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Isolation and Molecular Identification of Some Blue-Green Algae (Cyanobacteria) from Freshwater Sites in Tokat Province of Turkey

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ABSTRACT

Collected blue-green algae (cyanobacteria) from freshwater sites throughout Tokat province and its outlying areas were isolated in laboratory environment and their morphological systematics were determined and also their species identifications were studied by molecular methods. Seven different species of blue-green algae collected from seven different sites were isolated by purifying in cultures in laboratory environment. DNA extractions were made from isolated cells and extracted DNAs were amplified by using PCR. Cyanobacteria specific primers were used to amplify 16S rRNA and phycocyanine gene regions using PCR. Phylogenetic identification of species were conducted by evaluation of obtained sequence analysis data by using computer software. According to species identification by sequence analysis, it was seen that molecular data supports morphological systematics.

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Introduction

Cyanoprokaryotes are unique among microorganisms due to having chlorophyll a and their ability to make photosynthesis (Singh et al., 2011). They have gram negative cell walls and their peptidoglycan layers are ranging in 10–200 nm of thickness (Gademan and Portman, 2008). Dominant pigments present in cyanobacteria are chlorophyll a, chlorophyll c and phycocyanine which contributing to their distinct blue-green colorization. Additionally they have different kinds of color pigments such as β-carotene and xanthophyll. Also some cyanobacteria species contains phycoerythrin pigments which is one of phycobilin bilanes and attributed to red colorization (Altuner, 2010).

Cyanobacteria can either be unicellular or aggregates of many cells forming a colony. Those of unicellular cyanobacteria mostly assume forms of bacilli, discoidal, actinoidal or fibrillary shapes whereas colony forming cyanobacteria mostly assume forms of spherical shapes (Whitton and Potts, 2000). Certain cyanobacteria species contain special cells called as heterocyst which are smaller than akinete and responsible for fixating free nitrogen of air. These cells are among cells forming Tallus fiber and only present in cyanobacteria (Sukatar, 2002; Altuner 2010). Morphological traits are taken as basis for classification of cyanobacteria. However, morphological traits might be altered due to changes in environmental and developmental conditions. Strain diversity in cultures can be inhibited by using elective culture conditions. These reasons are making the development of molecular techniques for identification of cyanobacteria a necessity (Lyra et al., 2001).

Most frequently used marker gene is 16S rRNA gene for both identification of microorganisms and evaluation of relationships between them. While 16S rRNA gene contains many evolutionarily conserved sequences, many species specific variable sequences are also present in 16S rRNA gene. Species level identification is possible by amplifying these variable sequences using PCR. Precise identifications of species of *Geitlerinema* strain PCC 9452 (*Microcoleus* sp. strain 10 MFX) and *Oscillatoria limnetica* have been shown by amplification of 16S rRNA genes (Boyer et al., 2001). *Cylindrospermum* Kutz. Ex. Bornet et Flahault differentiated from Nostocacea family due to their terminal heterocysts and paraheterocystic

akinetes and 45 taxa of this genus identified (Guiry and Guiry, 2014; Johansen et al., 2014). Even though less frequently used, some researchers are using protein coding gene regions of rpoB, rpoC1, recA, rpoD1 and nifH as alternative templates for phylogenetic analysis of cyanobacteria (Saker et al, 2005; Premanandh et al, 2006). In another study, strains of *Synechococcus* have been determined by phylogeny using phycocyanine gene sequences (Robertson et al, 2001). In this study it is aimed to isolate, purify and molecularly identify various cyanobacteria collected from different freshwater sites on riverbank of Yesilirmak in Tokat Province of Turkey.

Material and Methods

Sample collection

Seven different stations were chosen on pelagic sites of Yesilirmak riverbank throughout Tokat province of Turkey and its outlying freshwater branches. Coordinates of chosen sites were determined by using geographical positioning system (GPS) and presented in Table 1. Collected freshwater samples were transferred into 1 L plastic canisters and were delivered to microalgae culturing laboratory.

Cyanobacteria Isolation

Delivered freshwater samples were first tromped by filter paper (GF/C Filter, Whatman, GE Life Sciences, PA; USA) and transferred into petri dishes. Filtrated samples were sterilized under UV light for 10 min in a sterilization chamber. Samples in petri dishes were incubated in enrichment solution (F/2 solution) until the identification stage and dishes were kept in acclimation chamber (Sanyo MLR 351, Sanyo, Tokyo, Japan) at 26°C under 2465 lux illumination with 12:12 hours light/dark cycle (Andersen, 2005; Teneva et al, 2012). Morphological traits were examined under light microscope (Olympus CX31-P, Olympus, Japan). Species going to be isolated were determined and mechanically isolated under inverted microscope by using micropipette and microinjection (Rai and Rajashekhar, 2016). Following the mechanical isolation, strains were inoculated into 1.5% agar and appropriate nutritional media (Bristol BG11 and BG11°, HiMedia Labs, Mumbai, India) by using line inoculation method. Procedures were repeated until only one strain remain isolated and pured (Galhanoa et al, 2011).

Morphological Characterization

Morphological differentiations based on either strains were filamentous or unicellular and if filamentous strains contained heterocysts were conducted first. Widths and heights of examined strains were measured by using micrometer apparatus in microscope ocular and photographs were taken. Systematics of purified strains were done in accordance to algae database (accessed online at http://www.algaebase.org/).

DNA Purifications

Total DNA extractions from 50–100 mg wet cyanobacteria samples were conducted by using ZR Fungal/Bacterial DNA Extraction kit (Zymo Research, Irvine, CA; USA) in accordance with manufacturer instructions. Amount and purity of extracted DNA were quality checked by using nanodrop spectrophotometry (DeNovix, Wilmington, DE; USA) at 260–280 nm wavelength absorbance.

PCR Procedures

Obtained genomic DNAs were first amplified by using universal bacterial primers for 16s gene region for all the cyanobacteria samples. Universal bacterial primer that were used have sequences for forward primer as 27F: 5'-AGA GTT TGA TCC TGG CTC AG-3' and for reverse primer as 1492R: 5'-TAC GCG CTA CCT TGT TAC GAC-3'. Afterwards, 16s gene region PCR amplification, phycocyanine gene region was conducted by using forward primer of PCβF: 5'-GGC TGC TTG TTT ACG CGA CA-3' and reverse primer of PCαR: 5'-CCA GTA CCA CCA GCA ACT AA-3' just for *Anabaena oryzae* and *Nostoc linckia* taxon. Roche FastStartTaq DNA Polymerase kit (Roche Life Sciences, Istanbul, Turkey) was used for PCR reactions. PCR mixtures were prepared in 0.2 ml sterile PCR tubes by additions of following reagents: 2.5 µl 2 mM 10x PCR buffer; 0.75 µl 10 mM dNTP; 2.5 µl 0.2 µM 10x primers (separately for each primer); 0.25 µl 2 U Taq DNA Polymerase; 100 ng genomic DNA. Total mixture volume add up to 25 µl by filling with ddH_2O. DNA amplifications were done using thermal cycler (Bio-Rad Turkey, Istanbul, Turkey) in following configuration for 30 cycles: Initial denaturation at 95°C for 4 min; Denaturation at 95°C for 55 sec; Annealing at 60°C for 45 sec; Extension at 72°C for a minute. At the last step of amplification, a final extension at 72°C for 7 min was applied. PCR products were subjected to electrophoresis inside 1.5% agarose gel by applying 80 V current for 45 min. Then, stained by ethidium bromide and screened under UV transilluminator cabin (Saker et al, 2005; Premanandh et al, 2006; Green et al, 2008).

Sequencing and Phylogeny

The PCR products of 16s rRNA and phycocyanin gene regions were sequenced and edited at REFGEN (Gene Research and Biotechnology Ltd. Sti., Ankara, TURKEY). Additional sequences (nineteen phycocyanin gene and seventeen 16s rRNA gene sequences, Table 2) for further phylogenetic analyses were acquired from GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

All of 16s rRNA and phycocyanin genes sequences were aligned using the Clustal-W algorithm in GENEIOUS version 10.1.3 (http://www.geneious.com, Kearse et al., 2012).

Limnothrix redekei (HE974998.1) and *Cyanothece sp.* (CP002198.1) were used as outgroups in analyses. Phylogenetic trees were constructed using the maximum likelihood (ML) algorithm presented in RAxML plugin (http://www.geneious.com/plugins/raxml-plugin). The GTR+I+G evolutionary model of substitution was used for ML using parameters (base frequencies, rate matrix of substitution types, and shape of gamma distribution) estimated from the data and trees was assessed using 1000 bootstrap replicates.

Table 1 GPS coordinates of research stations in Tokat province of Turkey

Station numbers	Samples	Location Names	Coordinates
1	*Geitlerinema carotinosum*	TEIAS Building	49º 19' 49.12" N, 36º 34' 2.06" E
2	*Nostoc linckia*	State Hospital	40º 19' 45.655" N, 36º 33' 45.06"E
3	*Oscillatoria limnetica*	Songut Town Riverbank	40º 19' 51.27" N, 36º 23' 4.69" E
4	*Chroococcus minutus*	Kat Town Riverbank	40º 17 40.19" N, 36º 19 28.81" E
5	*Cylindrospermum sp.*	Campus NE region	40º 20' 4.39" N, 36º 28' 37.46" E
6	*Phormidium sp.*	Campus North region	40º 19' 50.85" N, 36º 28' 37.46" E
7	*Anabaena oryzae*	Campus Heat Center, North	40º 19' 43.77" N, 36º 28' 22.26" E

Table 2 The list of GenBank data that we used for analysing the sequence data sets obtained from our 7 cyanobacteria samples

	Organism name	Strain	Accession no
16s rRNA	*Chroococcus minutus*	SAG 41.79	KM019988.1
	Chroococcus minutus	CCALA 055	GQ375047.1
	Chroococcus sp.	JJCV	AM710385.1
	Cylindrospermum alatosporum	SAG 43.79	GQ287650.1
	Cylindrospermum muscicola	SAG 44.79	KM019946.1
	Cylindrospermum stagnale	PCC 7417	NR_114701.1
	Cylindrospermum stagnale	PCC 7417	NR_102462.1
	Geitlerinema carotinosum	AICB 37	AY423710.1
	Geitlerinema pseudacutissimum	ladakh27	KT315938.1
	Geitlerinema pseudacutissimum	P005	JQ712608.1
	Geitlerinema sp.	Sai004	GU935348.1
	Geitlerinema sp.	Sai001	GU935345.1
	*Limnothrix redekei (outgroup)**	CCAP 1459/29	HE974998.1
	Oscillatoria limnetica	MR1	AJ007908.1
	Phormidium autumnale	JR16	KT315936.1
	Pseudanabaena sp.	1tu24s9	AM259269.1
Phycocyanin gene region	*Nostoc azollae*	0708	CP002059.1
	Anabaena cylindrica	PCC 7122	CP003659.1
	Anabaena cylindrica	PCC 7122,	NC_019771.1
	Anabaena planctonica	NIES-816	AY702218.1
	Anabaena sphaerica	UTEX 'B 1616'	DQ439645.1
	Anabaena variabilis	ATCC 29413	AY768465.1
	Aphanizomenon flos-aquae	ST97	FN552349.2
	Aphanizomenon flos-aquae	AB2008/70	FN552369.1
	Aphanizomenon sp.	TR183	AY036900.1
	*Cyanothece sp. (outgroup)**	PCC 7822	CP002198.1
	Cylindrospermopsis raciborskii	-	AF426799.1
	Cylindrospermopsis raciborskii	Florida I	AY078438.1
	Cylindrospermopsis raciborskii	Florida F	AY078437.1
	Nostoc linckia	PACC 5085	AY466120.1
	Nostoc sp.	PCC 7107	CP003548.1
	Nostoc sp.	C21	JN646756.1
	Nostoc sp.	C19	JN646755.1
	Nostoc sp.	PCC 7120	AY768464.1
	Nostoc sp.	MCC2741	KT166439.1

Results

Morphological Observations

According to morphological traits observed under light microscopy, following cyanobacteria strains were purified and their identifications were made. Systematics of purified strains are summarized in Table 3 below.

Chroococcus minutus (Kutz.) Nägeli: Cellular shapes were either ovoid or spherical and they were observed as either unicellular or as colonies of 2–4 cells. All cells were colorless. Those cells with envelopes were measured to have 6–15 µm diameter and those without envelopes were measured to have 4–10 µm diameter as seen in Figure 1A.

Geitlerinema carotinosum (Geitler) Anagnostidis: Cells were organized as resembling fascicules and trichomes were observed as linear and slightly coiled with becoming thinner at the ends. Cells have sizes of 1.5–3 µm width and 3–9 µm height as seen in Figure 1B.

Anabaena oryzae F.E. Fritsch: Trichomes were observed as linear and without envelopes. Cells have sizes of 2.5–4 µm width and 4–8 µm height and have barrel shapes. Heterocysts have shapes of either spherical or ellipsoidal and positioned at or near to terminal ends. Heterocysts have sizes of 3.5–6 µm width and 6–8 µm height as seen in Figure 1C.

Oscillatoria limnetica Lemmermann: Trichomes were observed as either linear or slightly curved. Cells have sizes of 1.3–2.2 µm width and 4–12 µm height. Cell walls were notably become narrower in longitudinal plane and terminal cells were observed as circular. They contain no calyptra as seen in Figure 1D.

Phormidium sp. MBIC10025: Many trichomes were observed as gelatinous or strapped aggregates. They were observed as flowing or bound colonies. They have very thin envelopes or no envelopes. Cells have sizes of 1.2–2.3 µm width and 1.2–3 µm height as seen in Figure 1E.

Cylindrospermum sp. CENA33: Trichomes were observed as unclear aggregates. Heterocysts were observed at terminal ends and seen as spherical. Cells have sizes of 3–5 µm width and 4–6 µm height. Heterocysts have sizes of 5–7 µm width and 6–8 µm height. Akinetes were not observed as seen in Figure 1F.

Nostoc linckia PACC 5085: Cells have 3–4 µm width with barrel shapes and gelatinous with colorization of blue-green to brown. Heterocysts have 5–6 µm width. Akinetes have sizes of 6–7 µm width and 7–10 µm height and resembling spherical or slightly elongated shapes as seen in Figure 1G.

Cyanobacteria species require water, light, CO_2 and simple inorganic compounds for survival (Hageman, 2011). However, cyanobacteria species can grow much faster in certain environmental conditions (Nidhi Gupta et al, 2002). For this reasons, there are specialized growth media for cyanobacteria culturing. In this study we also have defined the best growth media for these seven taxon. Almost each cyanobacteria species grown efficiently in different growth media. Bristol broth, BG11 (w/ $NaNO_3$) and BG11° (w/o $NaNO_3$) growth medias were prepared for culturing of isolated cyanobacteria strains. Bristol broth medium was used for culturing of *Chroococcus minitus* and *Geitlerinema carotinosum* strains. BG11 medium was used for *Oscillatoria limnetica* and *Phormidium* sp. MBIC10025 strains. BG11° medium was used for nitrogen fixating strains of *Nostoc linckia* PACC 5085, *Anabaena oryzae* and *Cylindrospermum* sp. CENA33. All cultures were induced at 25°C under 2465 lux illumination.

Table 3 The systematics information of purified cyanobacteria in our study

Subclass	*Order*	*Family*	*Genus*
Oscillatoriophycideae	Chroococcales	Chroococcaceae	Chroococcus
Oscillatoriophycideae	Oscillatoriales	Coleofasciculaceae	Geitlerinema
Nostocophycideae	Nostocales	Nostocaceae	Anabaena
Oscillatoriophycideae	Oscillatoriales	Oscillatoriaceae	Oscillatoria
Oscillatoriophycideae	Oscillatoriales	Oscillatoriaceae	Phormidium
Nostocophycideae	Nostocales	Nostocaceae	Cylindrospermum
Nostocophycideae	Nostocales	Nostocaceae	Nostoc

Table 4 GenBank accession numbers of Cyanobacteria species in this study.

Phycocyanin sequence			
	Accession number	Species	Strain
1	MF443183	*Anabaena oryzae*	Ind3
2	MF461294	*Nostoc linckia*	PACC 5085
16s rRNA sequence			
	Accession number	Species	Strain
1	MF423476	*Oscillatoria limnetica*	MR1
2	MF423477	*Geitlerinema carotinosum*	AICB 37
3	MF423478	*Cylindrospermum sp.*	CENA 33
4	MF423479	*Chroococcus minutus*	CCALA 055
5	MF423480	*Phormidium sp.*	MBIC10025

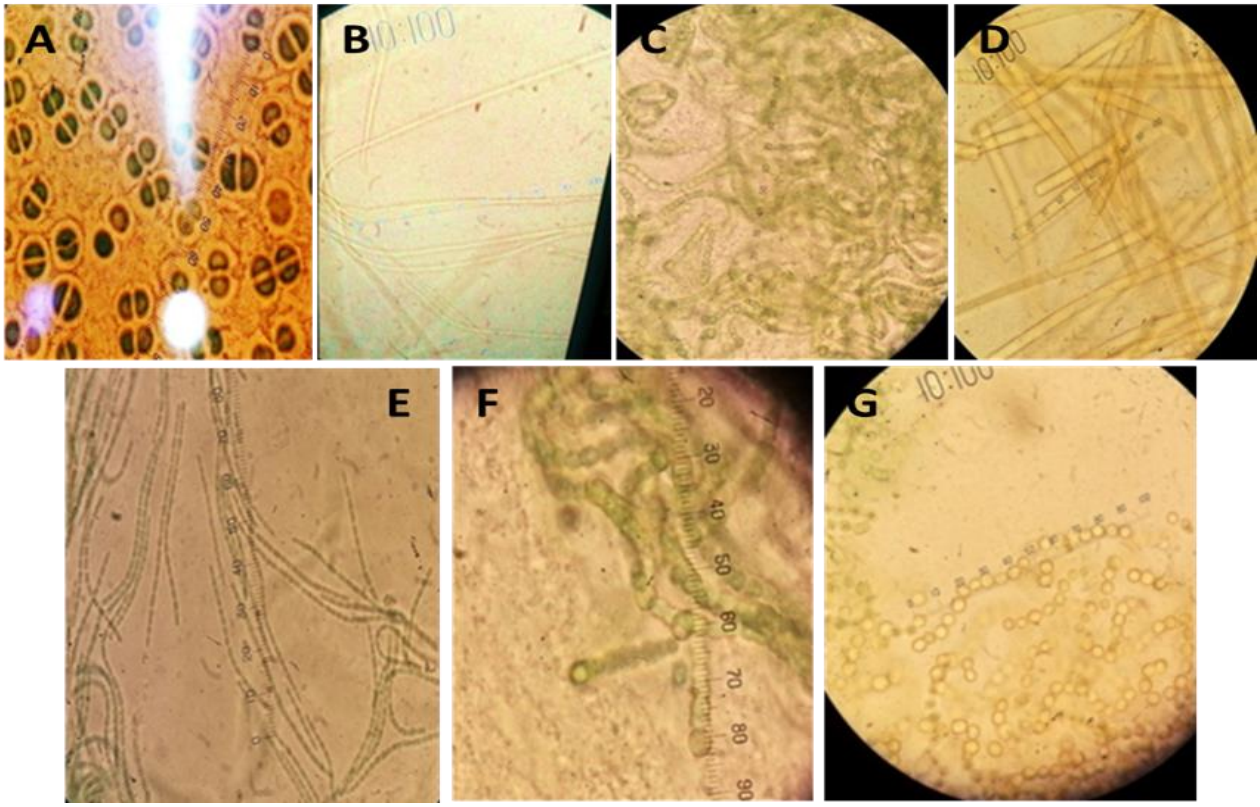

Figure 1 Light microscopy images of strains in our study. **A)** *Chroococcus minutus,* **B)** *Geitlerinema carotinosum,* **C)** *Anabaena oryzae,* **D)** *Oscillatoria limnetica,* **E)** *Phormidium sp*., **F)** *Cylindrospermum sp.,* **G)** *Nostoc linckia*

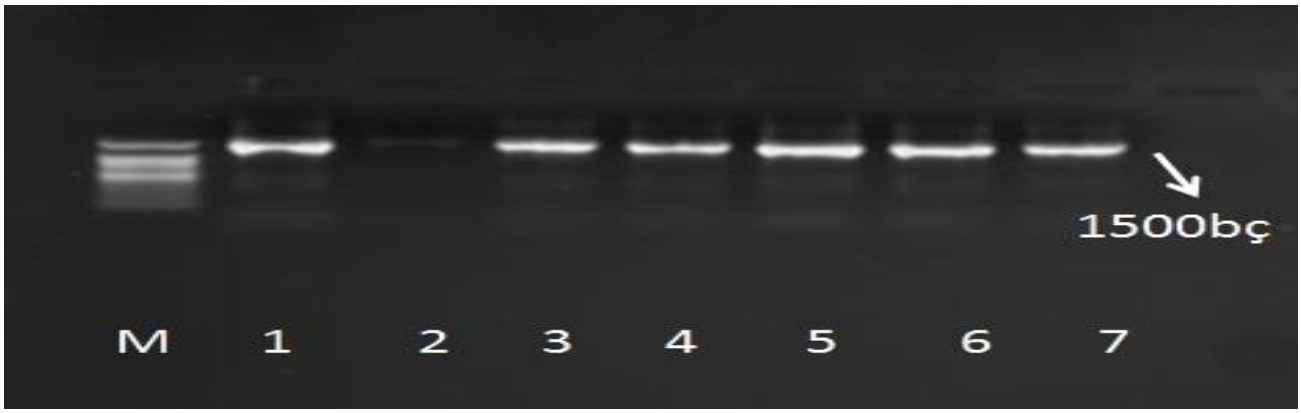

Figure 2: Agarose gel visualization of gene fragments amplified by 27F and 1492R primers. **M** stands for marker. **1:** *Chroococcus minitus,* **2:** *Oscillatoria limnetica,* **3:** *Geitlerinema carotinosum,* **4:** *Nostoc linckia,* **5:** *Phormidium sp.,* **6:** *Cylindrospermum sp.,* **7:** *Anabaena oryzae*

Results from Sequence and Phylogenetic Analyses

PCR amplifications from genomic DNAs of purified strains using cyanobacteria specific universal bacterial primers were conducted as seen in Figure 2. Size of PCR product obtained from primers 27F and 1492R was ≈1500 bp (Figure 2). Afterwards, PCR amplifications of cyanobacteria phycocyanine gene region were conducted by using phycocyanine specific primers for just *Anabaena oryzae* and *Nostoc linckia* species. PCR product sizes for phycocyanine gene regions were observed almost as ≈ 300 bp. Firstly our obtained sequences data were combined with the data from the GenBank database (Table 2). Later separate phylogenetic analyses were performed for the two gene regions by using this combined data set. The Maximum likelihood tree, obtained from the analysis of the 16s rRNA sequences, showed the distinction of genus at high accuracy (Figure 3). This tree also placed the species, morphologically diagnosed in our study, in to the expected correct clades (Figure 3). On the other hand, maximum likelihood tree, obtained from the phylogenetic analysis of the sequences belongs to the phycocyanin gene region, supported the morphological description of our cyanobacteria samples with a molecular point of view, although the clades were not fully separated (Figure 4). GenBank accession numbers of Cyanobacteria species in this study are given in Table 4.

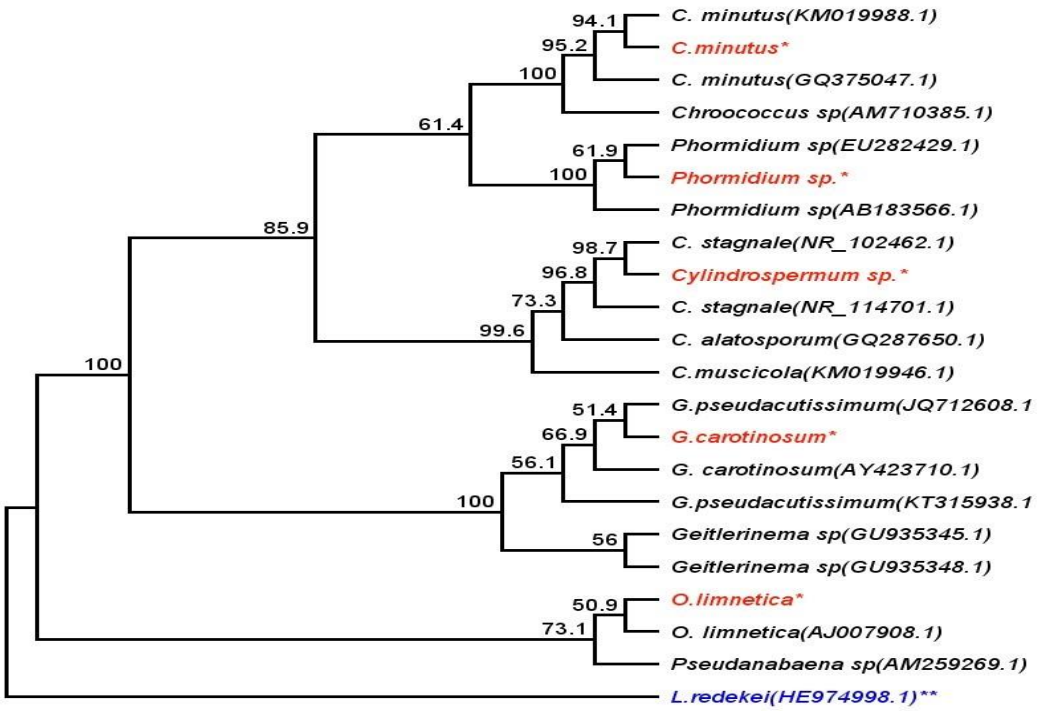

Figure 3 Maximum Likelihood tree based on 16S rRNA gene sequences (1500 bp). (Sequences from other species were obtained from GenBank. The examples signed with *and red colour are the data of our study from Tokat province. The example signed with ** and blue colour is out group, *Limnothrix redekei* CCAP 1459/29). Node robustness was assessed by performing 1000 bootstrap replications.

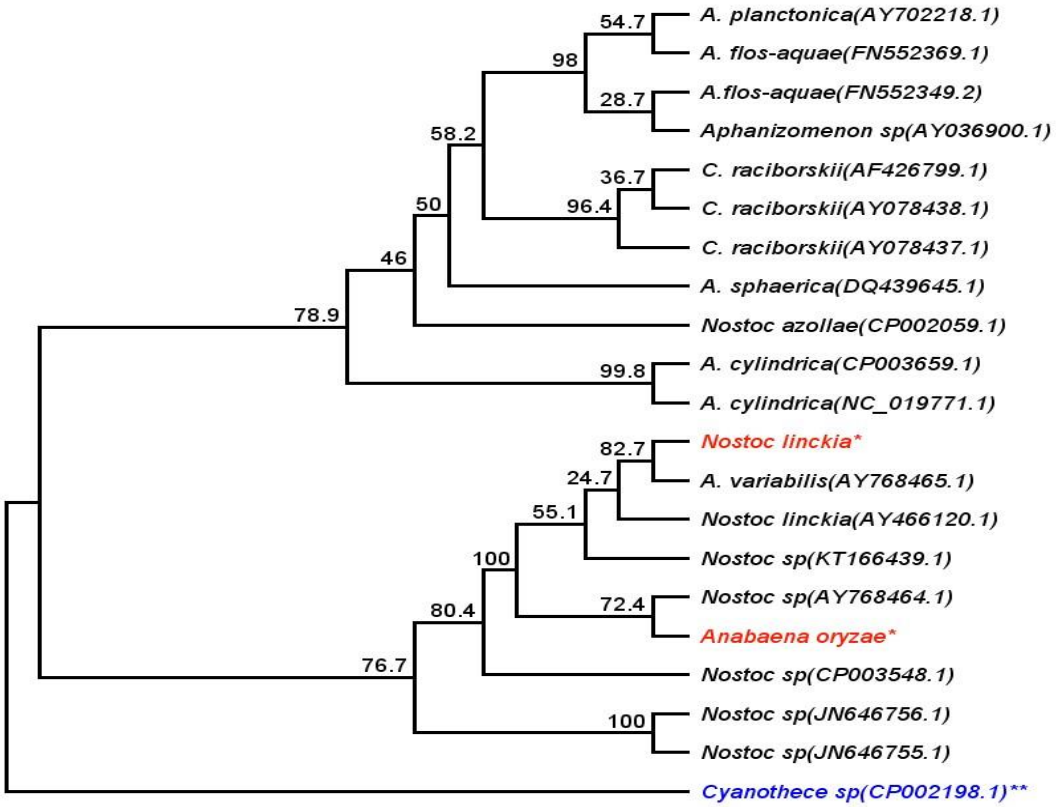

Figure 4 Maximum Likelihood tree based on Phycocyanin gene region. (Sequences from other species were obtained from GenBank. The examples signed with * and red colour are the data of our study from Tokat province. The example signed with ** and blue colour is out group, *Cyanothece* sp. (CP002198.1)).

Discussion

Taxonomy and classification of cyanobacteria species have been made according to cell morphology since 19th century. However, many species identified by morphological traits are not concise enough and numerous genus need major revisions. Recently, researchers are highlighting the importance of molecular characterization of bacteria for concise results (Wehr and Sheath, 2003; Hasler et al, 2012). Recent approaches on cyanobacteria taxonomy are multidisciplinary methods including combination of morphology and ecology coupled with molecular characterization methods. Therefore, molecularly identified species must be in accord with phenotypic and ecological data (Hoffmann et al, 2005; Komarek et al, 2010; Komarkova et al, 2010). Filamentous cyanobacteria such as Geitlerinema, Microcoleus and Phormidium species have been taxonomically identified using both morphological and molecular characterizations. Notably these genus and respective species are mistaken morphologically to each other and specie clarifications were made by comparing 16S rRNA sequences (Hasler et al, 2012).There are many studies conducted in Turkey in order to determine algae flora of Turkey (Ulcay, 2005). Nonetheless, molecular characterization studies of cyanobacteria species are quite few. Yuksel et al. (2009) were conducted a pioneer study in molecular characterization of cyanobacteria in Izmir province of Turkey. In their study researcher first isolated and purified 4 cyanobacteria species from thermal spring sites located in Izmir province of Turkey and molecularly identified these species on 16S rRNA sequences by using specific 27F and 809R primers for PCR amplifications. Nevertheless, there is still not enough studies present in molecular characterizations of cyanobacteria in Turkey and literature has mostly morphological systematic studies.

Conclusion

Our study is important as being an addition and a contribution to fill this gap about the molecular characterization of cyanobacteria in literature. With this study, isolation and purification of cyanobacteria species present in freshwater sites in Tokat province of Turkey were achieved for the first time. Morphological systematics of purified strains were conducted according to their morphological traits. Molecular systematic data of cyanobacteria species were obtained by using molecular characterization methods. Luckily, the results of molecular characterization have supported the morphological classification results of seven cyanobacteria species in this study. So the results related to the molecular analyses and the morphological description were in harmony, which did not create confusion in this study. This may not always be the case. Sometimes morphological systematic study results with molecular systematic study results may not support each other. It may be a little more difficult to do systematic and species diagnosis in this kind of situations. However, when doing classification studies, exploiting molecular genetic analysis as a kind of character is a necessity for the identification of suspicious taxa.

All in all, we believe that this study is a valuable addition to recent cyanobacteria literature by combining multidisciplinary approaches.

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