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Identification of new species record of Cyanophyceae in Diyala River, Iraq based on 16S rRNA sequence data

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Abstract. *Hassan FM, Mahdi WM, Al-Haideri HH, Kamil DW. 2022. Identification of new species record of Cyanophyceae in Diyala River, Iraq based on 16S rRNA sequence data. Biodiversitas 23: 5239-5246.* The biodiversity and water quality of the Diyala River require screening water in terms of biological contamination, because it is the only water source in Diyala City and is used for many purposes. This study aimed to identify a new species record of Cynaophyceae and emphasize the importance of using molecular methods beside classic morphological approaches, particularly in the water-shrinkage-aqua system. Five different sites along Diyala River were selected for Cyanophyceae identification. Morphological examination and 16S rRNA sequence analysis was conducted, and the phylogenetic tree was constructed using Mega 6 Programme. The morphological examination of samples showed a total of 28 species corresponds to Cyanophyceae, including one species of *Spirulina.* In our study of 28 identified species, three new species record were identified in Diyala River. The newly recorded species were confirmed by 16S rRNA and the phylogenetic tree construction. The species are registered in the National Centre for Biotechnology Information (NCBI) with the following accession numbers: *Arthrospira indica* (MW854667.1), *Arthrospira platensis* (MW854665.1), and *Limnospira fusiformis* (MW854666.1). Most notably, *Arthrospira platensis* is not listed in the checklist of Iraqi algae. Thus, these species are considered as a new record of Iraqi algal flora. The identification of new species record in Diyala River reflexes the impact of climate change on this river, and the necessity to use 16S rRNA to identify microalgae in all ecosystems.

Keywords: 16S rRNA, Cyanophyceae, Diyala River, phylogenetic tree

INTRODUCTION

Iraqi water resources face major water supply challenges and climate change's impact (Al-Ansari et al. 2014). The main impact of climate change is on the hydrological cycles through altering of precipitation and evapotranspiration (Winter et al. 2012; Xuan et al. 2014; Wang et al. 2021). The Diyala River is one of Iraq's most essential water supplies and the third most important tributary of the Tigris River. In addition, it is the sole water source for irrigation, domestic agriculture, municipal, and other uses (Hamza 2012). However, many issues, such as contamination and water shrinkage have arisen in the basin of the Diyala River, which may threaten water sources (Issa et al. 2014). According to Abbas et al. (2016), the Diyala River basin may undergo drought conditions as a result of climate change. Abdullah (2013) and Al-Jibouri et al. (2018) reported that the Diyala River suffers from the loading of different pollutants into the river. Adamo et al. (2020) also reported that dam construction in Iran caused a considerable change in the aquatic environment of the Diyala River, and the hydrological regime affects the aquatic biodiversity either as a positive or negative impact (Rolls et al. 2018).

Cyanophyceae are known for their important contribution to global carbon and nitrogen cycles and their ability for aerobic photosynthesis. Generally, they are found in all ecosystems and are important in most biotechnological applications (Wanigatunge et al. 2014). Study the diversity and distribution of Cyanophyceae in the Diyala River is important to assess the direct and indirect effect of climate change on the aquatic ecosystems (Muluneh et al. 2021). Many changes have been made in the classification of Cyanophyceae and the name of genera, families, and orders in addition to new taxonomic units based on molecular studies (Nowicka-Krawczyk et al. 2019; Kumari and Rai 2020).

The modern classification of Cyanophyceae places the genus *Arthrospira* within the family Microcoleaceae, which belongs to the order Oscillatoriales (Komárek et al. 2014). Both genera *Arthrospira* and *Spirulina* are similar and exchange their names. In 1932, Geitler (1932) reported the invalidation of *Arthrospira,* while in 1989, they were recognized as distinct genera by Castenholz. Both genera have some of the same morphological features (Komárek et al. 2014). *Arthrospira* was recorded in different aquatic systems (marine and freshwater), and is considered a cosmopolitan genus (Wan et al. 2021), and *Linmospira* is also mentioned correctly as *Spirulina* in many taxonomic studies (de Morais et al. 2015; Nowicka-Krawczyk et al. 2019).

The identification of Cyanophyceae is mostly performed by 16S rRNA sequencing, cloning, and phylogenetic assembly due to their variable distribution and morphological diversity. For example, identifying Cyanophyceae species in Sri Lanka was performed by polyphasic approach (morphology and 16S rRNA sequence analysis) (Wanigatunge et al. 2014). Wang and Zhao (2005); Komårek (2016); and Ozturk et al. (2018) reported that the morphological study of microalgae does not yield accurate results unless such molecular identification is performed.

This study aimed to identify a new species record of Cyanophyceae, and emphasize the importance of using 16S rRNA analysis beside classic morphological approaches, particularly in the water-shrinkage aqua-system.

MATERIALS AND METHODS

Study area

Diyala River has a northeastern and southwestern extension; it has many surface features, and its mouth is located in the Tigris River south of Baghdad City. Five sites were selected along the river, where site 1 represented the upper reaches, sites 2, 3 and 4 were mid-reaches while site 5 was lower reaches (Figure 1; Table 1)

Procedures

Algal sampling

Algal specimens were collected from the bank of the river monthly from September 2020 to April 2021. The wet surface with a depth of 2-3 cm and an area of 50 m^2 was placed in a nylon bag with a small amount of river water and kept in a sample box. The procedures of Eaton and Moss (1966) and Hassan et al. (2017) were used to isolate algae from sediment samples. A 40 g sediment sample in a petri dish was homogenously spread out and covered by lens tissue to trap the algae. The lens tissues were removed the next day and kept in vials. The algal samples were examined after shaking several times by a Genex compound light microscope (model GX-140105) in the laboratory of the Department of Biology, College of Science at University of Baghdad. The algal classification was performed according to Silva and Moe (2014), Komárek et al. (2014), and Wehr et al. (2015)

Table 1. Geographical coordinates by Global Positioning System of the study sites

Sites	Description	Coordinates Longitude (E)	Coordinates Latitude (N)
1	Al-Sudour Area	44° 58’ 54“	34° 39’ 44“
2	Al-Salam District	44° 43’ 17“	33° 55’ 07“
3	Baqubah City	44° 37’ 53“	33° 44’ 44“
4	Buhruz City	44° 36’ 18“	33° 40’ 55“
5	Khan Bani Saad District	44° 35’ 01“	33° 32’ 58“

Table 2. Composition of modified Chu-10 media

Number of stock solution	Chemical formula of each salt	Concentration (g/L)
1	MgSO4	10
2	K2HPO4	4
3	NaNO3	8
	CaCl2	16
4	FeCl3	0.32
5	EDTA-Na2	4
6	NaCl	30
7	Na2CO3	8
8	MnCl2·4H2O	0.02
	(NH4)6Mo7O24·4H2O	0.028
	ZnSO4·7H2O	0.224
	CuSO4·5H2O	0.08
	COCl2·6H2O	0.0004
	H3BO3	0.288
9	Na2SiO3	5.7

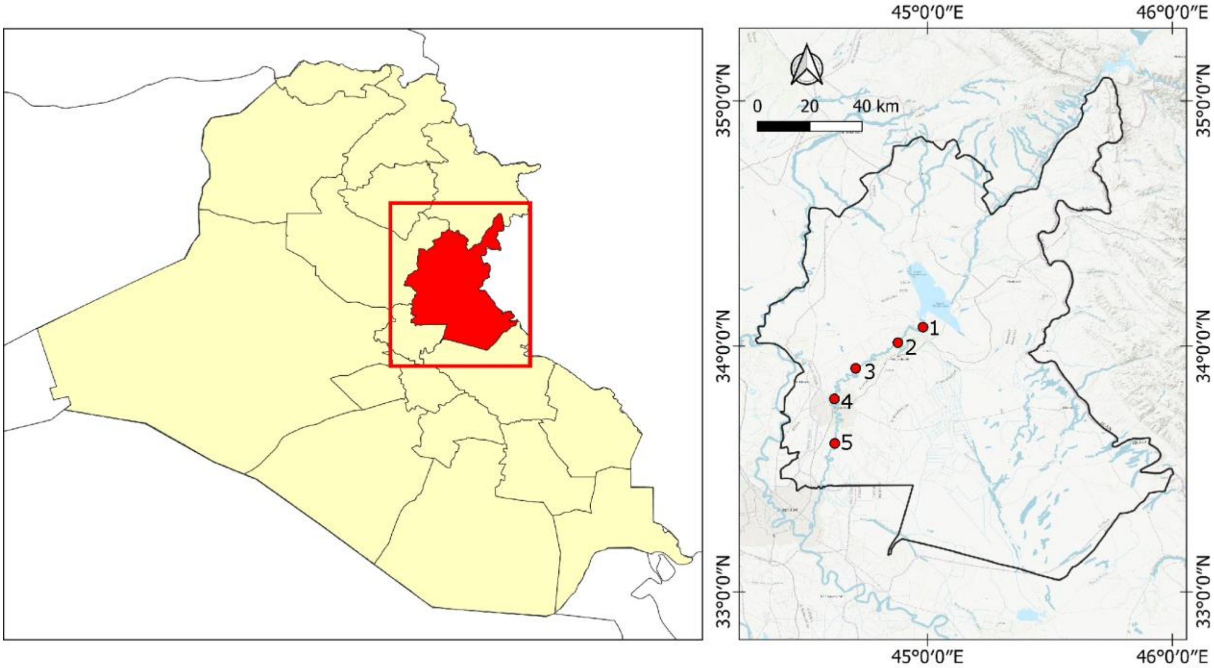

Figure 1. The study sites along the Diyala River, Iraq. The map was modified and adapted by Abdulla (2013)

Algal culturing

A liquid solution was prepared from the sediment sample by mixing of 1 part of sediment with 2 parts of distilled water. The algae were inoculated into a modified Chu-10 nutrient solution following the steps described by Hassan et al. (2013) and Al-Rawi et al. (2018) (Table2). These cultures were incubated in a cooled illuminated incubator at 30 ± 3°C, 300 µE/m^2/s and 16:8 light: dark for 21 days. The axenic culture was obtained by the streak plating method according to Katayama et al. (2020).

DNA extraction, amplification, electrophoresis, and sequencing

The genomic DNA of algal species was extracted according to the manufacturer's instructions using a Fast G-Spin Total DNA Extraction kit (Intron, Korea). The gDNA products of algal species were quantified and detected by 1% agarose gel and visualized under a UV transilluminator UltraBright Dual Wavelength 302/365nm, 21cm x 26 (transilluminators.com). The product of gDNA was stored at -20°C for further use.

The 16S rRNA-encoding gene was used to identify the isolated microalgae. The 16S rRNA region was amplified using universal primers, namely 16s RNA-F (5'-AGAGTTTGATCCTGGCTCAG-3') and R (5'-GGTTACCTTGTTACGACTT- 3') (Frank et al. 2008). The PCR of 16S rRNA amplification was performed according to the manufacturer's instructions (Gene Amp, PCR system 9700; Applied Biosystems). PCR mixtures were performed in a total volume of 25 µL containing 2.5 µL of each primer (10 pmol µL^{-1}), 12.5 µL of 2X Taq PCR PreMix DNA polymerase (Intron Korea), with assay buffer. Approximately 1 µL (2 ng µL^{-1}) gDNA was added to the reaction as a template prior to completing the volume with free nuclease-distilled water (6.5 µL). The amplification reaction mixture was performed in a thermal cycler with particular conditions: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94 °C for 45 s, followed by annealing at 52°C for 1 min and extension at 72°C for 1 min, and the mixture was subjected to final extension at 72°C for 7 min. The PCR products were resolved on 2% agarose using 1 X TBE buffer after they were mixed with 6 X loading dye to a final concentration of 1X. The electrophoresed products were detected by a transilluminator, and a digital camera captures images.

The amplified 16S rRNA gene was sent to the National Instrumentation Center for Environmental Management (NICEM)/ USA (http://nicem.snu.ac.kr/main/?en_skin=index.html). Sanger sequencing was conducted by ABI3730XL automated DNA sequences.

Data analysis

The resulting sequences of three new species were analyzed and aligned using the Basic Local Alignment Search Tool (BLAST) database and compared with the relative sequences of the gene database in NCBI. The expected value refers to the estimation of the number of times that is expected to get the same similarity coincidental and lower the value of E, indicating that the degree of similarity is high between sequences that give great confidence; a value close to zero indicates that these sequences are identical. The phylogenetic tree of aligned sequences was constructed and mapped using the MEGA 6 program (Al-Rawi et al. 2018).

RESULTS AND DISCUSSION

Species diversity and morphological identification of Cyanophyceae in Diyala River

A total of 28 species of Cyanophyceae were identified by light microscopy were belonging to four sub-classes, five orders, eleven families, and 17 genera (Table 3). The identified algae were classified as listed in Table 3.

The spatial variation in existence of Cyanophyceae taxa at different sites is illustrated in Table 3. Sites 1, 2, 4 and 5 recorded 20 taxa of Cyanophyceae, while 24 taxa were recorded in site 3 in the central Diyala Province (Baqubah City). Al-Jibouri et al. (2018) revealed that Cyanophyceae was the second most important algal group after Bacillaropphyceae, and their percentage was 26.37 %. Therefore, these results indicate the environmental alterations in the Diyala River. Al-Jibouri et al. (2018) and Bronmark and Hansson (2017) reported that the Cyanophyceae community is respond to environment changes. Wang et al. (2022), also reported that there is a relationship between the predominance of Cyanophyceae and environmental changes which can limit the growth and photosynthesis ability of Cyanophyceae. The Diyala River suffers from water scarcity, which affects water quality, alters the algal composition structure, and increases water pollution (Madhloom et al. 2017; Hassan et al. 2017).

Cyanophyceae play an indispensable role on earth in general by their photosynthetic ability and their role in aerobic respiration and multicellular life (Soo et al. 2017). The consumption of Cyanophytceae such as *Arthrospira platensis* has increased worldwide as a dietary supplement and for the production of the third-generation biofuels, as well as their role in heavy metal fixation due to their chelating properties for some metals, such as Al, Ba, Ni, and either Cd or fluoride (Kaldunska et al. 2020; Rubio et al. 2021). The genus *Arthrospira* is used in the food industry and is an important source of polyunsaturated fatty acids, phycobiliproteins, vitamins, minerals, and other important chemical compounds (Torzillo and Masojidek 2008). *Linmospira* is important in biotechnology, agriculture, bioremediation and biomanufacturing (Hicks et al. 2021; Tripathi et al. 2021). Most recently, *A. platensis* was used as a glycogen repository for biofuel production (Serra et al. 2020). This ability was enhanced by modification of growth conditions and growth medium, which led to increased polysaccharide production (Gonzalez Bautista et al. 2021). However, they can produce toxins that threaten the lives of humans and animals. Therefore, the rapid diagnosis of such toxic Cyanophyceae is essential, and the systematic morphological methods cannot distinguish between toxic and nontoxic Cynophyceae. Thus, molecular identification is reliable for accurate identification and has a high throughput (Yuan and Yoon 2021).

Table 3. Identified algae (Cyanophyceae) during the study period at five sites along the Diyala River, Iraq

Classification rank	Taxa	S.1	S.2	S.3	S.4	S.5
Phylum: Cyanobacteria						
Class: Cyanophyceae						
Sub-class: Nostocophycidae						
Order: Nostocales						
Family: Nostocaceae	*Nostoc* sp.	+	+	+	+	+
	Anabaena sp.	+	–	+	+	+
Sub-class: Synechococcophycidae						
Order: Synechococcales						
Family: Synechococcaceae	*Anathece endophytica* (W. & G.S.West) Komárek, Kastovsky & Jezberová	–	+	+	–	–
	Aphanocapsa elachista West & West	+	–	–	+	+
Family:Pseudanabaenaceae	*Pseudanabaena limnetica* (Lemmermann) Komárek	+	+	+	+	+
Family: Leptolyngbyaceae	*Planktolyngbya limnetica* (Lemmermann) Komárková - Legnerová & Cronberg	+	+	–	+	–
	3.2.4.Family: Synechococcales familia incertate sedis					
	Jaaginema angustissimum (West & G.S.West) Anagnostidis & Komárek	–	–	+	+	–
	J. minimum (Gicklhorn) Anagnostidis & Komárek	–	–	+	+	–
	Lyngbya sp.	+	+	+	+	+
Family: Merismopediaceae	*Merismopedia convoluta* de Brébisson ex Kützing	–	–	+	–	–
	M. elegans A.Braun ex Kützing	+	+	+	+	+
	M. glauca (Ehrenberg) Kützing	+	+	+	+	+
	M. tenuissima Lemmermann	+	–	+	–	+
Sub-class: Oscillatoriophycide						
Order: Chroococcales						
Family: Chroococcaceae	*Chroococcus dispersus* (Keis.) Lemmermann	+	–	+	–	+
	C. minor(Kützing) Nägeli	+	+	–	+	+
	C. turgidus (Kütz.) Nägeli	+	–	+	+	+
	Limnococcus limneticus (Lemmermann) Komárková, Jezberová, O.Komárek & Zapomelová	+	+	+	+	+
Family: Microcystaceae	*Microcystis* sp.	+	+	+	–	–
Order: Oscillatoriales						
Family: Microcoleaceae	*Kamptonema formosum* (Bory ex Gomont) Strunecký, Komárek & J.Smarda	+	+	+	+	+
	Microcoleus amoenus (Gomont) Strunecky, Komárek & J.R.Johansen	+	+	+	+	+
	Planktothrix agardhii (Gomont) Anagnostidis & Komárek	–	+	+	–	–
	P. prolifica (Gomont) Anagnostidis & Komárek	+	+	+	+	+
Order: Spirulinales						
Family: Spirulinaceae	*Spirulina major* Kützing ex Gomont	–	+	+	–	+
Family: Oscillatoriaceae	*Oscillatoria curviceps* C.A.Agardh ex Gomont	–	+	+	+	+
	O. limosa C.Agardh ex Gomont	+	+	+	+	+
	O. princeps Vaucher ex Gomont	+	+	+	+	+
	O. tenuis Agardh ex Gomont	+	+	+	+	+
	Phormidium ambiguum Gomont	–	+	–	–	–

Note: S1: Al-Sudour Area, S2: Al-Salam District, S3: Baqubah City, S4: Buhruz City, S5: Khan Bani Saad District, +: present, –: absent.

All above mentioned algae in the checklist were identified based on the morphological characters, which does not give an accurate identification and produces ambiguous results compared to the molecular classification (Al-Rawi et al. 2018). Five species of *Arthrospira* were identified in lakes and rivers in Iraq (Maulood et al. 2013), but the classification depends on morphological concepts. *Limnospira fusiformis* (Basionym: *Spirulina fusiformis*) is not recorded in Iraqi aquatic systems according to the last Iraqi algal flora (Maulood et al. 2013). Furthermore, Maulood et al. (2013) mentioned 15 species of *Spirulina* and 5 species of *Arthrospira.* They recorded the algae *A. platensis* according to the method of Bilbas (2004) but without any description and confirmed by the checklist of the Iraqi Kurdistan region (Aziz and Muhammed 2016). *Arthrospira platines* are characterized by their photosynthetic ability and filament shape with individual cells. Due to their growth conditions in subtropical alkaline lakes (pH 8.5-10) under a typical temperature above 35°C, they are considered as the main source for protein extraction and bioactive compounds such as phycocyanin (Cheng et al. 2019; Nematollahi et al. 2020).

Molecular identification and phylogenetic tree of new species record of Cyanophyceae

The molecular identification of microorganisms by 16S rRNA became a new standard for identifying a large scale of organisms, either prokaryotes or eukaryotes. Hence, they possess either 16S rRNA or 18S rRNA gene. Generally, 16S rRNA is universe among microalgae, around 1500 bp in length, with a variable and conserved region, allowing for distinguishing measurements (Clarridge 2004). Based on the morphological characteristics of 28 species of microalgae, three new recorded species were accurately identified by 16S rRNA sequence-base analysis. The 16S rRNA sequence analysis of three new recorded species showed that the PCR products were 1250 bp as shown in (Figure 2), which were then sent for sequence analysis.

Three new records species of Iraqi flora were recognized by molecular study and recorded in NCBI with the accession number as follows: *Arthrospira indica* Desikachary & N. Jeeji Bai (MW854667.1), *Arthrospira platensis* Gomont (MW854665.1) and *Limnospira fusiformis* (Voronichin) Nowicka-Krawczyk (MW854666.1).

The phylogenetic tree of each identified new species showed a convergence of approximately 99% and 100 % similarity with the universal species in the NCBI database for both *L. fusiformis* (Figure 3b) and *A. indica* (Figure 3c), whereas *A. platensis* showed a convergence of about 85 % and 100% similarity (Figure 3 a). The 16S rRNA sequences of three identified species of Cyanophyceae aligned with the relatives in NCBI revealed the polymorphism variations of the amplified gene (Table 4). The obtained results showed that *A. platensis* has two single nucleotide polymorphisms (SNPs) at nucleotides 441 and 742, whereas *L. fusiformis* showed three SNPs at nucleotides 289, 378, and 430. Similarly, *A. indica* showed four SNPs at nucleotides 186, 551, 649, and 685. The type of polymorphism in all identified species was variable and ranged from transition to transversion and thus is consistent with the constructed phylogenetic tree of all isolates.

The impact of climate change on ecosystem function and biodiversity is a global issue. Therefore, many species are threatened by extension. In Iraq, Diyala River is the main water source used for many purposes, and because this River undergoes climate change and water shortage, it may result in misdistribution of Cyanophyceae species. Here, the morphological methods were insufficient to identify the most economically important species in Diyala River, and needs for 16S rRNA sequence analysis became necessary.

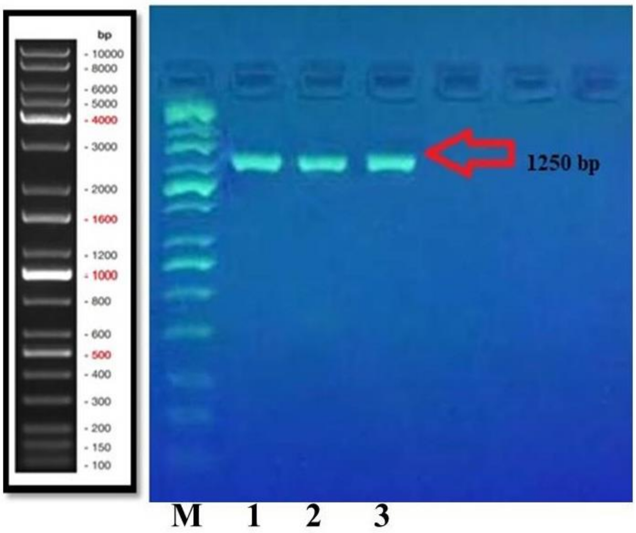

Figure 2. PCR amplification products of 16S rRNA. The genomic DNA of the identified species was used in PCR to amplify the 16S rRNA gene. The PCR products were analyzed on a 2% agarose gel and visualized. Lane M: DNA ladder SiZer^{TM}-1000 Plus; Lanes 1: refers to *Arthrospira platensis*, lane 2: refers to *Limnospira fusiformis*, and lane 3: refers to *Arthrospira Indica.* The 16S rRNA gene (~ 1250 bp) is represented by the red arrow

Table 4. Polymorphism variation in the 16S rRNA gene within the identified Cyanophyceae species

Type of substitution	Location	Nucleotide	Sequence ID with compare	Source	Identities
Transition	441	A\G	ID: MT426015.1	*Arthrospira platensis*	99%
Transition	742	C\T			
Transversion	289	G\C	ID: MT830373.1	*Limnospira fusiformis*	99%
Transversion	378	G\C			
Transition	430	A\G			
Transition	186	A\G	ID: AY575932.1	*Arthrospira indica*	99%
Transversion	551	G\C			
Transition	649	T\C			
Transition	685	A\G			

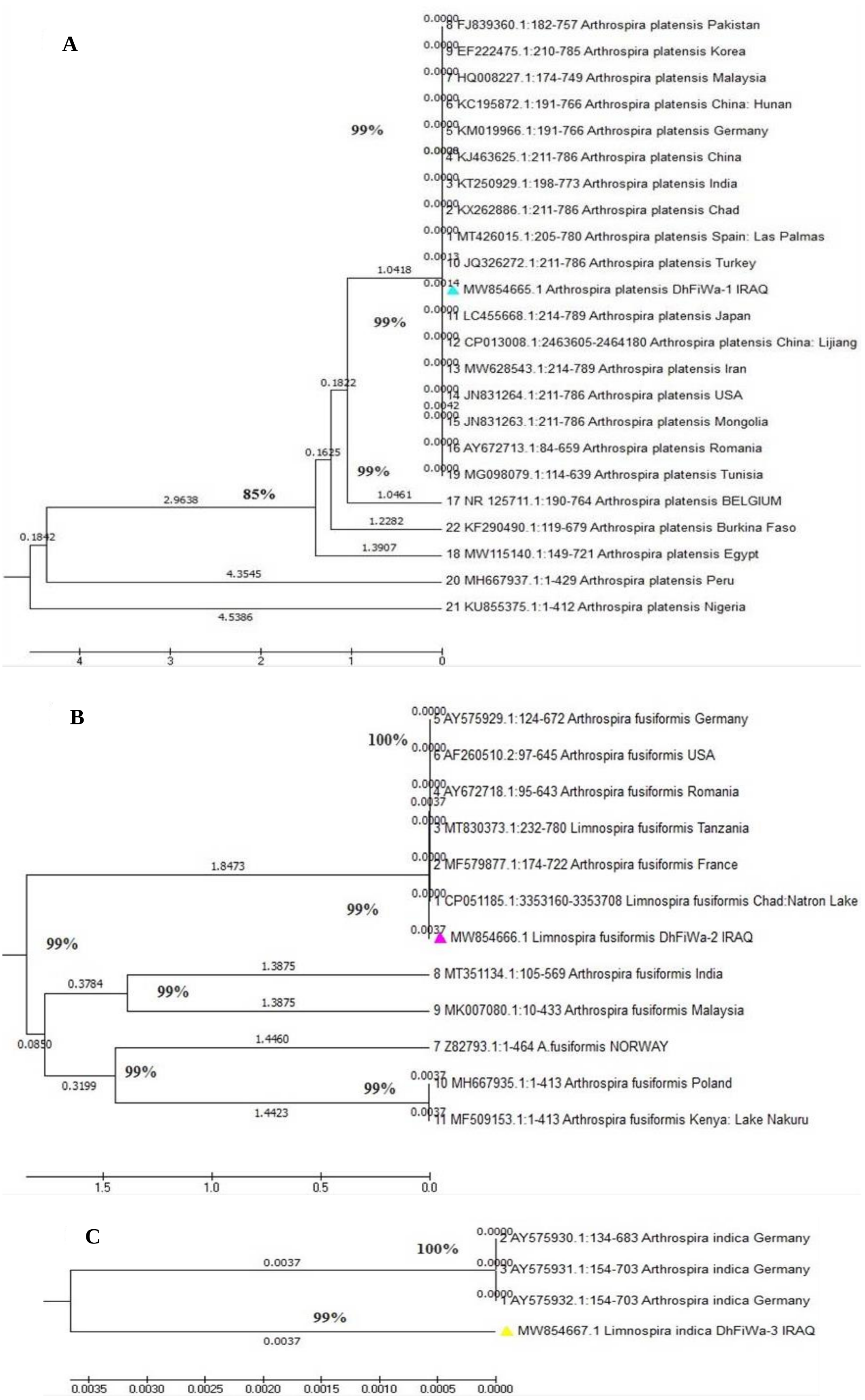

Figure 3. Phylogenetic relationship assembled for Cyanophyceae species. The phylogenetic tree algorithm revealed the overall degree of gene similarity for identified species evolved within the relative species in NCBI. The tree represents the position of three species corresponding to a: *Arthrospira platensis*; b: *Limnospira fusiformis; and c: Arthrospira indica*

The identification of three new species record of Cyanophyceae by this approach is consistent with the study of Shiels et al. (2019), where 25 species of Cyanophyceae algae were detected along the Irish coasts. The 16S rRNA analysis and phylogenetic tree revealed that these species belong to *Oscillatoria* spp., which was then renamed *Kamptonema okenii* and *Tychonema decoloratum*. Our results agree with those of Karan et al. (2017). They declared that the molecular approach works in harmony with the morphological characterization of Cyanophyceae and supports the description data. Using 16S rRNA sequence analysis, seven cyanobacteria species were identified and purified from freshwater sites in Tokat Province in Turkey (Karan et al. 2017). In addition, Wanigatunge et al. (2014), also revealed that both Nostocales and Pleurocapsales are monophyletic group by using morphological study and 16S rRNA sequence analysis. In the study of Ozturk et al. (2018), four isolates belonging to Cyanophyceae, and seven genera were identified in Turkey's lakes and Amazon River.

These findings strongly support the data obtained by the morphological characterization system. In contrast, in a previous study by Al Hassany et al. (2021), the identification and characterization of 27 species of Cyanophyceae from the University of Baghdad campus were performed only by a morphological approach.

Moreover, based on the abovementioned results, the detection of *L. fusiformis* in the Iraqi aquatic system paves the way for development a new techniques to operate this photoautotrophic species in different disciplines of biotechnology, agriculture, and economics. In addition, phylogenetic analysis revealed that *L. fusiformis* is placed in the Limnospira clade (Dvorak et al. 2021).

In conclusion, using advanced molecular-based techniques (16S rRNA) to identify new record species in the Iraqi environment is important, and imply the accuracy of identification of micro-algae that is not been identified by morphological study.

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