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Phylogenetic analysis of isolated *Phormidium* sp. and *Cyanobacterium aponinum* from Kor River

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Abstract

Cyanobacteria (blue-green algae) are a unique group of photosynthetic bacteria amongst the oldest forms of life, which widespread in aquatic environments including freshwater lakes, rivers and reservoirs. Furthermore, they have ability to produce a wide range of secondary metabolites with toxic property for aquatic animals, as well as human beings. Considering the importance of cyanobacteria in public health, the introduction of sensitive and reliable methods for their detection is the main aim of this study. In the present study, 30 water samples were collected from six locations of the Kor River, Iran. The samples were analyzed for the prevalence of cyanobacterial species using conventional culture methods and morphological tests. Then, molecular identification of the isolates was performed by the comparative evaluation of three sets of primers (CYA106/CYA781; CYA781/CYA359; and PCα/PCβ). Furthermore, their abilities to produce cyanobacterial toxins including microcystin and nodularin synthetase enzyme complexes were investigated by using polymerase chain reaction method. The results obtained from this study indicated that two isolates were identified as *Cyanobacterium aponinum* and *Phormidium* sp. none of the isolates had ability to produce selected toxins. In addition, the results indicated that the selected pair primers were proper for detection of the isolates. Hence, due to importance of cyanobacterial isolation from water samples and their effect on human health, periodic studies in different water resources and regions seem necessary. Furthermore, among the selected primers, CYA106/CYA781 could be a proper primer pair for detection of cyanobacterial isolates.

Keywords: *Cyanobacterium aponinum*, *Phormidium* sp., Microcystin, Nodularin, PCR

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Introduction

Cyanobacteria belong to a group of prokaryotic organisms known as variety of names, including cyanobacteria, blue-green algae, myxophyceans, cyanophytes, cyanophyceans, and cyanoprokaryotes (Hoehn and Long, 2008). These organisms with capability to produce oxygen are ubiquitous and can be found in various environmental habitats such as Soil, river, freshwater and springs (Tiwari *et al.,* 2005). It is well documented that cyanobacteria have a considerable flexibility to adapt to a wide range of environmental conditions (Iranshahi *et al.,* 2014). The growth of cyanobacteria depends on environmental factors such as nutrients and seasons (Mahdavi *et al.,* 2015). They are gram-negative bacteria, with diverse morphology including solitary, free-living cells, and colonies or filaments (Catherine *et al.,* 2013; Boopathi and Ki, 2014). In addition, their ability to produce different types of toxin made collaboration attractive among scientists worldwide (Håkanson *et al*., 2007). Such toxins are widely recognized as a potential risk for not only water ecosystem, but also for wildlife and humans. Therefore, scientists tried to focus on control of harmful blooms and monitoring of the toxins in water and food webs (Botana, 2014). Unfortunately, in some countries there is no systematic monitoring for the presence of toxigenic cyanobacteria and their related toxins in the water resources. It is noteworthy that the discharge of untreated or insufficiently treated industrial, urban and rural wastewaters into the water reservoirs has exacerbated water contamination by cyanobacterial toxins (Botana, 2014). Cyanotoxins are often classified into three groups based on their mode of action: Hepatotoxins, neurotoxins, and dermatotoxins (Boopathi and Ki, 2014). The most commonly encountered cyanobacterial toxicities are attributed to the monocyclic hepta- and pentapeptide hepatotoxins, microcystins and nodularins (Wiegand and Pflugmacher, 2005; Botana, 2014). In this regard, Nowruzi *et al*. (2013) worked on detection of Stigomena from Ali-Abad Lake in Iran. They found that the genus *Stigomena* has potency for producing highly toxic secondary metabolites. These authors characterized the genus based on the 16SrRNA sequencing and showed that the isolates carried the *mcyE* gene and a potential cryptic hassallidin gene cluster (Nowruzi *et al.,* 2013). Furthermore, Nikoei and Bahador (2017) showed that the Kor River in Fars province is a habitat for isolation of toxic cyanobacteria. They found that the isolates had capability to produce microcystin and sulfotransferase.

While a number of PCR-based methods for more rapid detection of cyanobacteria have been developed in recent years, a key question is which specific primer could provide more reliable and valid results. Thus, this study attempted to address this question by comparing the primers which are previously used by scientists and then introducing the appropriate one (Nübel *et al*., 1997; Maniglia *et al.,* 2010; Zarrini *et al.,* 2011).

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Materials and methods

Site description

The Kor River is one of the most important rivers located in Maharloo-Bakhtegan basin (28˚99'-31˚25' N, 51˚82'-54˚50' E) in Fars Province, Iran. The length, width and depth of the river are around 50 km, 15-20 km, and 20 m, respectively. It originates from the Zagros Mountain, joins with the Sivand River at Khan Bridge and finally discharges into Bakhtegan Lake. Throughout the various seasons the industrial, farm and domestic run-offs have already polluted this water source, which could be the main reason for accumulation of cyanobacteria (Ebrahimi and Taherianfard, 2010).

Sampling

In total, 30 water samples were collected from six stations along the river from September to October 2016. Samples were taken at the depths of 30 to 50 centimeters using 250 ml screw cap glass bottles, and then transferred to the laboratory at refrigerated temperature. The temperature and pH of water were recorded at each sampling sites.

Isolation and morphological identification of cyanobacteria

For isolation of cyanobacteria from the selected sites, the water samples were serially diluted (10^{-1}-10^{-6}) in saline-peptone water (1 g L^{-1} peptone and 8.5 g L^{-1} NaCl) and plated onto the BG11 medium (Himedia, Bombay, India) consisting of $MgSO_4.7H_2O$, $CaCl_2.2H2O$, $NaNO_3$, K_2HPO_4, Na_2CO_3, $Na_2EDTA.2H_2O$ and trace metal stock solution (H_3BO_3, $MnCl_2.4H_2O$, $ZnSO_4.7H_2O$, $Na_2MoO_4.2H_2O$, $CuSO_4.5H_2O$, and Co $(NO_3)_2.6H_2O$) (12). Then the plates were incubated at 28±2 °C under the illumination of 1500-2000 lux with 12:12 h light: Dark regime. After 2-3 weeks, the morphology of colonies was evaluated and one colony of each morphological type was purified on the same medium for further examination (Zare and Bahador, 2015). Partial identification of suspected isolates was evaluated based on cell morphology, Gram reaction and the presence of mucilaginous layer (Anagnostidis and Komarek, 1990; Zarrini *et al.*, 2011). Each isolate was purified and stored in trypticase soy broth (Merck, Darmstadt, Germany) containing 20% glycerol at freezing temperature (i.e. -20 °C) until analysis.

Molecular identification of isolates

Genomic DNA was extracted using DNG™-plus extraction Kit (CinnaGen, Tehran, Iran) as specified by the manufacturer and then quantified using spectrophotometer. 16S rRNA gene analysis was performed for molecular identification (Zarrini *et al.*, 2011; Zare and Bahador, 2015). The PCR assay was performed using three sets of primers. The lists of oligonucleotides, their sequences, target gene, size of amplification fragments, and amplification conditions illustrated in Table 1 (Maniglia *et al.*, 2010; Zarrini *et al.*, 2011; Zare and Bahador, 2015). Single PCR amplifications were conducted in a 25 µl reaction mixture consisting of the following: 2.5 µl of

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the 10×PCR buffer, 0.5µl (0.2 Mm) of each deoxynucleotide triphosphate, 1.5 mM of $MgCl_2$, 0.3 µl (1.5 unit) of *Taq* DNA-polymerase (SinaColon Company, Tehran, Iran), 0.5 µl (0.2 mM) of each primer (IGF Company, Tehran, Iran; Algae Biotechnology Center of Persian Gulf, Bushehr, Iran), and 5 µl of DNA template. *Microcystis aeruginosa* PCC7806 was kindly provided from Prof. Brett Neilan, School of Biotechnology and Bio Molecular Sciences, the University of New South Wales, Australia with the cooperation of Iranian Gene Fanavar Institute and used as a positive control (Massahi *et al.*, 2014). A phylogenetic tree was constructed to analyze the 16S rRNA sequences of the isolated strains with other relative bacteria species in the GenBank databases using MEGA 5.05 software.

Detection of selected toxin genes

In order to detect the genes involved in the production of microcystin and nodularin gene, the HEP primers (HEPF-5'-TTTGGGGTTAACTTTTTTGGGCATAGTC-3' and HEPR-5'-AATTCTTGAGGCTGTAAATCGGGTTT-3) were selected to amplify 472 bp products (Jungblut and Neilan, 2006). The primers have been designed based on the aminotransferase (AMT) domain, which is located on the modules *mcy*E and *nda*F of the microcystin and nodularin synthetase enzyme complexes, respectively (Jungblut and Neilan, 2006). The PCR reactions were performed as previously described, but using 0.5 pmol of forward and reverse primer and one µl of genomic DNA. The thermal cycling consisted of an initial denaturation step at 94 °C for 2 min, followed by 35 cycles at 94 °C for 20 secs, 57 °C for 30 sec (annealing), and 72 °C for 60 sec (extension), and a final extension step at 72 °C for 5 min. PCR products were analyzed on 1.5% Agarose gel in TAE buffer (Jungblut and Neilan, 2006). The electrophoresis was done for approximately 45 min at 70 V and visualized by ethidium bromide staining (0.5 µg ml^{-1}). The PCR product was investigated by the trans illuminator device.

Table 1: Cyanobacteria and toxin primers.

Gene	Primer	Sequence 5'–3'	Amplicon size	Amplification condition	Reference
16S rRNA	CYA-106	CGGACGGGTGAGTAACGCGTGA	664 bp	94°C-5 min 94°C-1 min 60°C-1 min 72°C-1 min 35 cycle	(Nübel *et al*., 1997)
	CYA-781	GACTACTGGGGTATCTAATCCCATT			
16S rRNA	CYA359	GGGGAATYTTCCGCAATGGG	487 bp	94°C- 3 min 94°C- 20 sec 50°C- 20 sec 72°C- 40 sec 72°C- 5 min 40 cycle	(Zarrini *et al*., 2011)
	CYA781	GACTACWGGGGTATCTAATCCCWTT			
Phycocyanin operon intergenic spacer (PC-IGS)	PCβ PCα	GGC TGC TTG TTT ACG CGA CA CCA GTA CCA CCA GCA ACT AA	650 bp	95°C-3 min 95°C-1min 56°C-40sec 72°C-1min 72°C-7min 35 cycle	(Maniglia *et al*., 2010)

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Results

Isolation and morphological identification of cyanobacteria

Two different types of colony morphology were observed on plated samples (Fig. 1). The first one (A) was blue-green, string-like, and filamentous. The other one (B) was blue-green, convex, circular, shiny, and smooth. Under light microscopy, they were elliptical with a mucilaginous sheath and showed a symmetrical division by binary fission.

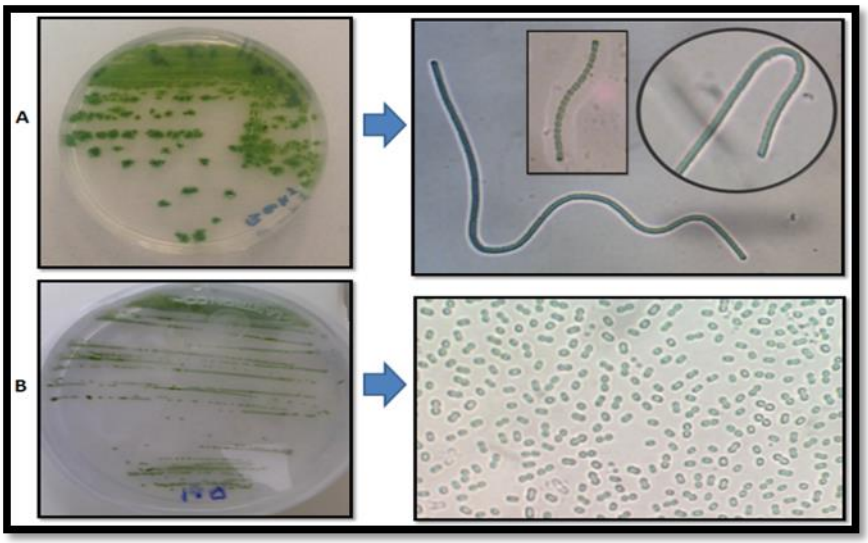

Figure 1: Colony and microscopic morphology of (A) *Phormidium* sp.; (B) *Cyanobacterium aponinum*.

Molecular identification of isolates

Among the primer pairs used for identification of cyanobacterial strains, CYA106/CYA781 primers showed more reliability and validity. As shown in Fig. 2, this primer pair could identify two cyanobacterial isolates, while none of the isolates was confirmed using PCβ/PCα primers (Fig. 3), and only one isolate was identified as a cyanobacterium by the third primer (CYA 359/CYA 781) (Fig. 4). In addition, phylogenetic analysis of data confirmed the obtained results (Figs. 5 and 6).

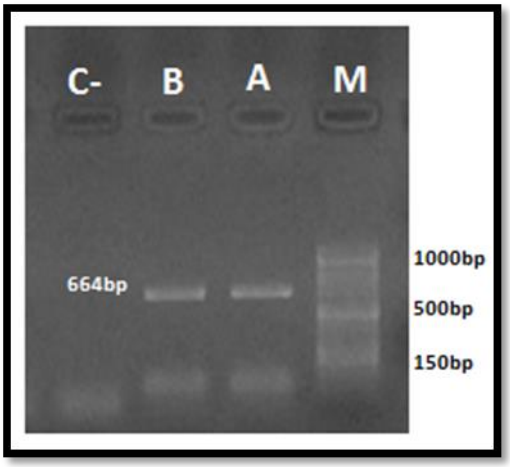

Figure 2: Agarose gel ladder (M), the negative control(C-). *Phormidium* sp. (A), *Cyanobacterium aponinum* (B). Fragment of 664 bp, using the CYA106 F, CYA781 R primers.

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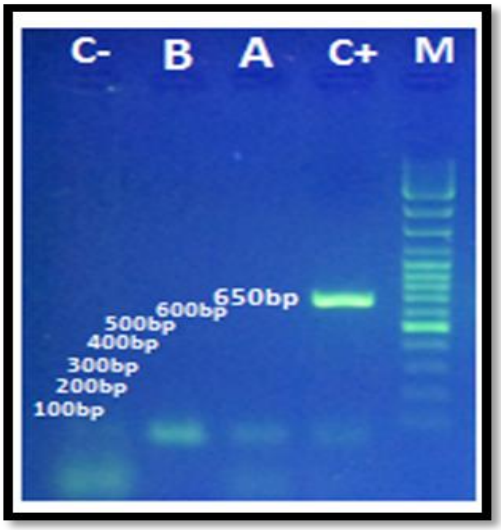

Figure 3: Agarose gel including a 100 bp ladder (M), the positive control *Microcystis aeruginosa* (C+) and negative control (C-). Fragment of 650 bp correspond to the amplification product the PC-IGS from sample of cyanobacteria bloom from KOR River, using the PCβ F and PCα R primers.

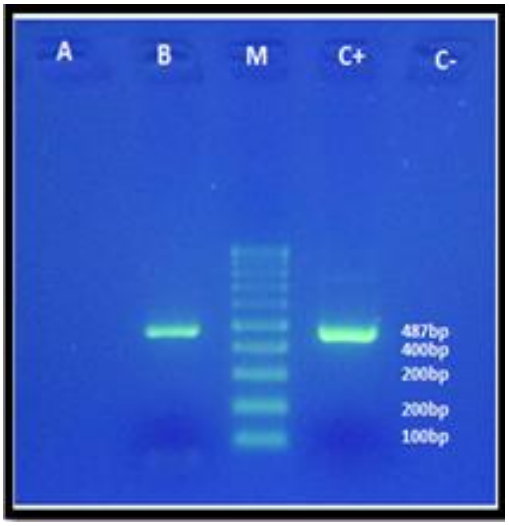

Figure 4: Agarose gel including a 100 bp ladder (M), the positive control *Microcystis aeruginosa* (C+), negative control (C-) and cyanobacterium (B). Fragment of 487, using the CYA781 R/CYA359 F primers.

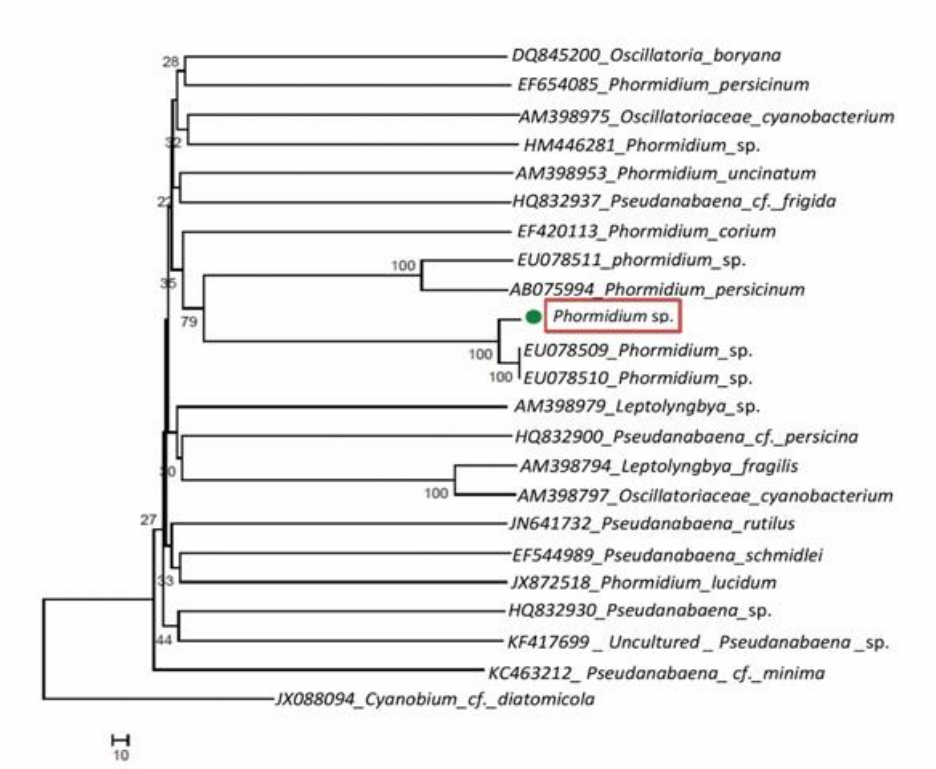

Figure 5: The Phylogenetic relationship of isolated *Phormidium* sp. among cyanobacterial strains using MEGA 5.05 software. The numbers above branches show the bootstrap values as percentage. The *Cyanobium cf. diatomicola* chosen as an outgroup.

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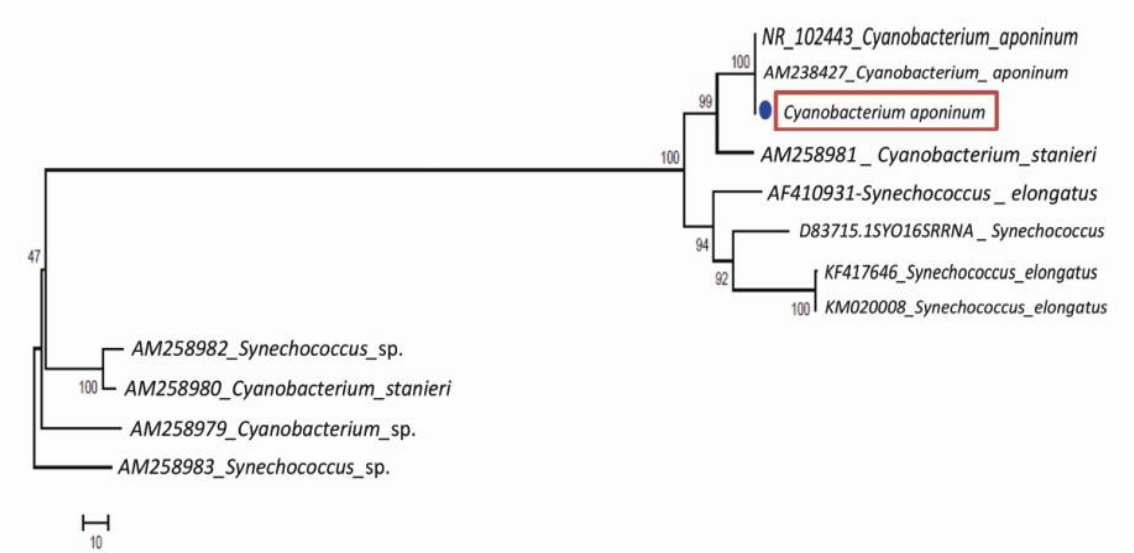

Figure 6: The phylogenetic relationship of isolated *Cyanobacterium aponinum* among cyanobacterial strains using MEGA 5.05 software. Bootstrap values are indicated in the point at nodes.

Detection of selected toxin genes

Although the positive control strain showed amplicon of 472 bp (expected size of amplicon), the results indicated that none of cyanobacterial isolates contain gene for the selected toxins (Fig. 7).

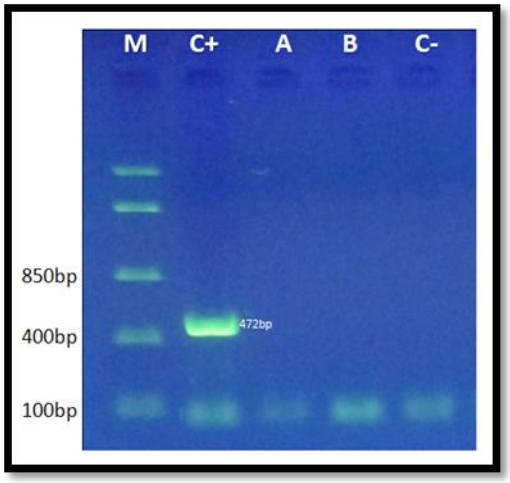

Figure 7: Agarose gel including Fermentas middler Rany DNA Ladder (M), the positive control *Micricystis aeruginosa* PCC7806 (C+) and negative control (C-). Sample of cyanobacteria bloom isolated from Kor River (A and B), using the HEPF, HEPR primers (472bp) for detection Microcystin / Nodularin toxin.

Discussion

Cyanobacteria are photosynthetic bacteria, which found naturally in lakes, streams, ponds, and other surface waters. The organism can rapidly multiply in surface water and cause "algal blooms". Depending on the light penetration and nutrient level, different groups of cyanobacteria found in aquatic habitats. Although some strains produce, harmful toxins for human being presence of the isolates with this ability are important for public health (Rantala *et al.*, 2006; Fortin *et al.*, 2010). Indeed, there are 65 isoforms of Microcystin, which are the most spreading types of reported cyantoxins (Bagu *et al.*, 1995). In addition, there is a report regarding growth of *Anabaena* and *Microcystis* in Bahia city in Brazil, which lead to the death of 88 children in the same geographical area (Hage, 1993).

For this purpose, the present study tried to isolate cyanobacteria from Kor River, then identified the isolates using

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molecular techniques by three sets of primers and finally evaluated presence of their toxins: Microcystin and nodularin synthetase enzyme complexes. There are several methods for detection of cyanobacteria, but each of these methods has their own limitations, while molecular method such as PCR is one of the most important techniques for identification. In PCR, researchers used different primers for detecting cyanobacteria (Neilan *et al.,* 1995; Maniglia *et al.*, 2010). The reports showed that PCβF/PCαR (Neilan *et al.,* 1995; Maniglia *et al.*, 2010; Zarrini *et al.*, 2011), and CYA359/CYA781R were used for detection of cyanobacteria from different geographical areas (Massahi *et al.,* 2014). Furthermore, Nübel *et al*. (1997) also used other primers. The results obtained from this study indicated that the primers which are used by Nübel *et al*. (1997) (CYA106 F/CYA781 R) for detection of cyanobacteria was more significant and useful compared to the other ones. In the present study, the isolates from Kor River were found to belong to the genera *Phormidium* sp. and *Cyanobacterium aponinum* using three primer sets. *In situ* observations from New Zealand rivers have shown that the greatest *Phormidium* cover usually occurs when dissolved inorganic nitrogen (DIN) concentrations are over a threshold of ca. 0.2 mg L^{-1} (Heath *et al.,* 2016). While, during the present study the authors did not check the amount of inorganic nitrogen in the detected area, but probably the presence of nitrogen in the sites could be an important factor for their existence. Although *Phormidium* can produce neurotoxic anatoxins, there are non-toxinogenic strains which can grow faster than the toxic strains. There are several reports which are explaining the relation between anatoxin concentration, their growth stage and concentration of nitrogen and phosphorus (Tiwari *et al.,* 2005). Indeed, urbanization has led to increasing nitrogen and phosphorus concentrations in aquatic ecosystems (Conley *et al*., 2009). Consequently, their increases resulted in greater *Phormidium* biomass (Tiwari *et al.*, 2005). As a result, although some strains of *Phormidium* sp. and *Cyanobacterium aponinum* are capable to produce toxins, the factors responsible for variations in toxin concentrations observed in the environment are unknown (Catherine *et al.,* 2013; Wood *et al.,* 2012). Hence, further research in this geographical area could help the scientists to evaluate the effect of environmental factors on the presence of toxigenic or non-toxigenic strains of Cyanobacteria, which is important for human health especially in summer season due to the swimming of the people in the explained location. Besides, in the present study, Neighbor-Joining method is used for analysis of the 16S rRNA and for the phylogenetic tree constructions. The 16S rRNA sequences were combined with other selected cyanobacterial species available in the CLUSTAL W analysis database and the BLAST search performed on the 16S rRNA primary

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sequences resulted in the highest similarity scores for cyanobacteria. Parallel with this work, Ezhilarasi and Narayanaswamy (2009) studied the molecular taxonomic of eight isolates from freshwater. They worked on genetic diversity among strains tested was determined with the sequences which were compared to those of representative heterocystous cyanobacteria available in GenBank and phylogenetic tree was inferred by NJ distance method. The clusters well supported by bootstrap analysis and partly reflect the morphological similarity of the organisms. Based on the results obtained from different researches on 16S rRNA variation from environmental samples, it suggests that culturable bacteria account for less than 0.1% of the species in a given environment (Cohan, 2011), the application of molecular methods with applying specific primers in the identification and phylogeny of cyanobacteria seem necessary.

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