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MORPHOLOGICAL AND PHYLOGENETIC DIVERSITY OF CYANOBACTERIA IN FOUR HOT SPRINGS OF IRAN

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Hot springs have been a subject of intense discussion for biologists in the last decades. Thermophilic cyanobacteria are scientifically valuable for their analogy to the ancient life forms on earth and also as a source of thermostable biocompounds. Exploration of their biodiversity is an important step towards these goals. In a revision of the cyanobacteria in hot springs of Iran, four hot springs in the Hormozgan (Bandar Abbas) and Mazandaran provinces of Iran were sampled from October 2011 to July 2012. In total 43 species belonging to 20 genera, 11 families and 5 orders of the planktonic cyanobacteria were identified. Among these taxa *Chroococcus* with 7, *Oscillatoria* and *Phormidium* with 5 species found to be more predominant and noticeable among other genera. All of these taxa are new records for studied hot springs. We developed a combined molecular and morphological approach to identification of cyanobacteria. In this study seventeen populations and 13 morphological characters were analyzed. Molecular study based on 16S rRNA gene sequence does not disrupt morphological information and it confirms the separation of studied taxa according to morphological characters.

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Key words. Cyanobacteria, hot spring, morphological diversity, 16S rRNA, phylogeny.

مطالعه تنوع مورفولوژیکی و فیلوژنی سیانوباکتری های ٤ چشمه آب گرم در ایران

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چشمه‌های آب گرم در سال‌های اخیر از سوی زیست‌شناسان بسیار مورد توجه واقع شده‌اند. سیانوباکتری‌های ترموفیل بدلیل شباهت با فرم‌های زندگی اجدادی در زمین و همچنین به عنوان منبعی از ترکیبات زیستی پایدار در برابر گرما، از ارزش علمی بالایی برخوردار هستند، و بررسی تنوع زیستی آنها گام مفیدی در جهت رسیدن به اهداف ذکر شده می‌باشد. در این تحقیق سیانوباکتری‌های موجود در ٤ چشمه‌ی آب گرم در ایران مورد مطالعه قرار گرفت. در این تحقیق ٤٣ گونه متعلق به ٢٠ جنس و ١١ تیره شناسایی شد. علاوه بر مطالعه بر اساس خصوصیات مورفولوژیک، جهت شناسایی دقیق‌تر سیانوباکتری‌های شناسایی شده، مطالعات مولکولی بر اساس توالی ژنومی 16S rRNA انجام شد. نتایج بدست آمده در هر دو روش بکار برده شده هم راستا و تایید کننده بکدیگر بودند.

INTRODUCTION

Cyanobacteria, the only oxygenic photosynthetic bacteria, are interested due to their ability to grow in high temperatures and in other extreme environments (Sompong & al. 2008). Inability to culture some cyanobacteria strains in laboratory conditions as well as the limited range of morphological features in this group of microalgae, complicate classification of these microorganisms (Ward & Castenholz 2000). During most of the 19th and 20th centuries, cyanobacterial

Fig. 1. Map of sampling locations and hot springs.

Table 1. Some physico-chemical parameters of four hot springs of Iran.

Parameters	Studied Sites			
	Geno	Chahahmad	Khamir	Ramsar
Location	Hormozgan province, Bandar Abbas city	Hormozgan province, Khamir city	Hormozgan province, Khamir city	Mazandaran province, Ramsar city
Latiude/Longitude	26º 56´ 55˝ N 55º 32´ 27˝ E	26º 56´ 31˝ N 55º 26´ 17˝ E	26º 56´ 46˝ N 55º 33´ 23˝ E	36º 55´ 20˝ N 50º 39´ 30˝ E
Temp (°C)	40	39	37	50
pH	7/2	6/9	7	6/8
EC (ms cm^{-1})	14/02	20	20	13.3
Na^{+}(mg L^{-1})	1600	2400	2200	410
K^{+}(mg L^{-1})	580	400	200	240
Ca^{++}(mg L^{-1})	800	3000	1440	1100
Mg^{+}(mg L^{-1})	475	950	710	1100
Cl^{-} (mg L^{-1})	4560	18520	9210	3504
PO_4^{-3} (mg L^{-1})	14/02	300	400	400
SO_4^{-2} (mg L^{-1})	1600	152	1374	1800
NO_3^{-} (mg L^{-1})	0.7-1.0	1.1- 1.3	1.3-1.4	0.8-13.6
Total alkalinity (mg L^{-1})	135	150	160	244

taxonomy was based on morphology (Geitler 1932; Elenkin 1938; Desikachary 1959; Starmach 1966; Kondrateva 1968). The taxonomic position of many morphologically defined species is unclear and some genera urgently need revision (Komárek & Anagnostidis 1998, 2005). Moreover, the situation of them is complicated by a conflict between bacteriological and botanical nomenclature and taxonomic practices (Stanier & al. 1978; Rippka & al. 1979; Castenholz 2001). The most progressive system utilize a polyphasic approach (Anagnostidis & Komárek 1985, 1988; Komárek & Anagnostidis 1986, 1989; Komárek 1994, 2003, 2011), which includes a combination of morphological, ecological and molecular characteristics. Resent molecular data support the validity of many genera, e.g. *Planktothrix*, *Pseudananabaena*, *Microcystis* and *Spirulina* (Willame & al., 2006; Komárek 2003, 2010) as defined by Komárek & Anagnostidis (1998, 2005), but at the species level we often have insufficient morphological, ecological and molecular data for reliable recognition of species diversity. Thermophilic cyanobacterial mat

Table 2. Morphological characters and their character states in studied taxa.

Number	Character	Character Code
1	Life form	0- unicellular, 1- filamentous, 2- pseudo filamentous
2	Vegetative cell shape	0- spherical, 1- rod shape, 2- disco shape, 3- other forms
3	Sheathe	0- without sheathe, 1- with thick sheathe, 2- with thin sheathe
4	Filament form	0- without filament, 1- flat, 2- spiral
5	Apical cell form	0- without apical cell, 1- flat, 2- headed
6	Duplicate form	0- dichotomy, 1- homogony
7	Cell form	0- longer than width, 1- shorter than the width, 2- equal length with a width
8	Cross wall	0- without cross wall, 1- thick cross wall, 2- thin cross wall
9	Granule wall	0- without granule, 1- with granule
10	Spiral form	0- without spiral, 1- spiral loose, 2- spiral massive
11	Filament width	0- without filament, 1- 10 to 45 µm, 2- 5 to10 µm, 3- 2 to 5 µm, 4- 0.6 to 2 µm
12	Cell width/length	0- 0.1, 1- 1, 2- 1 to 2
13	Cell width	0- 1 µm, 1- 1 to 3 µm, 2- 3 to 5 µm, 3- 5 to 10 µm

communities occur in geothermal springs of neutral/alkaline pH and at temperatures of up to ~ 74 °C. Sometimes shared simple morphological characters as well as limitations of cultivation have made microscopic studies usefulness. One way to better characterize these morphologically similar species is to use molecular diversity information (Sompong & al. 2008; Ferris & al. 1997; Nubel & al. 1997; Norris & al. 2002). It should also be noted that in studies where near-complete 16S rRNA gene sequences have been used, conflicts between morphological and molecular identification of some cyanobacterial sequences have been found (Hongmei & al. 2005). Although a few hot springs of the world have been studied, distinct phylogeographic groups have been extensively studied only in the continental USA, Iceland, New Zealand, and some countries in Asia (Papke & al. 2003; Yim & al. 2006). Studies of the cyanobacterial mats in hot springs in Iran have been minimally investigated. In present study, an attempt is to explain the morphological and phylogenetical diversity of cyanobacteria in four hot springs of Iran using numerical taxonomy and molecular methods based on 16S rRNA gene sequences.

MATERIALS AND METHODS

Sampling

Water samples were collected from four hot springs in Geno, Khamir, Chahahmad and Ramsar (Table 1, Fig. 1). The physico-chemical properties of the water from each sampling site were measured. At each hot spring, the sites were surveyed to characterize the temperature profile range, and water temperature clines were established in the range of 37-50°C.

Physico- chemical characteristics of the spring water

Water samples were collected in a 1 liter polyethylene bottle from depth 10-30 cm and 30-50 cm from the water edge (Masoudi & al. 2011). Spring water temperature was measured at the site by laboratory thermometer (mercury thermometer).The pH of the water samples was determined by digital pH meter (DENVER pH meter) and electrical conductivity determined by a conductivity bridge. Mineral content of water samples such as potassium (K^+), calcium (Ca^{++}) and sodium (Na^+) were determined by flame photometer. Concentration of nitrate (NO_3^-) and sulphate (So_4^{-2}), dissolved oxygen (DO), chloride (Cl^-) and inorganic phosphate (PO_4^{-3}) also were determined for water samples following the methodology outlined by Clescerl & al. (1995) (Table 1).

Morphological study

Samples were streaking on agar nitrate free BG-11 medium (Stanier & al. 1971), and incubated in a culture chamber at 25° and a 12/12 h light-dark cycle at artificial illumination (37- 46 µmol m^{-2} s^{-1}) for two weeks. Isolation involved from removing colonies that developed in medium and observed under light microscope. Taxonomic determination was carried out by light microscopy (Olympus, Model BM-2) and based on Desikachary (1952) and Prescott (1962). Identification was carried out by morphometric method. Seventeen morphological characters and numerical taxonomic studies were used for classifying the various species of cyanobacteria. Taxonomic analysis was performed with cluster analysis and principal component analysis. A data matrix based on

the coded multiple states of characters were used in this study (Table 2). Cluster analysis using the UPGMA method (unweighted pair-group method with arithmetical averages) was carried out. Phenetic relationships among the species were constructed. All analyses were carried out using SPSS (Ver.16).

DNA extraction

The bulk of each cyanobacterial culture isolation was extracted by genomic DNA extraction kit AccuPrep (Bioneer). 25 mg of each sample in a 1.5 ml tube with 200 µl lysis buffer and 20 µl of Proteinase K were homogenized. The tubes were incubated for 1 h at 60 °C. 200 µl Binding Buffer was added. After mixing gently for 10 minutes, the samples were incubated at 60 °C. 100 µl Isopropanol was added. The supernatant was then transferred to a 2 ml tube. After a final gentle mixing centrifugation for 5 min at 8,000 rpm, 150 µl elution buffer was added. Extracted DNA was harvested at -20 °C temperature.

PCR amplification of cyanobacterial 16S rRNA

For DNA amplification, the 16S rRNA gene regions, approximately 600 bp in length, were amplified by PCR using the A2 (5- AGAGTTTGATCCTGGCTCAG-3) and S8 (5- TCTACGCATTTCACCGCTAC-3) primers (Giovannoni & al. 1988). Each reaction contained 2.8 µl $MgCl_2$, 150 mM dNTPs, 0.5 µM of each primer, approximately 50 ng template DNA, 3 µl *Taq* polymerase in a total volume of 100 µl. For PCR amplification cycle using the cyanobacterial primers was 4 min at 95 °C, then 30 cycles of 1 min denaturation at 95 °C, 1 min annealing at 59 °C, 2 min extension at 72 °C, and a final extension of 8 min at 72 °C (Fig.2).

Phylogenetic analysis

Multiple alignments were created with reference to the selected GenBank sequences using BioEdit which implements the Clustal W multiple alignment algorithm. Sequences were aligned with the software MEGA, version 5. The Neighbor-joining method was used to compute evolutionary distances in present study. In this program, bootstrap analysis was used to evaluate the tree topologies by performing 1000 resamplings. The tree was rooted using the *Bacillus subtilis* 16S rRNA sequence as an out-group.

RESULTS

Morphological analysis

In this study, 43 distinct morphospecies of cyanobacteria were characterized using light microscopy (Fig. 5). All morphospecies and their distribution are listed in Table 3.

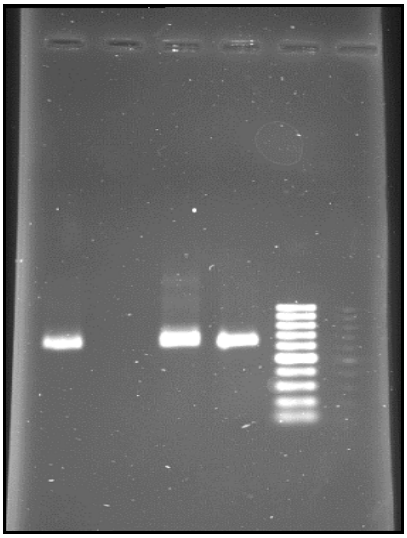

Fig. 2. GE banding patterns of O.sub (Oscillatoria subbrevis), J.met (Jaaginema metaphyticum) and S.aqu (Synechocystis aquatilis).

The lowest species diversity was observed in Nostocales and the highest species diversity belonged to Oscillatoriales. Among the genera were identified, *Oscillatoria*, *Phormidium*, *Chroococcus* and *Spirulina* showed the highest number of species. *Oscillatoria subbrevis*, *Jaaginema metaphyticum* (Syn.: *Oscillatoria angusta*) and *Spirulina subsalsa* was observed in 3 stations of each hot spring. In present study, 32 taxa from Geno hot spring, 16 taxa from Chahahmad, 14 taxa from Khamir and 9 taxa from Ramsar were identified. The highest diversity of cyanobacteria was found in the Geno hot spring. The result also revealed that seasonal variation can affect the species diversity. Highest species diversity was recorded in autumn. Several taxa of these microorganisms could not be grown in the enrichment cultures and in the laboratory condition. The present study has been done by focusing on the importance of morphological characters to recognize cyanobacterita, by numerical taxonomic study. In total 17 morphospecies of cyanobacteria from 4 hot springs by using 13 most stable quantitative and qualitative characters used for clustering analysis. Results of this study showed that the selective morophological characters separate genera but they did not separate them in species rank. Although, single cell strains were separated completely from filamentous forms. Some of filamentous samples due to their morphological similarities were not completely separated from each other. In numerical taxonomic study according to morophological characters, it was consedered that the species *Jaaginema metaphyticum* (Syn: *Oscillatoria angusta*) and *Geitlerinema*

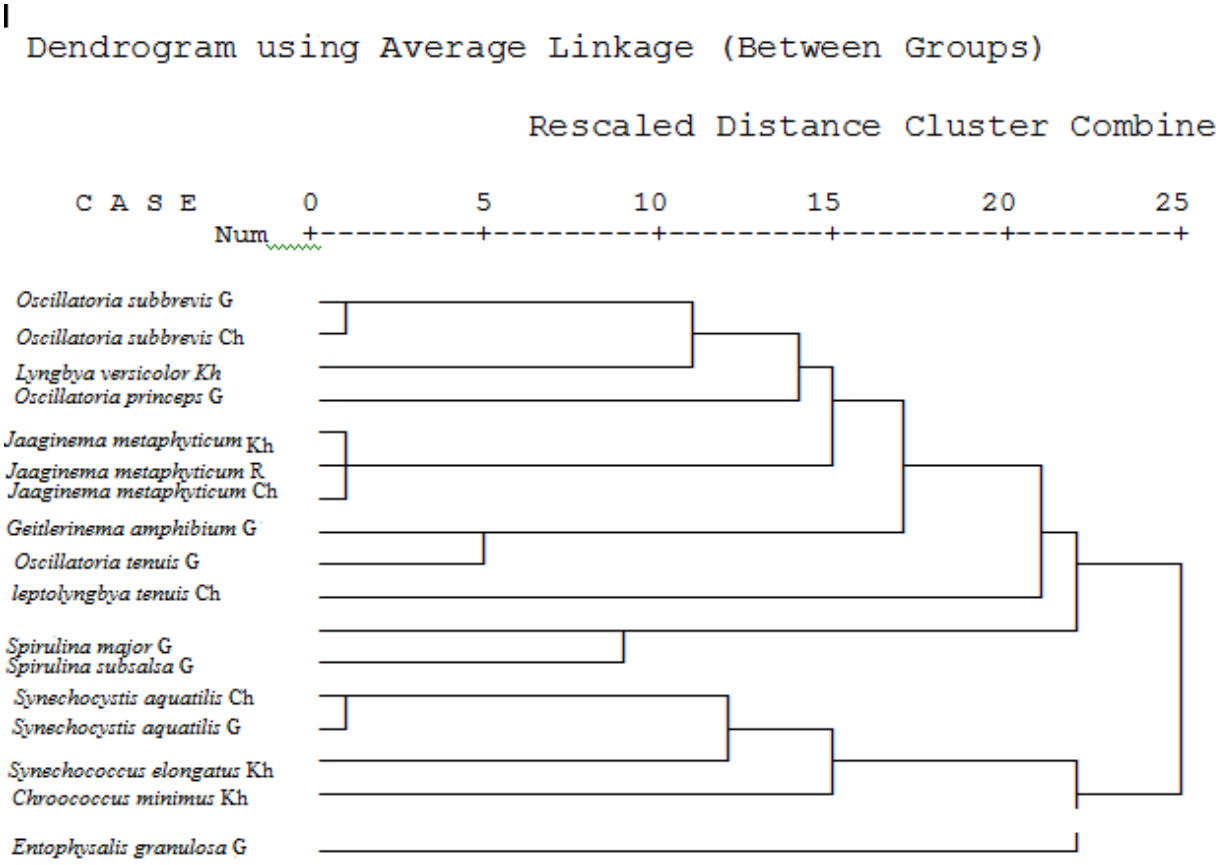

Fig. 3. Hierarchical cluster analysis dendrogram of cyanoprokaryota taxa based on morphological characters using UPGMA method.

amphibium (Syn: *Oscillatoria amphibian*) are grouped to their pervious genus *Oscillatoria* (Fig. 3).

To discover the most variable characters among the several morphological features, PCA carried out. The analysis revealed that the first five factors comprise about 88.2% of total variance. In the first factor with about 25% of total variance, characters of trichome, vegetative cell, form of spiral and kind of proliferation possessed the highest positive correlation. In the second factor with about 24% of total variance, characters of apical cell shape and filament width possessed the highest positive correlation (Table 4, 5).

Phylogenetic analysis

In this section, for drawing cladogeram by using the analysis of data of sequence of genome in 16S rRNA region, the genome sequences of 22 samples were used (Table. 6). Among the studied samples, 11 samples belonged to cyanobacteria of hot springs from four regions of Geno, Chahahmad, Khamir and Ramsar. In the phylogenetic tree by algorithm neigbhor-joining based on gene sequence 16S rRNA the distinctive primary clustering of the sample of out-group from taxa of photosynthetic prokaryotes from cyanobacteria were analyzed. In clusters, the cluster containing all of the analyzed cyanobacteria, two minor groups are recognizable. In one of minor clusters, all the unicellular samples belonged to *Synechococcus*, *Synechocystis*, *Chroococcus* with the bootstrap value of 99% placed in a unique group, and in other cluster *Spirulina* and *Oscillatoria* were presented (Fig. 4).

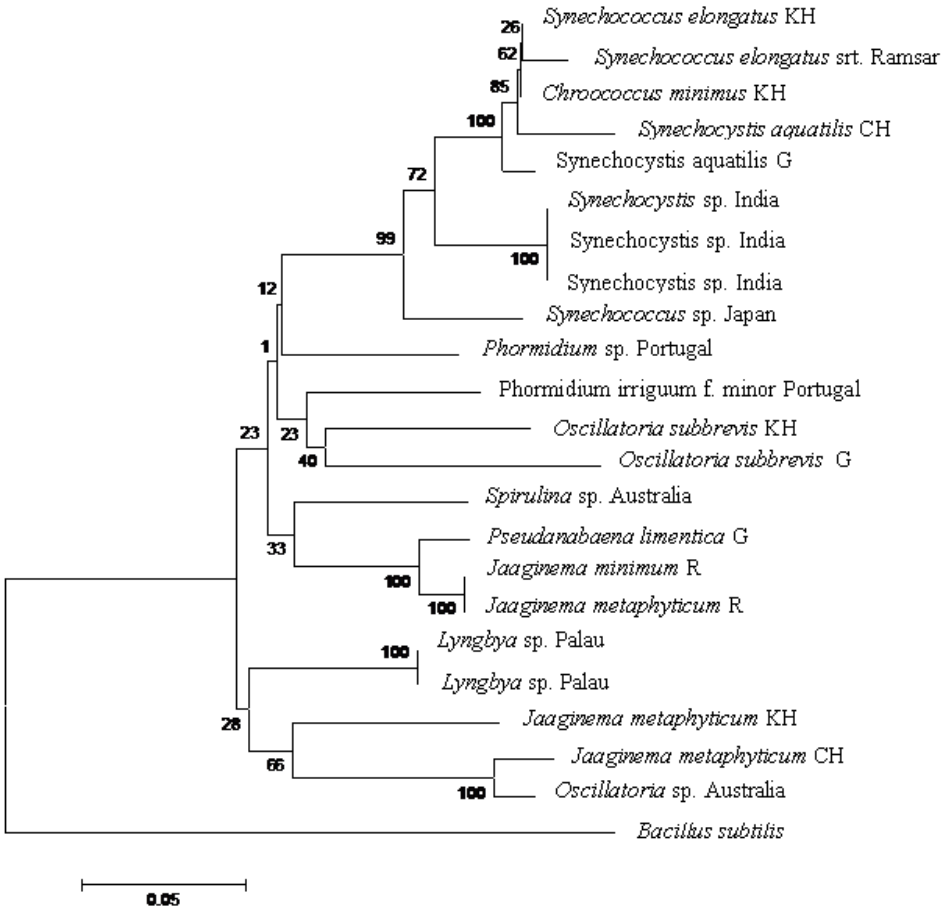

Fig. 4. Phylogenetic relationship for the thermophilic cyanobacteria was constructed using partial 16S rRNA gene sequences.

DISCUSSION

Temperature is one of the most important parameter for cyanobacterial species diversity in microbial mat of hot springs. The studies revealed that cyanobacterial diversity and complexity decreased with increasing temperature (Ferris 1996; Ferris & Ward 1997; Ward 1998). Skimisdottir (2000) showed that in thermal gradients from 50 ˚C to 75 ˚C, the layered mats are characterized by the presence of unicellular forms like *Synechococcus*. The cyanobacterial mats occurring at the lower end of thermophily (40-50˚C) are often dominated by morphologically defined filamentous cyanobacteria like *Phormidium, Oscillatoria, Pseudanabaena, Calothrix* and *Fischerella* (Ward & Castenholz 2002; Sompong & al. 2005). However, Norris & al. (2002) reported that cyanobacteria such as *Synechococcous* also co-occurs with other unicellular and filamentous forms at lower temperature. In the present study, *Synechococcous* have been found in the mats that grow between 30-40 ˚C. Some workers also focus on the role of pH and combined nitrogen (especially ammonium), on the species distribution in cyanobacterial mat community below 60 ˚C (Ward & Castenholz 2002; Sompong & al. 2005). Results of this study also support the previous studies. Diazotrophic cyanobacteria are able to colonize in the springs where nitrogen levels are lower than proper condition for the other taxa. Conversely, they may be out-competed by non diazotrophic cyanobacteria in spring with sufficient combined nitrogen (Ward & Castenholz 2002). In this study in hot springs with high levels of combined nitrogen, diazotrophic cyanobacteria are absent. Results of this study showed that cyanobacterial community reduced in high temperature. In Ramsar hot spring with 50 ˚C temperature, the lowest species diversity was observed. In addition cyanobacterial species diversity

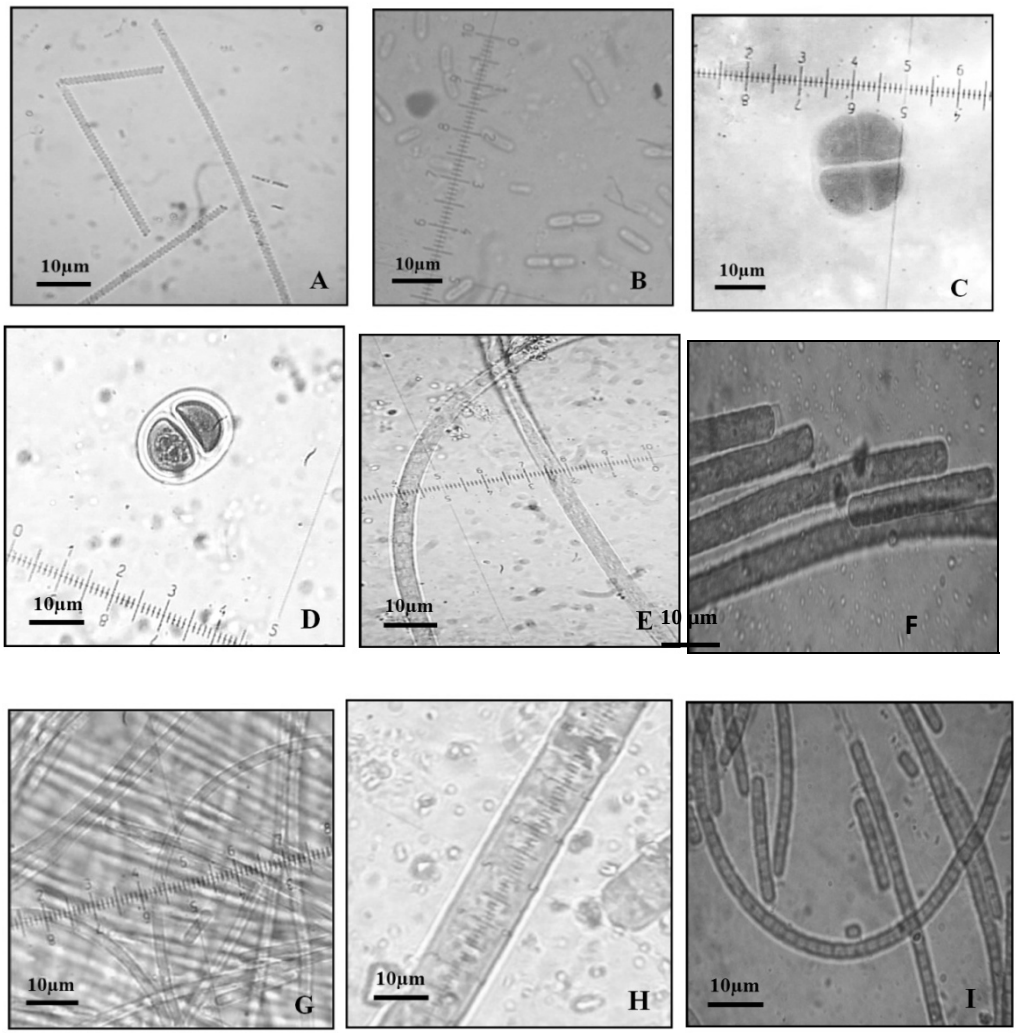

Fig. 5. **A.** *Spirulina subsalsa*, **B.** *Synechococcus elongatus*, **C.** *Chroococcus* sp., **D.** *Chroococcus minimus*, **E.** *Planktotrix rubescens*, **F.** *Oscillatoria subbrevies*, **G.** *Phormidium* sp., **H.** *Phormidium chalybeum*, **I.** *Phormidium articulatum*.

can be affected by physico-chemical characteristics of the spring waters, temperature and the regional distribution of springs. In this study, because of the temperature was below 60 °C, high species composition similarity was observed. Ramsar hot spring showed the least of diversity due to high temperature and different geographical location from the other springs.

According to this fact that the grouping of taxa according to morphological characters is not sufficient, especially in complex taxa such as *Jaaginema metaphyticum*, 16S rRNA gene sequencing was used for better sample recognition. The phylogenetic tree according to neighbor-joining algorithm, in appropriate way separates the studied genera and which is a confirmation on the morphological studies. The cladogram according to 16S rRNA gene sequencing well separated filamentous taxa from single cell taxa. In filamentous taxa the polyphyly of oscillatorials were determined. But even molecular study cannot obviously separate *Jaaginema metaphyticum* (Syn: *Oscillatoria angusta*) from its previous group.

In conclusion, we report here that the molecular study does not disrupt results of morphological classification. Genomic sequences also does not provide the necessary information for separation of taxa from pervious groups (*Oscillatoria angusta*, *Oscillatoria amphibian*, *Oscillatoria limentica* and *Phormidium tenue*) and the separated taxa remain close to their pervious groups. More exact recognition of them need more detail study on polyphasic.

Table 3. List of Cyanoprokaryota species recorded from Iran and their distributions in studied sites (1.Geno hot spring, 2. Chahahmad hot spring, 3. Khamir hot spring, 4. Ramsar hot spring.*New Record from Iran).

	Taxon	1	2	3	4
1	*Chroococcus dispersus* (Keissler) Lemmermann		+	+	
2	**Chroococcus limenticus* Lemmermann	+	+	+	
3	**Chroococcus macrococus* (Kützing) Rabenhorst	+			
4	*Chroococcus minimus* (Keissler) Lemmermann	+		+	
5	*Chroococcus minor* (Kützing) Nägeli	+			
6	*Chroococcus turgidus* (Kützing) Nägeli	+			
7	**Chroococcus varius* A. Braun		+		
8	**Cyanobacterium cedrorum* (Sauvageau) Komárek, Kopecky & Cepak			+	
9	**Entophysalis granulosa* Kützing	+		+	
10	**Geitlerinema acutissimum* (Kufferath) Anagnostidis	+			
11	**Geitlerinema amphibium* (C. Agardh ex Gomont) Anagnostidis	+			
12	**Jaaginema metaphyticum* Komárek	+	+	+	+
13	**Jaaginema minimum* (Gicklhorn) Anagnostidis & Komárek				+
14	*Johannesbaptistia pellucida* (Dickie) W. R Taylor & Drouet	+			+
15	**Komvophoron schmidlei* (Jaag) Anagnostidis & Komárek	+			
16	**Leptolyngbya tenuis* (Gomont) Anagnostidis & Komárek	+	+		
17	**Lyngbya versicolor* Wartmann ex Gomont			+	
18	*Microchaete* sp.	+			
19	*Nodularia spumigena* Mertens ex Bornet & Flahault	+			
20	*Oscillatoria anguina* Bory ex Comont		+		
21	*Oscillatoria limosa* C. Agardh ex Gomont			+	
22	*Oscillatoria princeps* Vaucher ex Gomont	+		+	
23	*Oscillatoria subbrevis* Schmidle	+	+	+	+
24	*Oscillatoria tenuis* C. Agardh ex Gomont	+		+	
25	*Phormidium articulatum* (Gardner) Anagnostidis & Komárek	+	+		+
26	*Phormidium chalybeum* (Mertens ex Gomont) Anagnostidis & Komárek			+	
27	**Phormidium formosum* (Bory de Saint – Vincent ex Gomont) Anagnostidis & Komárek	+			
28	**Phormidium nigrum* (Vaucher ex Gomont) Anagnostidis & Komárek		+		+
29	**Phormidium ornatum* (Kützing) Anagnostidis & Komárek		+		
30	**Planktothrix agardhii* (Gomont) Anagnostidis & Komárek	+	+	+	+
31	**Planktothrix prolific* (Gomont) Anagnostidis & Komárek	+			
32	**Planktothrix rubescens* (De Candolle ex Gomont) Anagnostidis & Komárek	+			
33	**Pseudanabaena catenata* Lauterborn	+			
34	*Pseudanabaena limnetica* (Lemmermann) Komárek	+			+
35	**Pseudanabaena mucicola* (Naumann & Huber-Pestalozzi) Schwabe	+			
36	*Spirulina labyrinthiformis* Gomont	+			
37	*Spirulina major* Kützing ex Gomont	+			
38	**Spirulina nordstedtii* Nordstedt ex Gomont	+			
39	*Spirulina subsalsa* Oerstedt ex Gomont	+	+	+	+
40	**Synechococcus elongatus* (Nägeli) Nägeli	+	+		
41	*Synechocystis aquatilis* Sauvageau	+	+		
42	**Trichodesmium lacustre* Klebahn	+			
43	**Tychonema bornetii* (Zukal) Anagnostidis & Komárek		+		

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Table 4. Total variance of factors according to principal component analysis of morphological characters.

Total Variance Explained

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	5.144	39.566	39.566	5.144	39.566	39.566	3.256	25.045	25.045
2	2.131	16.392	55.958	2.131	16.392	55.958	3.172	24.401	49.446
3	1.696	13.046	69.004	1.696	13.046	69.004	1.730	13.305	62.751
4	1.372	10.557	79.561	1.372	10.557	79.561	1.690	12.997	75.748
5	1.135	8.734	88.295	1.135	8.734	88.295	1.631	12.547	88.295
6	.875	6.730	95.025						
7	.282	2.167	97.192						
8	.230	1.770	98.962						
9	.086	.659	99.622						
10	.041	.316	99.938						
11	.007	.055	99.993						
12	.001	.007	100.000						
13	-7.371E-16	-5.670E-15	100.000						

Extraction Method: Principal Component Analysis.

Table 5. Rotated component matrix according to PCA analysis of morphological characters.

Rotated Component Matrix[a]

	Component				
	1	2	3	4	5
VAR00001	.267	.309	.844	-.027	.045
VAR00002	.872	.367	.110	-.009	.210
VAR00003	-.072	.030	-.110	.938	.004
VAR00004	.943	.203	.178	.035	.148
VAR00005	.092	.973	.051	-.030	.076
VAR00006	.685	.599	.181	.097	.345
VAR00007	-.041	-.345	.660	.335	.370
VAR00008	.097	.898	.105	.275	.156
VAR00009	-.045	.075	.169	-.179	.694
VAR00010	.836	-.463	.064	-.061	-.175
VAR00011	.459	.625	.598	.001	-.081
VAR00012	.101	.181	.291	.755	-.174
VAR00013	.347	.157	-.064	.061	.854

Extraction Method: Principal Component Analysis.
Rotation Method: Varimax with Kaiser Normalization.
a. Rotation converged in 9 iterations.

Table 6. GenBank code of samples.

	Sample	GenBank code
1	*Synechocystis* sp.	HQ900669.1
2	*Synechocystis* sp.	AB039001.1
3	*Synechocystis* sp.	HQ900668.1
4	*Synechococcus elongatus* str. *Ramsar*	JQ771323.1
5	*Synechococcus* sp.	AF448077.1
6	*Oscillatoria* sp.	EF150796.1
7	*Phormidium irriguum* f. *minor*	FN813342.1
8	*Lyngbya* sp.	AY049752.1
9	*Lyngbya* sp.	AY049751.1
10	*Spirulina* sp.	DQ058861.1
11	*Phormidium* sp.	HM217057.1

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