Phylogenetic analysis of isolated \textit{Phormidium} sp. and \textit{Cyanobacterium aponinum} from Kor River

Zare M.\textsuperscript{1,2}, Bahador N.\textsuperscript{1,*}; Baseri Salehi M.\textsuperscript{3}

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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 \textit{Cyanobacterium aponinum} and \textit{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: \textit{Cyanobacterium aponinum}, \textit{Phormidium} sp., Microcystin, Nodularin, PCR

1-Department of Microbiology, Faculty of Science, Agriculture and Modern Technology, Shiraz Branch, Islamic Azad University, Shiraz, Iran.
2-Department of Microbiology, Faculty of Science, Islamic Azad University, Tehran (Fars) Science and Research Branch, Iran.
3-Department of Microbiology, Faculty of Science, Kazeroon Branch, Islamic Azad University, Kazeroon, Iran

*Corresponding author’s Email: nimabahador22@gmail.com
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).
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_2·2H_2O, 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^{TM}-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
the 10×PCR buffer, 0.5µl (0.2 Mm) of each deoxynucleotide triphosphate, 1.5 mM of MgCl₂, 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 (HEP-5'-TTTGGGGTTAACTTTTTTGCCAT

AGTC-3' and HEPR-5'-AATTCTTGA GGCTGTAATCGGTTT-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 meyE and ndaF 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.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence 5'-3'</th>
<th>Amplicon size</th>
<th>Amplification condition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>CYA-106</td>
<td>CGGACGGGTGAGTAACGCCTGA</td>
<td>664 bp</td>
<td>94°C-5 min</td>
<td>(Nübel et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>CYA-781</td>
<td>GACTACTGGGGTATCTAATCCATT</td>
<td></td>
<td>94°C-3 min</td>
<td>(Zarrini et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>CYA359</td>
<td>GGGGAATTTCCGCAATGGG</td>
<td>487 bp</td>
<td>94°C-5 min</td>
<td>(Maniglia et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>CYA781</td>
<td>GACTACWGGGGTATCTAATCCWTT</td>
<td></td>
<td>95°C-5 min</td>
<td></td>
</tr>
<tr>
<td>Phycocyanin</td>
<td>PCβ</td>
<td>GGC TGC TGG TTT ACG CCA CA</td>
<td>650 bp</td>
<td>95°C-1 min</td>
<td>(Mingiglia et al., 2010)</td>
</tr>
<tr>
<td>operon spacers</td>
<td>PCα</td>
<td>CCA GTA CCA CCA CCA CCA ACT AA</td>
<td></td>
<td>95°C-1 min</td>
<td></td>
</tr>
<tr>
<td>(PC-IGS)</td>
<td></td>
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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*.](image)

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.](image)
Phylogenetic analysis of isolated Phormidium sp.

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.
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).

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 cyanotoxins (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
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
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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