

Isolation and Identification of Tersakan Stream (Amasya, Suluova, Türkiye) Cyanobacteria and Investigation of the Presence of the Microcystin-LR

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Abstract

Cyanobacteria are a concern worldwide due to their blooms and the toxins they produce. The aim of this study is to reveal the cyanobacterial diversity of Tersakan Stream and toxin analysis. For these purpose, water samples were taken from Tersakan Stream in 2018 and cyanobacterial diversity was determined by culture-dependent and independent methods. PCR amplification of microcystin genes (*McyB* and *McyE*) and Microcystin-LR (L:Leucine, R:Arginine) (MC-LR) analysis in water sample was performed. While 10 different cyanobacteria were identified by light microscopy examination and 16S rRNA + ITS sequence, less diversity was revealed by culture-independent methods. Sequences of the isolates were associated with *Pseudanabaena lonchooides*, *Leptolyngbya* sp, *Toxifilum* sp, *Cylindrospermum moravicum*, *Nostoc* sp, *Oscillatoria* sp, *Synechocystis* sp, *Synechococcus* sp, *Cyanobacterium aponinum* and *Cyanobium* sp. Culture-independent sequences were associated with *Microcystis* sp, *Nostoc* sp, *Cyanobium* sp, *Dolichospermum* sp, *Anabaena* sp, *Leptolyngbya* sp and Uncultured cyanobacterium. Microcystin analysis was performed by both molecular and analytical methods. PCR results of *McyB* and *McyE* genes supported the HPLC-DAD analysis result. 0.467 µg L⁻¹ microcystin LR was detected in Tersakan Stream by HPLC-DAD analysis. Although the microcystin concentration is low, Tersakan Stream is an important area to be monitored with its eutrophic character and cyanobacterial diversity.

Introduction

Cyanobacteria are gram-negative aquatic bacteria, which are capable of aerobic photosynthesis. The origin of aerobic photosynthesis is based on cyanobacteria. These microorganisms, known to have existed for more than 3.5 billion years, represent the largest and most important bacterial group responsible for atmospheric oxygen generation (Chaurasia, 2015). While cyanobacteria usually spread in freshwater (Tunç et al., 2021) and sea (Celepli et al., 2017), they can also be found in very different habitats such as glaciers (Anesio

et al., 2017), deserts (Hagemann et al., 2017), and geothermal fields (Bennett et al., 2020). Their ability to adapt to these extreme environments attracts the attention of researchers, who examine many sources in Türkiye (Yılmaz Cankilic, 2016; Yılmaz Cankilic and Arık Berk, 2016; Ozbayram et al., 2021) and the world (Wood et al., 2017; Kurobe et al., 2018) in terms of cyanobacterial diversity.

Pollutants in aquatic ecosystems are of critical ecological and economic importance (Ma et al., 2018). Cyanobacterial blooms are commonly found in alkaline, warm, and stagnant waters. Bloom-forming

cyanobacteria affect the taste, smell, and appearance of water due to the toxins they synthesize and cause toxic effects on animals and humans (Pantelić et al., 2013; Ammar et al., 2014; Sadegh et al., 2021). One of the most common toxins globally is microcystin with the cyclic peptide structure. It is a hepatotoxin that promotes liver damage and tumor formation (Wu et al., 2019). While microcystin is generally produced by *Microcystis*, whereas *Anabaena* and *Planktothrix* are also known to produce microcystin (Li et al., 2011). It is impossible to distinguish between toxic and non-toxic strains using morphological features. Therefore, molecular methods are important for facilitating the distinction between morphologically similar species and providing information about their toxic potential (Scholin et al., 2003). Primers have been defined for different regions of *Mcy* genes, and they are specific for any producer of microcystins (Ramya et al., 2018; Radkova et al., 2020; Yuan et al., 2020).

Identification of cyanobacteria is traditionally based on morphological features. However, cyanobacteria may have similar morphological features, which leads to problems in determining diversity in an environment. For this reason, molecular methods are preferred as well as morphological features (Rippin et al., 2018; Jasser et al., 2020). Therefore, it is observed that molecular methods have been frequently preferred to determine cyanobacterial diversity in recent years (Silva et al., 2014; de Lima et al., 2021). The most widely applied strategy to determine cyanobacterial biodiversity in nature has been sequencing 16S rDNA of isolates (Rudi et al., 2000; Pope & Patel, 2008). The relationship of non-culturable species with the environment is revealed by culture-independent methods (Affe et al., 2018). The importance of culture-independent studies has increased due to the inability to fully meet the requirements for culturing microorganisms in the laboratory environment. However, it is recommended to use culture-dependent and independent methods together since culture-independent methods significantly affect the negative consequences of DNA extraction, PCR, and other enzymatic reactions (Kisand & Wikner, 2003; Rego et al., 2019).

This study aimed to determine the cyanobacterial diversity in the Tersakan Stream using culture-dependent and culture-independent methods. In this context, the sequence analysis of the isolates 16S rRNA + ITS + 23S30R gene region, clone library from eDNA and the metagenomic analysis of 16S V3-V4 were performed. In addition to the cyanobacterial diversity, we aimed to determine potentially toxic cyanobacteria and the concentration of MC-LR in water samples.

The Tersakan Stream, one of the three major branches of the Yeşilirmak, arises from the foothills of Akdağ, reaches the Havza from Ladik Lake and then joins the Yeşilirmak when reaching Amasya (Tekin, 1997). Tersakan Stream has borders with Samsun and Amasya provinces and is 100 km long in total. There are

industries around the Tersakan Stream that can be important sources of pollutants. Wastes obtained from animal farms in Suluova district are used indiscriminately as fertilizer in agriculture fields and the excess is discharged to Tersakan Stream. In addition, this source, which is used for irrigation purposes in agriculture, dries up in dry periods with low rainfall and is fed again with drainages opened from agricultural lands. This situation causes an increase in the pesticide-induced pollution load (Tübitak MAM 2010). In addition, since there is no wastewater treatment plant in Suluova, domestic wastes are discharged directly or indirectly into the Tersakan Stream. For these reasons, Tersakan Stream turns into a waste channel carrying animal, agricultural and industrial wastes within the borders of Suluova. Soil structure consists of alluviums, 3rd time sediments, flysch, pre-tertiary sediments and crystal. Tersakan Stream dissolves the rocks in the fields it passes through, and therefore, the sediment load also includes substances such as calcium, chloride, sodium and sulfate. This nutrient-rich area is heading towards eutrophication and provides a suitable ground for the growth of cyanobacteria. Moreover, there are significant knowledge gaps in terms of molecular identification of cyanobacteria in the Yeşilirmak Basin. Previous studies conducted at the sampling point have performed the identification of cyanobacteria using only morphological features (Maraslıoğlu et al., 2016 a, b). This study is important as it is the first study in which morphological and molecular methods are used together in the identification of Tersakan Stream (Amasya, Suluova) cyanobacteria.

Materials and Methods

Study Site, Sampling and Physicochemical Analysis of Water

The water samples used in the study were collected from the Tersakan Stream (Figure 1) Suluova/Cürlü (40°47'32"N 35°36'44"E-40°47'15"N 35°36'44"E) from an area 500 m long, located in the province of Amasya (Türkiye), in sterile glass bottles in June 2018. The water samples were kept in the dark and brought to the laboratory on the same day.

The physicochemical properties of the water samples were analyzed. The pH values of the water samples were measured and recorded by an Isolab handheld pH meter at the sampling points. The Fe⁺⁺, Ca⁺⁺, Na⁺, K⁺ and Mg⁺⁺ contents of the water samples were determined using an inductively coupled plasma optical emission spectrometer (ICP-OES) Optima 8000 (Perkin-Elmer) device. Water samples were prepared according to ISO 5667-6 (ISO 5667-6:2005) and analyzed according to ISO 11885:2007 (ISO 11885:2007).

Additionally, the fluoride⁻, chloride⁻, sulfate⁻, phosphate⁻, nitrite⁻, nitrate⁻, and bromide⁻ contents of the water samples were determined chromatographically by a Dionex ICS-3000 (Thermo



Figure 1. Tersakan Stream sampling points Amasya/Suluova/Cürlü **a)** 40°47'32"N 35°36'44"E **b)** 40°47'15"N 35°36'44"E

Scientific, Sunnyvale, CA, USA) device. Anions were identified according to their retention time, and their amount was determined according to their conductivity degree (Ronkart 2012).

Isolation and Morphological Identification of Cyanobacteria

Water samples were inoculated into BG11 and BG11₀ (is standard BG11 but omitting NaNO₃) nutrient media (Nitnaware et al., 2021). 100 ml of water sample was concentrated by passing through sterile filters with 0.22 µm pore diameter (Menezes et al., 2020) and these filters were placed in liquid media and left for incubation. In addition, 150 mL of water samples were transferred to sterile flasks to ensure the growth of cyanobacteria in their own environment without any nutrient media contribution. The flasks were incubated at room temperature in a continuous light (3000 lux) for 3 weeks. Pure cultures were obtained by the streak plate method (Waterbury 2006). The purified isolates were stored in liquid nitrogen in BG11 medium containing 5% DMSO (Gaget et al., 2017).

A Leica DM 500 model light microscope was used for the morphological examination of pure isolates. Photographs of the isolates were taken by the Leica ICC50 HD camera integrated into the microscope. The isolates were evaluated according to whether they were unicellular or filamentous, the shape of the cells and whether they contained cell differentiations, such as akinete and heterocyst (Komárek and Anagnostidis, 1998; Moro et al., 2007; Komárek et al., 2014)

DNA Extraction from Pure Isolates and Environmental Samples

Genomic DNA extraction from pure isolates was performed using the EcoSpin Bacterial Genomic DNA Kit (EcoTech Biotechnology, Türkiye) according to the manufacturer's instructions. For total eDNA extraction, 200 ml of the water sample was passed through a 0.22 µm diameter polycarbonate 47 mm filter (Merck Millipore, USA). Total eDNA extraction from the filters was performed according to the method described by Boutte et al. (2005) and Calusinka (2005). The obtained DNA samples were stored at -20°C.

PCR Amplification of 16S rRNA+ ITS + 23S30R rRNA

CYA 359F (5'-GGGGGAATTTCCGCAATGGG-3') (Nübel et al., 1997) and 23S30R (5'-CTTCGCCTGTGTGCCTAGGT-3') (Taton et al., 2003) primer sets were used for the amplification of the 16S rRNA + ITS + 23S30R rRNA gene region. The reaction content in a Peqlab PeqStar thermal cycler was as follows: 1 X of Taq Buffer with (NH₄)₂SO₄, 1 mg ml⁻¹ of BSA (bovine serum albumin), 200 µM of dNTP mix, 0.5 µM of the forward and reverse primers, 1 U µl⁻¹ of Taq DNA Polymerase (Thermo Scientific™ EP0701) with proofreading activity. The reaction conditions were optimized according to Nowicka-Krawczyk et al. (2019) and the following conditions were adjusted: 5 min at 94°C; 30 cycles of 45 s at 94°C, 45 s at 57°C, and 2 min at 72°C; and a final elongation step of 7 min at 72°C. PCR products were run on a 1% agarose gel, then stained

with ethidium bromide and examined under UV light. PCR products were purified using the GeneJET PCR Purification Kit (Thermo Scientific™) and stored at -20°C.

Culture Independent Cyanobacteria Diversity

Cloning of 16S rRNA+ ITS + 23S30R Gene Region

Total eDNA was used as template and amplification of 16S rRNA + ITS + 23S30R rRNA gene region was performed as previously mentioned. Ligation of 16S rRNA+ ITS + 23S30R rRNA PCR product to the pGEM-T vector was performed at +4°C for 18 hours in line with the manufacturer's instructions. By transforming the ligation product into a potent *E. coli* DH5 α strain, cells were spread on LB agar plates containing ampicillin (100 $\mu\text{g ml}^{-1}$), X-gal (80 $\mu\text{g ml}^{-1}$), and 0.5 ml IPTG (100 mM) and incubated at 37°C for 18 hours. At the end of the incubation period, blue and white cells were selected. The randomly selected white clones were used as templates and 16S rRNA+ ITS + 23S30R PCR products were obtained with 359F 23S30R primers under the previously specified conditions. The products were run in a 1% agarose gel in electrophoresis at a current of 100 V for 1 hour.

16S rRNA Metagenomic Analysis

16S metagenomic analysis was performed in BM Labosis (Ankara, Türkiye) laboratory. Total eDNA was used as template and the 16S V3-V4 gene region was selected for metagenomic analysis, universal primer set for bacteria (341F-CCTACGGGNGGCWGCAG and 805R-GACTACHVGGGTATCTAATCC) (Herlemann et al., 2011) and universal primer set for cyanobacteria (CYA359F-GGGGAATTTCCGCAATGGG, CYA-781R(a) GACTACTGGGGTATCTAATCCATT and CYA781R(b)-GACTACAGGGGTATCTAATCCCTTT) (Nübel et al., 1997). In the index PCR step, Illumina binary indices and adapters were added using the Nextera XT index kit and then purified. The concentration of the libraries created by real-time PCR was measured and normalized by dilution to 4nM. The normalized samples were combined with the pooling method, and sequencing was performed after the library was prepared. The raw data were analyzed for chimeric reads with the Dada2 pipeline. Reads with Phred scores less than 20 bp, primers and barcodes, chimeric reads were filtered out. These sequences were analyzed in the QIIME2 2019.4 environment (Callahan et al., 2016; Bolyen et al., 2019).

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

For ARDRA, 10 μl of the PCR products (which obtained isolates and clones) was incubated in the recommended buffer medium with 1 U enzyme (*MspI*) at 37°C for 16 hours and incubated at 65°C for 20 minutes to stop the reaction. The products obtained by

mixing with 6X loading dye were loaded onto the agarose gel prepared at a 2% concentration. Electrophoresis was performed for 3 hours by applying a 5V/cm electric current.

Sequencing and Phylogenetic Analysis

Clones and isolates with different restriction profiles were selected and re-amplified with the same primer sets, and the PCR products were sent to MacroGen (Netherlands) for sequence analysis after purification with the Thermo GenJet PCR purification kit. 23S30R and 1492R (GGTTACCTTGTTACGACTT) primer sets were used for sequence analysis. The sequence data of isolates (accession numbers MZ621158-64 and MZ790588-96) and clones (accession numbers MZ812834-43) are stored in GenBank.

Sequences from isolates and clones were analyzed in Chromas Pro software. Sequences were aligned with the NCBI Nucleotide BLAST program to obtain the most closely related organism sequences. In addition, a library was created by selecting sequences from cyanobacteria databases (Cydrasil). ClustalW alignment program was used in Mega 11 software to align the sequences in the library. Phylogenetic tree was constructed according to Neighbor-Joining and Maximum Likelihood methods. It was found that the most suitable method for Maximum Likelihood analysis was the Kimura 2 + G + I model (for heterocyst forming cyanobacteria and clones) and General Time Reversible + G + I model (non-heterocyst forming cyanobacteria) as a result of the Maximum Likelihood fit test of 24 different nucleotide substitution models. The statistical accuracy of the branches formed was determined by "bootstrap" analysis by sampling 1000 times (Taton et al. 2003; Boutte et al. 2005).

Amplification of *Mcy* genes and Microcystin Analysis

In this study, PCR amplification of *McyB* and *McyE* genes from microcystin synthetase gene clusters were performed. For the amplification of the *McyB* and *McyE* gene regions, primers *McyB*-F (5'-TGGGAAGATGTTCTTCAGGTATCCAA-3'), *McyB*-R (5'-AGAGTGGAACAATATGATAAGCTAC-3') *McyE*-F2 (5'-AATTGTGTAGAAGGTGC-3') and *McyE*-Ra4 (5'-AATTCTAAAGCCCAAAGACG-3') were used respectively (Rantala et al., 2004; Glowacka et al., 2011). The reaction content was as follows: 1 X of Taq Buffer with (NH₄)₂SO₄, 1 mg ml⁻¹ of BSA (bovine serum albumin), 200 μM of dNTP mix, 1 μM of the forward and reverse primers, 1 U μl^{-1} of Taq DNA Polymerase (Thermo Scientific™) with proofreading activity. The following reaction conditions were adjusted: 3 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C; and a final elongation step of 10 min at 72°C. PCR products were run on a 1% agarose gel, then stained with ethidium bromide and examined under UV light. PCR products were purified using the GeneJET PCR Purification Kit (Thermo Scientific™) and stored at -20°C.

HPLC-DAD was used for the quantitative analysis of MC-LR in water samples. 1 liter of water sample was passed through a sterile filter with 0.22 pore diameter and these filters were used for microcystin extraction. Extraction of microcystin was performed according to Lawton (1994). All the reagents were of high-performance liquid chromatographical (HPLC) grade. Elution mode was used: injection volume 25 μL , flow rate 1 mL min^{-1} , and column temperature 40°C. The mobile phases were Milli-Q water and acetonitrile both containing 0.1% TFA. Detection wavelength was set to be at 238 nm.

Results

Physicochemical Properties of Water

At our sampling point, described as freshwater, the pH level of the water was measured as 8.05, and it was evaluated as slightly alkaline water. When the results of ICP OES and ion chromatography are examined, it is seen that calcium, chloride, nitrate, nitrite, and sulfate values are very high (Table 1).

Identification of Culturable Cyanobacteria of Tersakan Stream

A total of 34 cyanobacteria (24 isolates were purified from BG11 medium and 10 isolates were purified from BG11_o medium) were isolated. As a result of light microscope examinations, 10 morphotypes were observed in the Tersakan Stream and it was found that the number of filamentous isolates (20) was higher than single-cell isolates (14). While unicellular cyanobacteria were associated with *Synechococcus* sp. (Figure 2a) *Cyanobium* sp. (Figure 2b), *Synechocystis* sp. (Figure 2c) and *Cyanobacterium aponinum* (Figure 2d), whereas filamentous cyanobacteria were associated with *Cylindrospermum* sp. (Figure 2e), *Nostoc* sp. (Figure 2f), *Pseudanabaena* sp. (Figure 2g), *Leptolyngbya* sp. (Figure 2h), *Oscillatoria* sp. (Figure 2i) and *Toxifilum* sp. (Figure 2j).

As a result of the ARDRA analysis, performed to identify isolates with different restriction profiles, seventeen different cut profiles were determined from the 34 isolates obtained from the Tersakan Stream with the *MspI* restriction enzyme. Upon examining the spread of ARDRA profiles among the isolates, the sequence analysis results showed that the most common profile was similar to *Pseudanabaena*

lonchoides at a rate of 98.94%. The other profiles were *Cyanobium* sp. (99.31%), *Leptolyngbya* sp. (99.78%), *Synechocystis* sp. (98.42%), *Synechococcus* sp. (99.21%), *Cyanobacterium aponinum* (99.36%), *Cylindrospermum moravicum* (98.47%), *Nostoc* sp. (98.50%), *Toxifilum* sp. (97.25%), *Oscillatoria* sp. (97.31%) and Uncultured cyanobacterium clone (98.06%) (Table 2).

Culture Independent Cyanobacterial Diversity of Tersakan Stream

Ten different profiles were detected in the *MspI* ARDRA profiles of 100 white colonies in the 16S rRNA + ITS + 23S30R clone library. The BLAST results of clones sequences were associated with *Dolichospermum affine*, *Dolichospermum smithii*, *Anabaena* sp., *Microcystis aeruginosa* and uncultured *Anabaena* clone (Table 3).

16S rRNA V3-V4 region metagenome analysis was performed with two different primer sets. With primer set universal for bacteria (341F-805R), 191868 reads with a length of 250 bp were obtained. It was determined that there were members with a 100% bacterial domain at the regnum level. Bacteria domain members were identified as 5 branches. Upon examining the percentage distribution of these branches, the most common branch is Verrucomicrobia at a rate of 42.56%. There are 37.89% Proteobacteria, 14.99% Bacteroidetes, 2.83% Cyanobacteria and 1.74% other branches. In this analysis, *Nostoc punctiforme* was represented at a rate of 4% and *Microcystis aeruginosa* was represented at a rate of 2.83% but no other cyanobacterial species could be identified (Figure 3a). With primer set universal for cyanobacteria (359F-781R), 97162 reads with a length of 250 bp were obtained. The cyanobacteria clade was represented by 99.04% and when the sequences obtained were analyzed at the species level, 85.44% *Microcystis aeruginosa*, 8.63% *Anabaena cylindrica*, 2.7% *Cyanobium gracile*, 2.01% *Nostoc punctiforme* and 1.22% other species were determined (Figure 3b).

Phylogenetic Analysis

In this study, 16S rRNA sequencing was performed to examine the phylogenetic relationship between cyanobacteria isolated with each other and with other strains in the world. Similar results were obtained when NJ and ML phylogenetic trees were examined. Therefore, the results are presented based on the ML tree.

Table 1. Physicochemical properties of water.

ICP-OES (ppm)						
Fe ⁺²	Ca ⁺²	Na ⁺	K ⁺	Mg ⁺²		
<10	178.9	<10	<1	<10		
ION CHROMATOGRAPHY (ppm)						
Fluoride	Chloride	Sulfate	Nitrate	Nitrite	Phosphate	Bromide
< 1.0	153.3	273.0	7.0	< 5.0	< 10.0	< 5.0

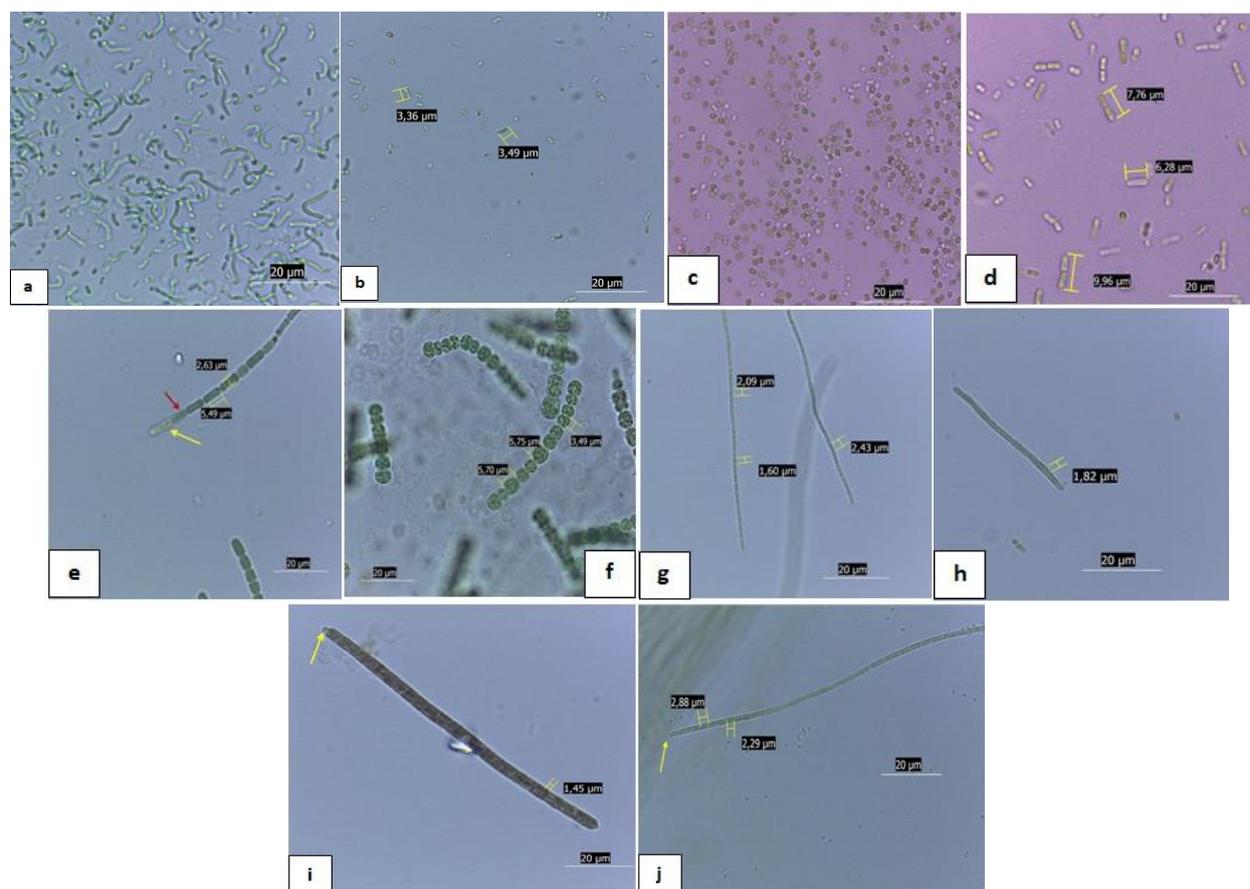


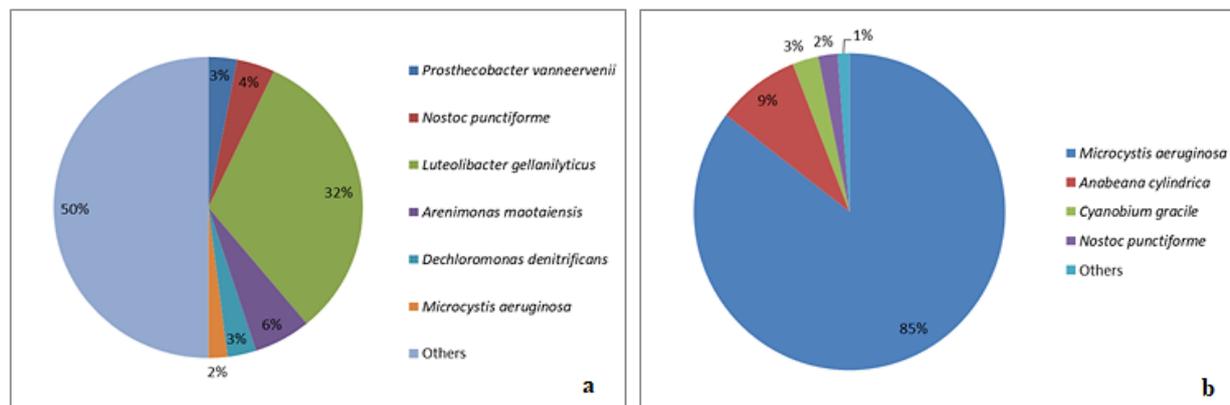
Figure 2. Light microscope images of some isolates **a)** *Synechococcus* sp. strain 11TAC 1 **b)** *Cyanobium* sp. strain OTAC 2 **c)** *Synechocystis* sp. strain 11TAC 14 **d)** *Cyanobacterium aponinum* strain OTAC 3 **e)** *Cylindrospermum* sp. strain OTAC 4 **f)** *Nostoc* sp. OTAC 5 **g)** *Pseudanabaena* sp. strain 11TAC 8 **h)** *Leptolyngbya* sp. strain 11TAC 13 **i)** *Oscillatoria* sp. strain 11TAC 24 **j)** *Toxifilum* sp. strain OTAC 6. Scale bar 20 µm.

Table 2. Cyanobacterial isolates and their closest genbank matches.

ARDRA Profile	Sample Code Accession No	% Of Identity With The Closest Relative	Closest Relative According To BLAST Search / Accession No/Source
14	OTAC 2 MZ621158	99.65%	<i>Cyanobium</i> sp. CHAB 6568 clone 03/ MT488300.1 Qinghai-Tibet Plateau
2	11TAC 9 MZ790590	99.31%	<i>Cyanobium</i> sp. CHAB 6568 clone 03/ MT488300.1 Qinghai-Tibet Plateau
7	11TAC 16 OL 241795	99.24%	<i>Cyanobium</i> sp. CHAB 6568/ MT488300.1 Qinghai-Tibet Plateau
4	OTAC 3 MZ621159	99.90%	<i>Cyanobacterium aponinum</i> HG3/ KY172946.1 Hammat Gader hot springs
13	OTAC 8 MZ621164	99.90%	<i>Cyanobacterium aponinum</i> PCC 10605/ CP003947.1 Fresh water
11	11 TAC 23 MZ790595	99.36%	<i>Cyanobacterium aponinum</i> HG3/ KY172946.1 Hammat Gader hot springs
15	OTAC 4 MZ621160	98.85%	<i>Cylindrospermum moravicum</i> CICALA 993 / KF052608.1 Cave sediment
16	OTAC 5 MZ621161	98.50%	<i>Nostoc</i> sp. ACT703 / MK247970
10	OTAC 6 MZ621162	97.81%	Uncultured <i>Toxifilum</i> sp./ MH492757.1 Estuary
17	OTAC 7 MZ621163	98.06%	Uncultured bacterium clone/ JX521379.1 Terrestrial sulfidic spring
1	11TAC 1 MZ790588	99.21%	<i>Synechococcus</i> sp. CCAP 1479/14/ HE975008.1 Russia: Lake Baikal, Siberia
8	11TAC 17 MZ790593	98.42%	<i>Synechococcus</i> sp. MW99B6/ AY151247.1 Lakes Mondsee and Hallstättersee
3	11TAC8 MZ790589	98.94%	<i>Pseudanabaena lonchoides</i> PltPIn33/ KM438180.1 Hot-spring mats of Greece
5	11TAC 13 MZ790591	99.64%	<i>Leptolyngbya</i> sp. BN22/ HM151383.1 Lake Taihu
6	11TAC 14 MZ790592	98.42%	<i>Synechocystis</i> sp. ElfSCS31/ KM438202.1 Hot-spring mats of Greece
12	11 TAC 24 MZ790596	97.31%	<i>Oscillatoria</i> sp. CHAB TP201735.1 clone/ MT488229.1 Qinghai-Tibet Plateau

Table 3. Cyanobacterial clones and their closest genbank matches.

Clone Code Accession Code	% Of Identity With The Closest Relative	Closest Relative According To Blast Search / Accession No/ Source
TACK1 MZ812834	98.95%	Uncultured <i>Anabaena</i> sp. clone Okaro10 / EU402397/ Lake Okaro
TACK2 MZ812835	99.17%	<i>Dolichospermum affine</i> 04-44 / FN691907/ Svět fishpond
TACK3 MZ812836	99.34%	<i>Dolichospermum affine</i> 05-03/ FN691906/ Staňkovský fishpond
TACK4 MZ812837	98.39%	<i>Anabaena</i> sp. WA102/ CP011456/Anderson Lake
TACK12 MZ812838	99.46%	<i>Microcystis aeruginosa</i> AICB618/ KJ746513/ KJ746513
TACK18 MZ812839	99.16%	<i>Dolichospermum affine</i> 04-44 / FN691907/ Svět fishpond
TACK36 MZ812840	99.18%	<i>Dolichospermum affine</i> CHAB5815/ MN813778/ Zhelin Lake
TACK51 MZ812841	99.63%	<i>Microcystis aeruginosa</i> FD4/ CP046973/Caloosahatchee River
TACK61 MZ812842	98.78%	<i>Dolichospermum smithii</i> 05-05/ FN691923/ Dubněnský fishpond
TACK97 MZ812843	99.89%	Uncultured cyanobacterium partial 16S rRNA gene, isolate cln_OTU34C109/ AM259251/Lake Tuusulanjärvi

**Figure 3.** a) Species-level bacteria graphic as a result of 16S rRNA metagenome analysis with 341F-805R primers. b) Species-level cyanobacteria graphic as a result of 16S rRNA metagenome analysis with 359F-781R primers.

The phylogenetic tree of heterocyst-forming isolates was drawn separately in other isolates and in this group, isolates OTAC 4 and OTAC 5 were located in two separate branches. OTAC 4 isolate was clustered together with *Cylindrospermum stagnale*, while OTAC 5 isolate clustered together with *Nostoc* sp (Figure 4a).

When the phylogenetic tree of the isolates that non heterocyst forming cyanobacteria was examined, it was observed that the isolates clustered in 5 different branches. While the isolate 11TAC 14, which branched closest to the first group, clustered together with *Synechocystis* sp from the unicellular cyanobacteria, isolates OTAC 3, OTAC 8 and 11TAC 23 clustered together with *Cyanobacterium aponinum* in the other branch. In the third group, there are members of Pseudanabaenales. In this group, isolate OTAC 7 was associated with *Thermoleptolyngbya* sp, 11TAC 13 and 11TAC 18 were associated with *Leptolyngbya* sp in different closely related branches. In the same group, the isolates 11TAC 8 and OTAC 6 were clustered together

with *Toxifilum* sp. In the fourth group, isolates 11TAC 1, 11TAC 9, 11TAC 16 and 11TAC 17 from unicellular cyanobacteria clustered together with *Cyanobium* sp. In the fifth group, the isolate 11TAC 24 was clustered together with the Oscillatoriales members (Figure 4b).

The phylogenetic tree obtained from the sequences of the selected clones was clustered in 2 main branches. Most of the clones clustered in the branch with heterocyst-forming cyanobacteria, while two clones clustered together with *Microcystis aeruginosa* (Figure 5).

MC-LR Analysis Results

As a result of the PCR carried out with the genomic DNA of the cyanobacteria isolated from Tersakan stream, positive PCR product could not be obtained for *McyB* and *McyE* gene regions. However, positive PCR product was obtained for *McyB* and *McyE* gene regions with total eDNA sample.

HPLC-DAD analysis, the limit of detection value for MC-LR was found to be $0.022 \mu\text{g mL}^{-1}$. In Tersakan Stream, MC-LR was detected at a concentration of $0.467 \pm 0.003 \mu\text{g L}^{-1}$.

Discussion

Cyanobacteria are photosynthetic bacteria commonly found in freshwater. The stagnant, nutrient-rich waters are abundant with cyanobacteria. Anthropogenic activities affect the physical and chemical parameters of water (Shakir et al., 2013) and are also closely related to the cyanobacterial diversity in aquatic systems (Takarina et al., 2017). Therefore, it is very important to determine the physicochemical properties of sampling points in diversity studies. The Tersakan Stream was characterized as weak in terms of ecological status according to the Yeşilirmak Basin Management Plan reports (TVOB 2021), and no improvement could be detected according to the seven-year observation results. A previous study reported that the sampling point was under a serious pollution threat due to the discharged animal wastes and was in transition from mesotrophic structure to eutrophic structure in terms of trophic structure (Maraşlıoğlu et al., 2016a). Our study also supports these results. The Tersakan Stream was found to have high chloride and sulfate values (Table 1). This situation may be caused by

mixing inorganic fertilizers, garbage leachate, industrial wastewater, animal feed, and irrigation drainage (WHO, 2003) into the Tersakan Stream.

The cultivation and molecular identification of the Tersakan Stream cyanobacteria have not been performed previously. This study is the first to use culture-dependent and culture-independent methods to investigate cyanobacterial diversity in the Tersakan Stream (Amasya). Previous studies have determined the cyanobacterial diversity in the Tersakan Stream based on morphological features and *Gloeotheca rupestris*, *Merismopedia punctata*, *Dolichospermum spiroides* (Maraşlıoğlu et al., 2016a), *Chroococcus minutus*, *Dolichospermum flosaquae* and *Spirulina nordstedii* (Maraşlıoğlu et al., 2016a, b) were identified. In another study carried out in the Yeşilirmak River basin, it was reported that *Anabaenopsis milleri* Woron and *Planktothrix isothrix* (Skuja) Komárek & Komárk species were found (Maraşlıoğlu et al., 2022). It was observed that the cyanobacterial diversity isolated in this study was different from the other study carried out at the sampling point (Maraşlıoğlu et al., 2016a). At the same time, it was observed that the physicochemical properties of the water deteriorated during this time, and the cyanobacterial diversity most likely have been affected by this situation. Although there are studies on Cyanobacteria morphologies at the sampling points, there is no data on the molecular identification of these

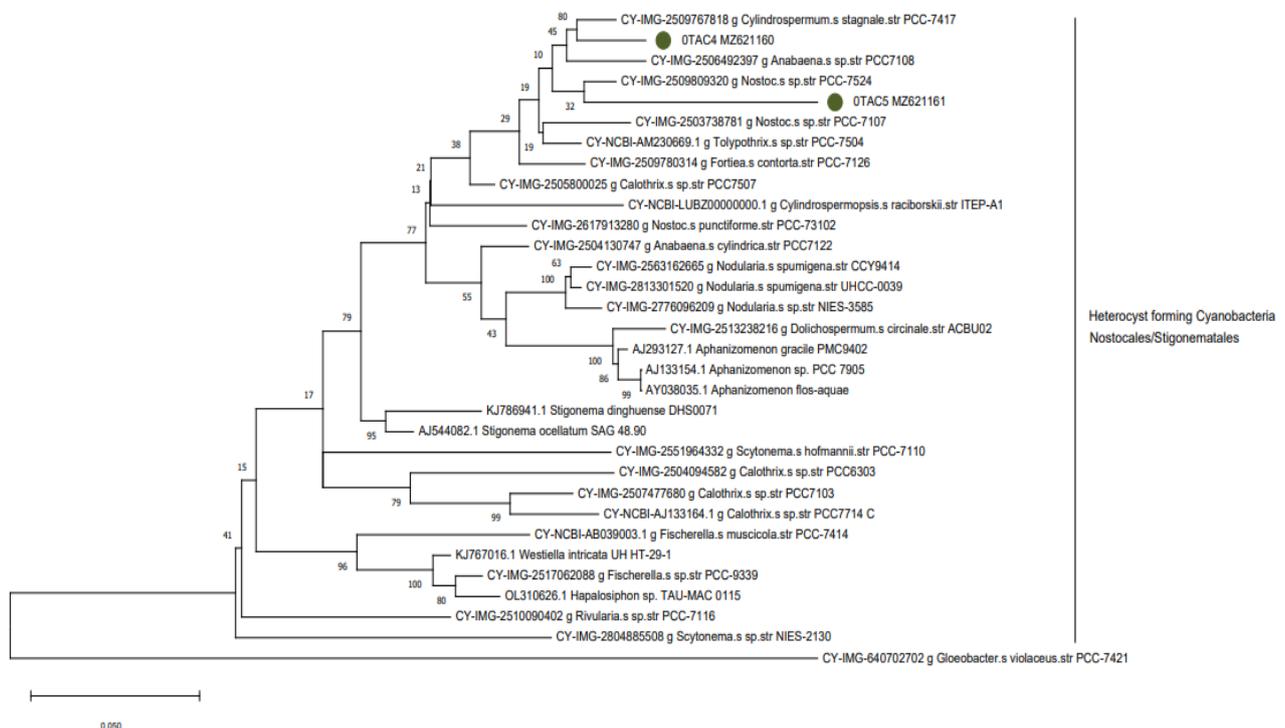


Figure 4. a) Maximum likelihood tree based on 16S rRNA of heterocyst forming isolates (indicated green circle). The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. The tree with the highest log likelihood (-4426.11) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1662)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 36.69% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 32 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 879 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

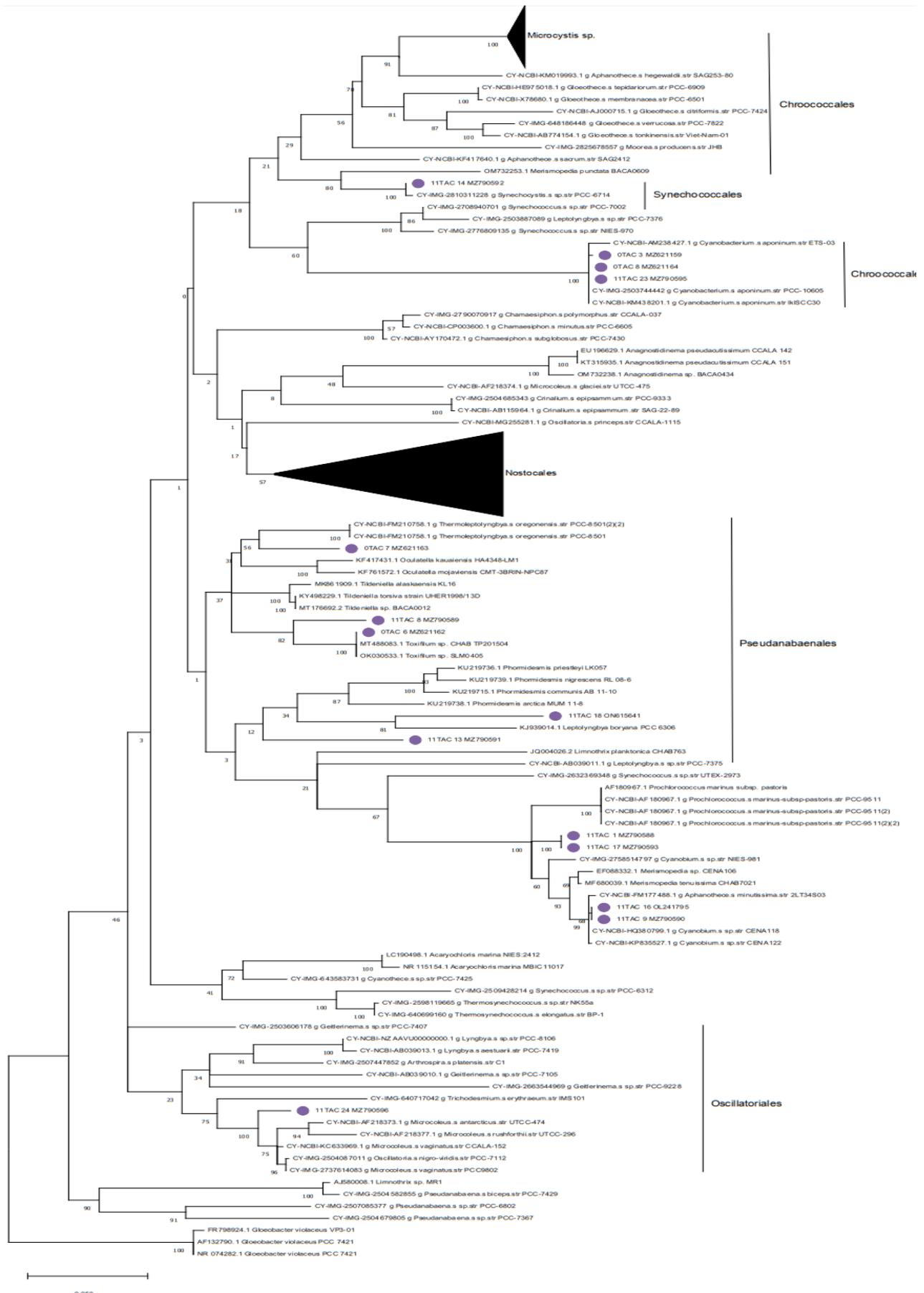


Figure 4. b) Maximum likelihood tree based on 16S rRNA of non-heterocyst forming isolates (indicated purple circle). The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model. The tree with the highest log likelihood (-8636.38) is shown. The percentage of trees in which the associated taxa clustered together is shown below the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2482)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 33.29% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 100 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 844 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

cyanobacteria species that are known to exist in the environment but cannot be cultured (*Microcystis aeruginosa*, *Dolichospermum* sp. and *Anabaena* sp.) were determined. This situation most likely be caused by unsuitable cultivation conditions. In addition, this situation probably be caused by species that are active in different seasons but not in large numbers at the time of sampling or DNA residues in the environment. In our study, this situation could not be clarified because sampling could not be carried out in different seasons due to lack of budget.

This study observed that the species diversity obtained by cloning and metagenomic studies was lower than that obtained in isolation studies. As a result of the isolation study, 10 different cyanobacteria genera (Table 2) were identified, while 3 different cyanobacteria genera (Table 3) were identified by cloning and 4 different cyanobacterial genera (Figure 3) were identified by 16S metagenome. The main reason for this most likely be that efficient extraction cannot be performed due to the use of manual methods in environmental DNA extraction. Efficient DNA extraction could not be performed due to their exopolysaccharides (Motham et al., 2014). A similar study reported that the results of metabarcoding were inadequate compared to the results of isolation, and it has been indicated that taxonomic assignment is difficult and reduces the resolution of analyses due to the quality of eDNA samples and the need to obtain molecular data from cultured species to support identification (Psifidi et al., 2015; Fabrin et al., 2020). Another reason probably be that the selected primer sets are not suitable for all cyanobacteria. In the present study, we observed that PCR-based metagenomic libraries established with different primer sets reflected different cyanobacterial diversity. While only *Microcystis aeruginosa* (Figure 3a) could be identified in the metagenomic library created with the 341F-805R primer set, *Microcystis aeruginosa*, *Anabaena cylindrica*, *Cyanobium gracile* and *Nostoc punctiforme* were identified in the metagenomic library created with the 359F-781R primer set (Figure 3b). Although cyanobacterial diversity is demonstrated in more detail with the 359F-781R primer set, isolation studies still show that it is insufficient. One of the reasons why metagenomic results do not reflect true diversity is the short read regions of 250 bp in length. The short read sequences of 16S hypervariable regions typically allow only secure sequence classification at the genus or sometimes species level (Alvarenga et al., 2017). For these reasons, culture-dependent and culture-independent results can differ from each other.

Another objective of this study is to screen the microcystin producing cyanobacteria in sample region. Microcystins are the most prevalent cyanobacterial hepatotoxins in freshwaters (Rantala et al., 2004) and pose a danger to pets and humans (Douma et al., 2009; Plaas & Pearl, 2020). *Microcystis aeruginosa* producing microcystin has been reported in many freshwaters in Türkiye (Albay et al., 2005; Taş et al., 2006; Sömek et al.,

2008; Yilmaz et al., 2019). In this study, although the presence of *Microcystis aeruginosa* strain was detected by cloning and metagenomic studies, this strain could not be isolated. This probably be due to the low biomass of the *Microcystis aeruginosa* strain due to competitive growth inhibition in mixed cultures (Ngwa et al., 2014). However, sequences belonging to members known to produce microcystin (*Anabaena*, *Dolichospermum*, *Nostoc*, *Pseudanabaena* and *Oscillatoria*) were found in our study. These cyanobacteria probably also contribute to the amount of microcystin in Tersakan stream.

Even if the microcystin concentration is very low, microcystin producers can be confirmed by amplifying the *Mcy* gene regions (Stoyneva-Gärtner et al., 2021). In this study, no isolates with the *McyB* and *McyE* genes were obtained, however, positive PCR products were obtained for the targeted gene regions from the eDNA sample. PCR can only show the potential for toxin synthesis and does not provide information on actual toxin production. However, in monitoring studies, this method ensures early warning of the potential to produce toxins. Therefore, PCR amplification of the *Mcy* gene is very important for predicting the presence of microcystin in aquatic systems. In our study, PCR results of *Mcy* genes supported the analysis of microcystin. As a result of HPLC-DAD analysis, microcystin LR was detected at a concentration of 0,467 $\mu\text{g L}^{-1}$. The presence of *Mcy* genes and MC-LR has been reported in many wetlands in Türkiye (Gurbuz et al., 2009; Koker et al., 2017; Yilmaz et al., 2019; Köker et al., 2021; Köker et al., 2022). However, there are no studies on the investigation of cyanotoxins in the Yeşilirmak basin, which also includes the Tersakan Stream. In this study, Tersakan Stream microcystin analysis was carried out for the first time.

It is very important to determine the toxin-producing cyanobacteria diversity due to the location and use of this stream, which originates from Ladik Lake in Samsun and pours into the Yeşilirmak, for irrigation purposes and angling in the region. PCR results of the microcystin synthetase *McyB* and *McyE* gene regions indicate the presence of species that can produce toxins in the environment. In addition, the HPLC results supported the PCR results and the presence of MC-LR was determined in the sampling point. Periodical mass fish deaths in the Tersakan Stream can be associated with this toxin.

Conclusion

This is the first study in which culture-independent methods, such as cloning and metagenomic analysis, and culture-dependent methods were used together to identify cyanobacteria in the Tersakan Stream (Suluova). At the same time, the *McyB* and *McyE* gene regions responsible for the production of microcystin and HPLC-DAD analysis of MC-LR were investigated for the first time in the Tersakan Stream (Suluova). The results of this study increased our understanding about the

cyanobacterial composition and MC-LR concentration of Tersakan Stream, one of the largest tributaries of Yeşilirmak. In addition, the sequence information obtained as a result of this study has been added to the international database and this information will shed light on other studies planned for the Yeşilirmak basin in the future.

Considering the biotechnological potential of cyanobacteria, cyanobacteria have metabolites with significant antimicrobial (Saurav et al., 2019; Nainangu et al., 2020) antioxidant (Parida et al., 2021) and anticancer (Sampathkumar and Halith, 2020) activities. For this reason, the metabolite profiling and biological activities of these isolates, which are purely isolated and maintained in our culture collection, will be investigated in further studies.

Ethical Statement

This article does not contain any studies with animals performed by any author.

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Author Contribution

All authors contributed to the study conception and design. Authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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