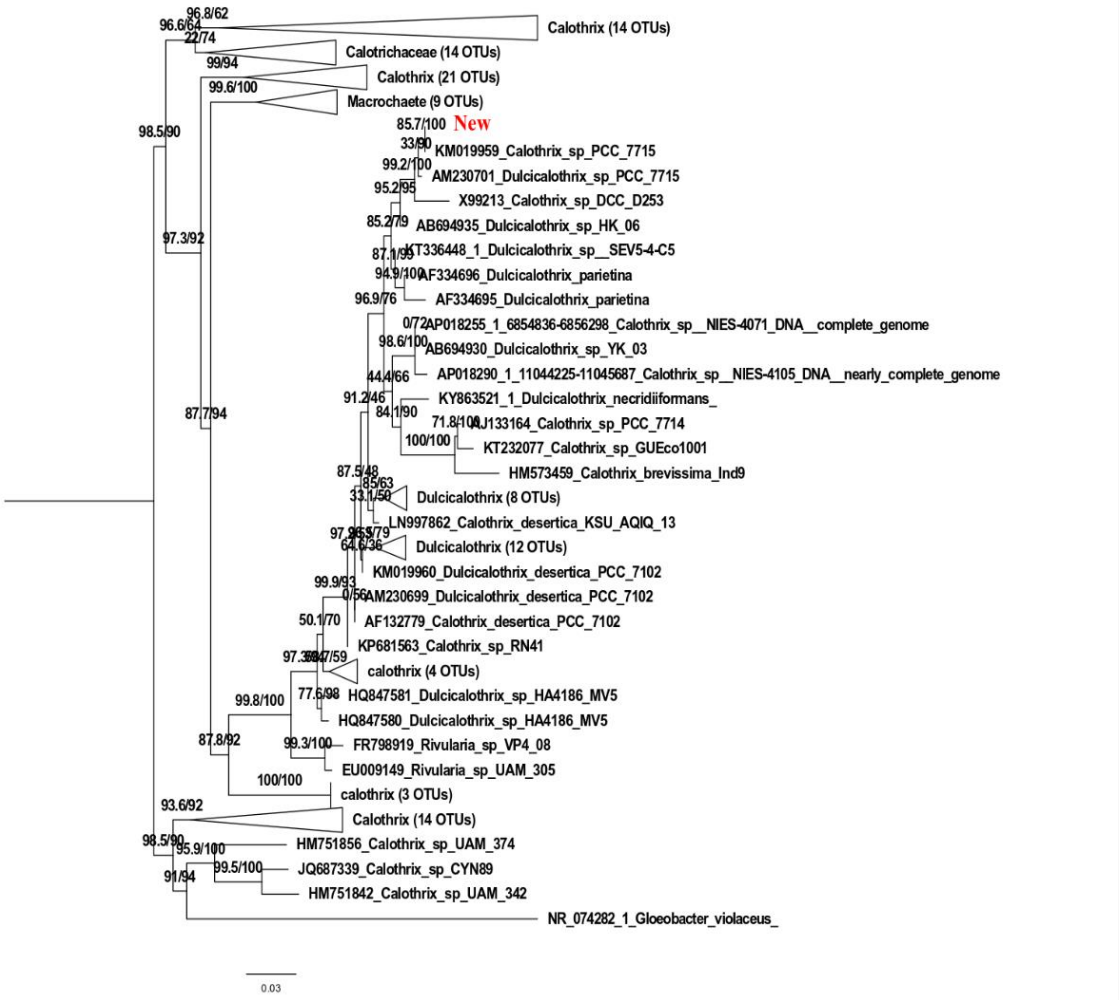

Figure 6. Phylogenetic tree constructed based on the 16S rRNA gene. The resulting scale of 0.03 indicates the number of nucleotide substitutions *per* site.

RESULTS OF THE IDENTIFICATION OF THE STUDIED STRAIN

The morphological analysis of the strain confirmed its classification within the genus *Calothrix*. The characteristic features identified for this genus are illustrated in Figure 7.

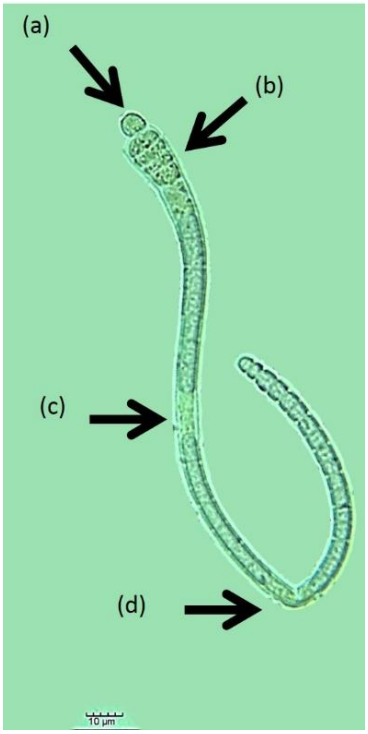

Figure 7. Light microscopy image of the studied strain at 10 µm magnification. (a) Hemispherical heterocyst located at the terminal end of the trichome; (b) Cylindrical akinetes adjacent to the heterocysts, aligned in series; (c) Formation of hormogonia in the trichome, with fragmentation facilitated by necridial cell formation for reproduction; (d) Tapering at the terminal end of the trichome, a defining feature of the genus *Calothrix*.

SECONDARY STRUCTURE ANALYSIS OF ITS USING THE MFOLD PROGRAM

The ITS region was analyzed to identify the helical regions: D1-D1 (yellow), D2 (red), D3 (pink), *trnI* gene, *trnA* gene (blue), BOX B (green), and BOX A (turquoise). The comparative analysis of ITS region lengths between the studied strain and other phylogenetically related strains revealed differences in the lengths of the D1-D1 helix and BOX B regions. Additionally, the predicted secondary structures of these regions (D1-D1 helix and BOX B) displayed structural variations (Figures 8 and 9, Tables 4 and 5).

Table 4

Comparison of ITS region lengths between the studied strain and phylogenetically related strains

Studied strain and related sequences	D1-D1' helix	D2 with spacer	D3 with spacer	trRNA^{Ile} gene	TrRNA^{Ala} gene	Pere BOX B spacer	BOX B	Post BOX B spacer	BOX A
Studied strain	71	20	11	74	73	137	30	15	11
Calothrix parietina	72	21	10	74	73	77	34	18	11
Calothrix sp. HA4395-MV3	99	20	11	74	73	78	34	17	11
Calothrix sp. Asko	61	-	-	74	76	31	27	17	11

Table 5

Comparison of ITS secondary structures (D1-D1 helix and BOX B) between the studied strain and phylogenetically related strains

Studied strain and related sequences	**D1-D1' helix**							**BOX B**			
	Terminal Bilateral Bulge (A)	Bilateral Bulge (B)		Unilateral Bulge (C)		Basal Bilateral Bulge (D	Basal Unilateral Bulge (E)	Terminal Base (F)	Terminal Bilateral Bulge (A)	Bilateral Bulge (B)	Unilateral Bulge (C)
	Nucleotide count	Loop count	Total Number of Nucleotides	Loop count	Total Number of Nucleotides	Nucleotide count					
New strain	6	1	12	1	5	13	12	11	13	7	-
Calothrix parietina	6	1	12	-	-	13	12	11	6	7	5
Calothrix sp. HA4395-MV3	6	-	-	-	-	20	26	13	6	-	-
Calothrix sp. Asko	6	1	11	1	5	13	11	9	7	7	-

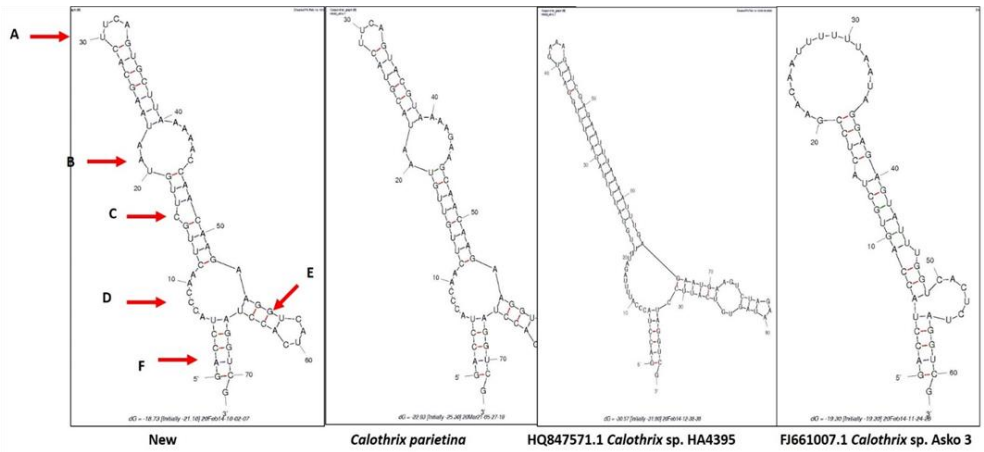

Figure 8. Identification of the various regions of the D1-D1′ helix: A: Terminal Bilateral Bulge; B: Bilateral Bulge; C: Unilateral Bulge; D: Basal Bilateral Bulge; E: terminal unilateral Bulge; F: terminal base.

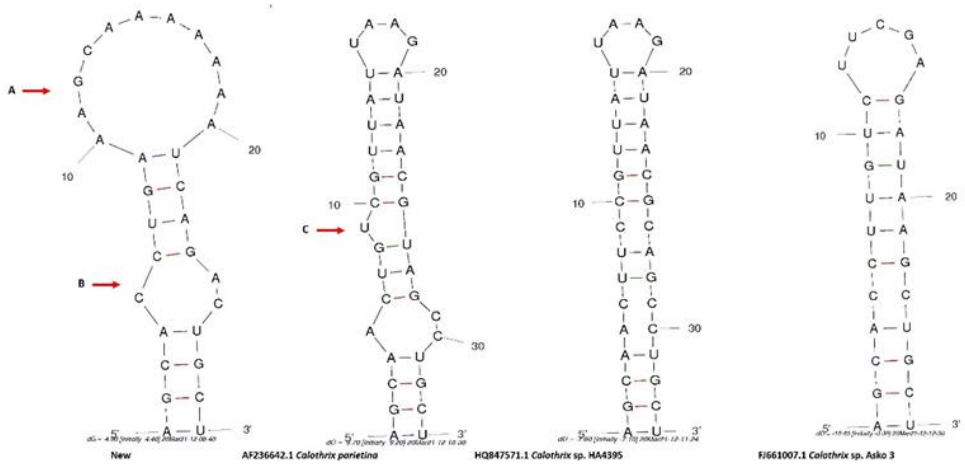

Figure 9. Identification of various regions of the BOX B helix: D1-D1′ helix, A: terminal bilateral bulge; B: bilateral bulge; C: unilateral bulge.

IDENTIFICATION OF THE PRESENCE OR ABSENCE OF *RPOC1* AND *RBCL* GENES IN THE PATHOGENIC STRAIN

The results of the identification of the structural genes *rpoC1* and *rbcL* were clearly observable on the electrophoresis gel. Phylogenetic analysis based on the *rbcL* gene revealed that the studied strain clustered with *Dulcicalothrix*

necridiiformans V13, *Calothrix desertica* PCC 7102, *Calothrix* sp. NIES-4105, and *Calothrix* sp. NIES-4071. However, it also formed a distinct clade with high phylogenetic relatedness, supported by a bootstrap value of 99.9% (Figure 10).

Similarly, phylogenetic analysis based on the *rpoC1* gene showed that the studied strain clustered with *Calothrix* NIES-4105 (GenBank accession: AP018290) and *Calothrix* NIES-4071 (GenBank accession: AP018255). Despite this, it also formed a separate branch with high phylogenetic affinity, supported by a bootstrap value of 99.7% (Figure 11).

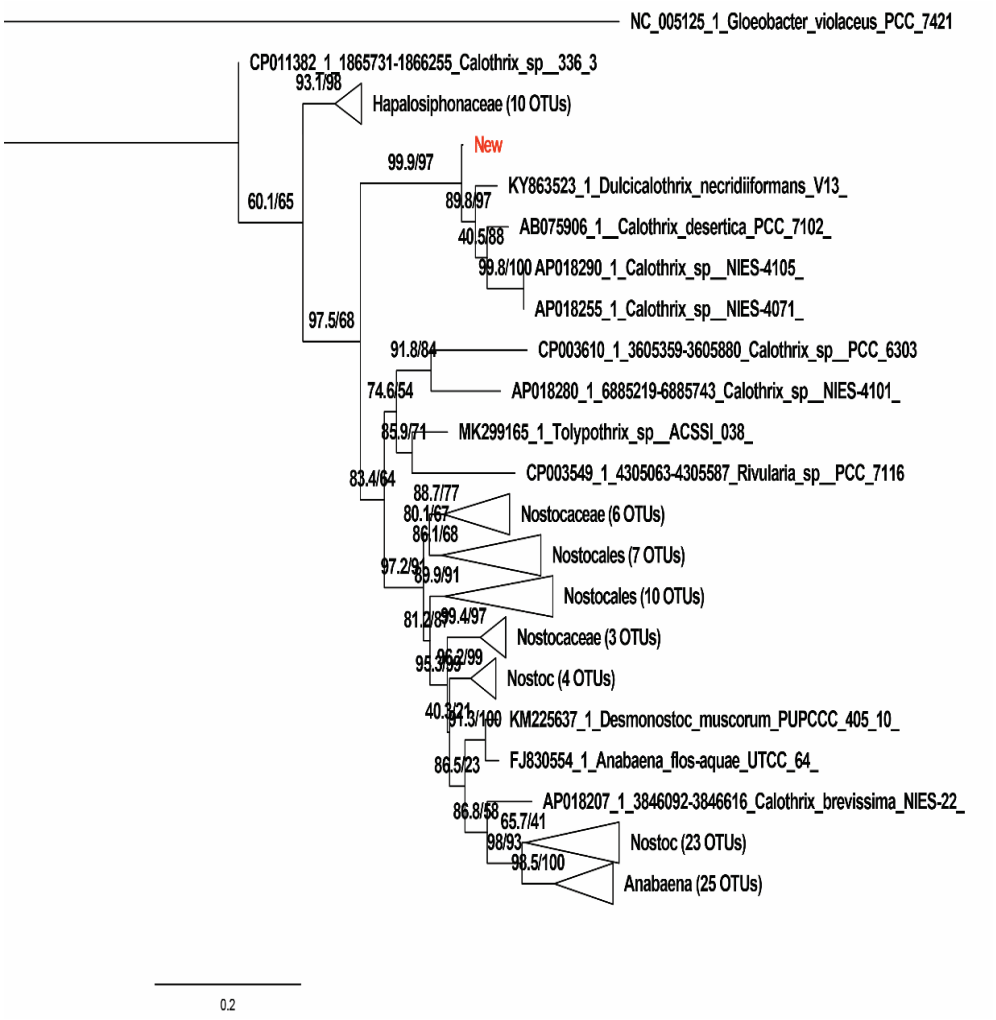

Figure 10. Phylogenetic tree constructed using the *rbcL* gene. The resulting scale of 0.2 indicates the number of nucleotide substitutions *per* site.

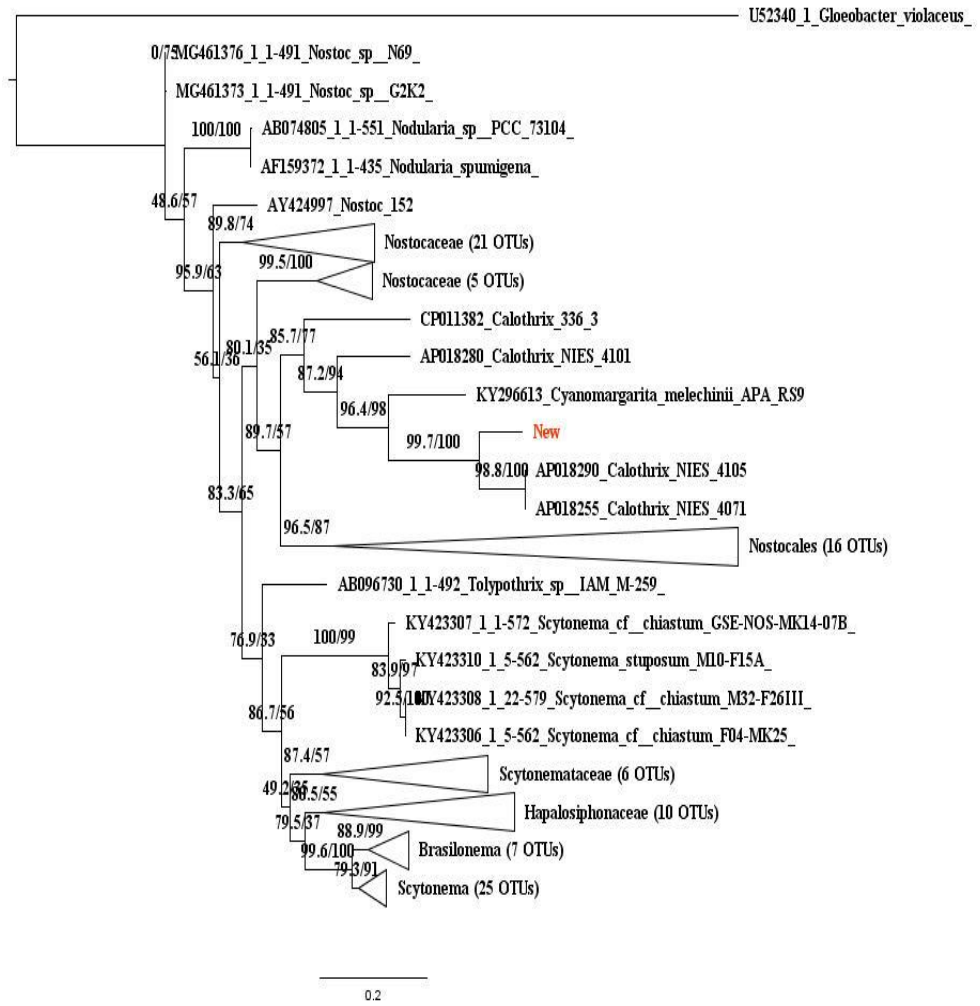

Figure 11. Phylogenetic tree constructed using the *rpoC1* gene. The resulting scale of 0.2 indicates the number of nucleotide substitutions *per* site.

DISCUSSION

The morphological diversity of cyanobacteria in culture conditions presents numerous challenges for their taxonomic classification. Investigations of isolates grown in different culture media using classical identification keys, such as dichotomous keys and morphological characteristics of type specimens, have

encountered substantial difficulties due to variability during experiments and the instability of certain identification features (Dvořák, Jahodářová *et al.* 2023).

Many identifications based solely on morphological features are often superficial and inaccurate, leading to frequent errors in laboratory studies (Komárek, 2014). Studies have shown that even under similar culture conditions, identical strains have been identified differently by various researchers. Accurate taxonomy should integrate morphological characteristics with polyphasic approaches, encompassing phenotypic, chemotaxonomic, and genotypic data. Consequently, the morphological variation among cyanobacterial strains and the issues arising in their molecular phylogeny have led researchers to adopt molecular techniques that focus on genetic sequences using gene markers. This shift is necessitated by the fact that many morphological characteristics are heavily influenced by environmental conditions and cannot be reliably used as taxonomic indicators (Komárek 2014, Teneva, Belkinova *et al.,* 2019).

In the present study, we applied a polyphasic approach to investigate a toxic cyanobacterial strain isolated from Tushan Lake in Gorgan (Golestan Province). This approach incorporated morphological characterization and molecular analyses utilizing short tandem repeat regions (STRR), structural genes (16S rRNA and ITS), and functional genes (*rbcL* and *rpoC1*).

Genomic fingerprinting using the STRR1a primer demonstrated genetic heterogeneity among the tested colonies. The dendrogram generated from STRR cluster analysis clearly highlighted differences between colony number two and the other colonies. This led to the selection of colony number two for further analyses, including the identification of pathogenic and other structural and functional genes.

Valerio *et al.* (2009) reported a significant correlation between STRR-based fingerprinting clusters and the taxonomic classification of cyanobacteria, emphasizing the utility of this method for strain identification and typing. In general, palindromic STRR sequences are considered powerful molecular markers for revealing genomic relationships and distinguishing between cyanobacterial strains (Valerio, Chambel *et al.* 2009).

In this study, we examined the presence of microcystin biosynthesis-related genes (*mcyG*, *mcyD* and *mcyE*) in colony number two. Among these, only the *mcyG* gene, approximately 247 bp in length, was detected. The phylogenetic tree based on *mcyG* showed that the identified gene in the studied strain belonged to the nonribosomal peptide synthetase (NRPS) gene cluster. Although *mcyG* is a hybrid gene composed of nonribosomal peptide synthetase and polyketide synthase elements, the amplified 247 bp fragment confirmed the presence of the NRPS portion. Further phylogenetic analysis using NRPS genes revealed that the studied strain clustered with *Calothrix parasitica* NIES-267, a known microcystin synthetase-producing strain, with 89.9% phylogenetic similarity. This finding indicates the potential of the isolated strain from Tushan Lake to produce microcystin, although further confirmation through additional investigations is required.

To achieve more precise molecular identification of the strain, amplification of the 16S rRNA and ITS gene regions was performed. The expected bands of approximately 1500 bp for 16S rRNA and about 500 bp for ITS were observed on the electrophoresis gel, indicating high DNA quality and successful PCR amplification. The 16S rRNA gene is recognized as a valuable molecular marker for cyanobacterial identification. Studies have demonstrated that this gene offers high resolution for discriminating between genera and higher taxonomic levels within cyanobacteria. While generally less variable than ITS, it provides a robust framework for broad phylogenetic relationships. The phylogenetic tree derived from 16S rRNA showed consistency with the *mcyG*-based tree, placing the studied strain in a clade with *Calothrix* sp. PCC 7715, supported by a bootstrap value of 85.7%, thereby confirming the genus-level classification of the studied strain. Other studies have also employed 16S rRNA gene sequences for distinguishing between cyanobacterial genera.

Indeed, both partial and full sequences of the 16S rRNA gene provide valuable insights for determining relationships among prokaryotic organisms. Previous research has shown that, in many cases, sequence-based rRNA information corresponds well with traditional taxonomy based on morphological analysis in cyanobacteria.

In the study by Sihvonen et al, the 16S rRNA gene sequence was employed for the differentiation of various cyanobacterial genera. By analyzing 42 cyanobacterial cultures belonging to the morphologically similar genera *Calothrix*, *Rivularia*, *Gloeotrichia* and *Tolypothrix*, they found that these genera do not form a monophyletic group and exhibit significant genetic diversity (Sihvonen, Lyra *et al.* 2007). The results of this study indicate that the 16S rRNA gene is an effective marker for genus-level discrimination in cyanobacteria.

The Internal Transcribed Spacer (ITS) region is a suitable marker for studying intraspecific diversity among cyanobacteria. The ITS sequence is much more variable than the 16S rRNA gene and has been widely used for distinguishing species within a genus. For example, Rehakova *et al.*, (2007) used variations in ITS secondary structure to compare *Nostoc commune* and *Nostoc punctiforme* (Řeháková, Johansen *et al.* 2007). Similarly, Bohunicka *et al*., (2015) ITS sequences and Box-B and V3 helices to identify four species within the genus *Roholtiella* (Bohunická, Pietrasiak *et al.* 2015). In the present study, variable regions within the ITS sequence were also used to further differentiate the toxin-producing strain isolated from Tushan Lake. While the B-box region showed no variation among the isolated strains, the D1–D1′ helix exhibited significant differences, enabling discrimination among the studied strains.

In addition, numerous studies have employed structural and functional genes for identifying new genera and species. For instance, Martins et al, (2016) described a novel genus *Ancylothrix* gen. nov. in the family Phormidiaceae based on polyphasic analyses using D1–D1′, box-B, and V3 secondary structure regions (Martins, Rigonato *et al.* 2016). Cai *et al.*, (2018) reported a new species

Desmonostoc danxiaense sp. nov. from the order Nostocales, isolated from the Danxia mountains in China (Cai, Yang *et al.* 2018). Villanueva *et al.* (2018) identified two novel cyanobacterial strains – *Brasilonema geniculatum* and *Calothrix dumus* – from limestone rocks in Florida, USA (Villanueva, Hašler *et al.* 2018). Mahansaria *et al.* (2018) reported a new genus *Oxynema aestuarii* from India, while Vaz *et al.* (2015) introduced two new genera, *Pantanalinema* gen. nov. and *Alkalinema* gen. nov., from a saline and alkaline lake. Jahodarova *et al.* (2018) described *Elainella* gen. nov., and Gonzalez-Resendiz *et al.* (2018) identified two new *Phyllonema* species in the family Rivulariaceae, all based on 16S rRNA and ITS gene sequences (Vaz, Genuario *et al.* 2015, González-Resendiz, Johansen *et al.* 2018, Jahodářová, Dvořák *et al.*, 2018, Mahansaria, Jaisankar *et al.* 2018).

Moreover, Brito *et al.* (2017) used 16S rRNA, ITS, and 23S rRNA regions to identify a new genus of the order Pleurocapsales, *Hyella patelloides* LEGE 07179, from the Atlantic coast (Brito, Ramos *et al.,* 2017). Similarly, 16S rRNA and ITS gene regions were used to review the genus *Geitlerinema* and describe the new genus *Anagnostidinema* gen. nov. (Oscillatoriophycidae, Cyanobacteria) (Johansen, Strunecký *et al.,* 2017).

Nowruzi *et al.* (2017), through a polyphasic approach, investigated morphological and genotypic differences between two cyanobacterial strains isolated from agricultural fields and freshwater sources in Kermanshah Province. The identification and classification of both *Calothrix* sp. N42 and *Scytonema* sp. N11 were challenging and confusing. Morphologically, both strains appeared to be *Calothrix*, as their initial filaments were unbranched and narrow; however, branching occurred upon further growth. Consequently, molecular analysis was performed. Phylogenetic comparison of 16S rRNA gene sequences revealed that the two strains belonged to distinct phylogenetic clades and were identified as *Calothrix* sp. N42 and *Scytonema* sp. N11, respectively (Nowruzi, Fahimi *et al.,* 2017).

In the present study, functional genes *rpoC1* and *rbcL* were also examined. The *rpoC1* gene, encoding the γ-subunit of DNA-dependent RNA polymerase, is a valuable molecular marker in cyanobacterial phylogenetic studies and enables the discrimination of closely related species. The *rbcL* gene, encoding the large subunit of the RuBisCO enzyme, is also considered a powerful molecular tool for studying diversity and classification in cyanobacteria, providing high resolution at the genus and species levels. Electrophoresis results confirmed the presence of expected bands for *rpoC1* (~723 bp) and *rbcL* (~950 bp) genes. The phylogenetic tree based on the *rbcL* gene showed that the studied strain clustered with various strains of *Dulcicalothrix* and *Calothrix*, yet formed a distinct branch with strong bootstrap support. Similarly, the *rpoC1*-based phylogenetic tree revealed that the strain clustered with *Calothrix* strains, but again formed a separate, well-supported branch. These findings indicate that the *rpoC1* and *rbcL* genes also contribute to differentiating the studied strain, in agreement with the results based on the structural genes 16S rRNA and ITS.

Nowruzi and Shalygin successfully identified a new cyanobacterial strain, *Dulcicalothrix alborzica* sp. nov., isolated from agricultural fields in Kermanshah Province. This identification was based on the molecular markers *rbcL*, *rpoC1* and 16S-23S ITS. Morphological analyses suggested that the strain belonged to the genus *Calothrix*; however, 16S rRNA gene analysis placed it within the *Dulcicalothrix* cluster. To confirm this classification, *rbcL* and *rpoC1* markers were also analyzed, and their results supported the identification of the new strain as *Alborzica* based on 16S rRNA. The secondary structure analysis of 16S-23S ITS showed that the *Alborzica* strain possesses a unique structure compared to *Dulcicalothrix* (Nowruzi and Shalygin 2021). In this study as well, the genes 16S rRNA and ITS were evaluated as structural markers, while *rbcL* and *rpoC1* served as functional markers, and the resulting dendrograms corroborated the phylogenetic tree findings.

Additionally, extensive research has been conducted to identify new genera and species using both structural and functional genes. For instance, Nowruzi and Soares, (2021) introduced a new genus, *Alborzia kermanshahica* gen. nov., isolated from agricultural lands in Kermanshah Province. This identification was based on molecular markers including 16S rRNA–23S ITS and *mcy* genes (Nowruzi and Soares, 2021).

Shokraei *et al.*, (2019) applied a polyphasic approach to investigate morphological and genotypic variations among different species. They conducted the first documented genomic fingerprinting study on seven closely related cyanobacterial strains in Iran using three types of palindromic sequences – HIP, ERIC and STRR – as molecular markers (Shokraei, Fahimi *et al.,* 2019).

CONCLUSION

Accurate taxonomy should be based on a combination of morphological characteristics of the specimens and polyphasic approaches, including phenotypic, chemotaxonomic, and genotypic data. Many morphological traits are highly influenced by environmental conditions and, therefore, cannot be reliably used as definitive taxonomic features. The use of DNA sequences enables the inference of phylogenetic relationships among organisms. The present study concludes that the repetitive sequences identified in the genomes of cyanobacteria – particularly the (STRR) palindromic sequences – are highly useful in uncovering genomic relationships and strain-level differentiation among the studied cyanobacterial isolates. These sequences can serve as diagnostic markers for distinguishing between different cyanobacterial strains. The use of two genetic markers, 16S rRNA and ITS, for identifying the studied strain and constructing a phylogenetic tree via the IQ-TREE web server was consistent with the results obtained from the phylogenetic trees based on functional genes (*mcy*) and structural genes (*rbcL* and *rpoC1*). The results demonstrated that strains belonging to the same genus

clustered together within a common clade with strong bootstrap support, although the studied strain formed a distinct, separate branch.

DECLARATIONS

Ethics approval and consent to participate: All methods were carried out in accordance with relevant guidelines and regulations. Authors don't do any experiments on humans and/or the use of human tissue samples.

Consent for publication: Not applicable**Availability of data and materials:** The datasets used and/or analysed included in this published article .

Availability of data and materials: The datasets generated and/or analysed during the current study are available in the datasets generated and/or analysed during the current study.

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Live vertebrates and/or higher invertebrates: this study is not on live vertebrates and/or higher invertebrates

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