

# Morphological and phylogenetic diversity of thermophilic cyanobacteria in Algerian hot springs

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**Abstract** Geothermal springs in Algeria have been known since the Roman Empire. They mainly locate in Eastern Algeria and are inhabited by thermophilic organisms, which include cyanobacteria forming mats and concretions. In this work, we have investigated the cyanobacterial diversity of these springs. Cyanobacteria were collected from water, concretions and mats in nine hot springs with water temperatures ranging from 39 to 93 °C. Samples were collected for isolation in culture, microscopic morphological examination, and molecular diversity analysis based on 16S rRNA gene sequences. Nineteen different cyanobacterial morphotypes were identified, the most abundant of which were three species of *Leptolyngbya*, accompanied by members of the genera *Gloeocapsa*, *Gloeocapsopsis*, *Stigonema*, *Fischerella*,

*Synechocystis*, *Microcoleus*, *Cyanobacterium*, *Chroococcus* and *Geitlerinema*. Molecular diversity analyses were in good general agreement with classical identification and allowed the detection of additional species in three springs with temperatures higher than 50 °C. They corresponded to a *Synechococcus* clade and to relatives of the intracellularly calcifying *Candidatus* Gloeomargarita lithophora. The hottest springs were dominated by members of *Leptolyngbya*, *Synechococcus*-like cyanobacteria and Gloeomargarita, whereas Oscillatoriales other than *Leptolyngbya*, Chroococcales and Stigonematales dominated lower temperature springs. The isolation of some of these strains sets the ground for future studies on the biology of thermophilic cyanobacteria.

**Keywords** Cyanobacteria · Hot spring · Thermophilic · Microbial mat · Biomineralization · Carbonate

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## Introduction

Cyanobacteria constitute a phylum of photosynthetic bacteria of crucial importance in ecology and evolution. Their ancestor developed oxygenic photosynthesis, thus leading to the oxygenation of the Earth's atmosphere and imposing novel environmental selective constraints to other groups of organisms (Herrero and Flores 2008). The chloroplasts of eukaryotic plants and algae derived from ancestral cyanobacterial endosymbionts, which led to another major diversification event in the history of terrestrial life (Nelson et al. 1995; Cavalier-Smith 2002). From an ecological point of view, cyanobacteria are also extremely successful, being able to thrive in a wide variety of ecosystems. These include not only oceans and freshwater systems, but also many extreme environments. Essentially,

except for acidic environments ( $\text{pH} < 5$ ), they have colonized most extreme habitats, such as hypersaline settings, hyperarid desert areas, UV and ionizing radiation-exposed settings and even rock interiors (Garcia-Pichel et al. 1998; Wierzbos et al. 2006; Warren-Rhodes et al. 2006; Gorbushina and Broughton 2009; Ragon et al. 2011).

Cyanobacteria are also able to live at the upper limit temperature for photosynthesis, up to 74 °C, in thermal springs and their associated microbial mats (Castenholz 2001). Thermophilic cyanobacteria from the Yellowstone National Park were the first, and possibly the most extensively studied (Dyer and Gafford 1961; Castenholz 1969; Brock 1967; Ward et al. 1998). But, although cyanobacteria from other thermal areas have also been explored to some extent, many remain unstudied. Classical morphological studies reveal the conspicuous presence of some genera in this kind of environments, such as *Synechococcus*, *Phormidium*, *Calothrix* or *Mastigocladus* (Castenholz 2001). However, molecular phylogeny studies show that many cyanobacteria with relatively simple morphotypes (e.g., *Synechococcus*) are polyphyletic (Robertson et al. 2001). Accordingly, more recent molecular diversity exploration approaches suggest that simple morphotypes may conceal a previously unsuspected diversity and include some very distant lineages (Ferris et al. 1997; Nubel et al. 1997; Miller and Castenholz 2000; Norris et al. 2002; Bhaya et al. 2007). Some of these lineages can indeed possess unique properties, as was recently demonstrated by the enrichment of a small, early-branching cyanobacterium capable of forming intracellular Mg–Ca–Sr–Ba–carbonates, whose closest relatives seem to thrive in thermophilic microbial mats (Couradeau et al. 2012).

The Algerian territory has about two hundred hydrothermal resources localized mainly in the alpine orogenic belt between the littoral and the Southern pediment of the Saharan chain. The curative properties of spring waters and their spatial distribution have facilitated the advent of a long-term resort tradition with recognized effects in care and therapies. Algerian thermal springs have been studied mostly from the perspective of geothermal resources (Kedaid 2007; Saibi 2009), hydrogeological aspects and physico-chemical composition (Verdeil 1982; Dib 1985; Issaadi 1992). The biological study of these ecosystems in Algeria started more than 74 years ago with the expedition of a French colonial explorer who studied the fauna of one of the hottest springs, “Meskoutine spring” (Masson 1939). However, only a few studies on thermophilic bacteria inhabiting these hot springs, with the isolation of new extremophile species have been conducted since then (Kecha et al. 2007; Bouanane-Darenfed et al. 2011), and the cyanobacteria of Algerian hot springs have remained unexplored so far.

In this work, we report the results of a study of the cyanobacterial communities in nine hot springs in Algeria. We combined classical morphological studies done with microscopy examination with molecular diversity studies based on the amplification, cloning and sequencing of 16S rRNA genes from enrichment cultures and natural samples.

## Materials and methods

### Sampling sites

Nine hot springs from different regions in Algeria, mainly in the East of the country, were investigated (Table 1; Figure S1). Sampling was carried out in May 2011. Hot spring water was collected in proper containers as close as possible to the spring discharge point. Standard physico-chemical parameters such as water temperature, pH, conductivity, total dissolved solids (TDS) and dissolved oxygen were measured in the field using a WTW multi-parameter probe. Water samples for metal and cation concentration analysis were collected in 250-ml polyethylene bottles after filtration through 0.45- $\mu\text{m}$  pore-diameter membranes and acidified (1 % v/v  $\text{HNO}_3$ ). Microbial mats, concretions and sediments for the study of cyanobacterial enrichments were picked with sterile forceps and spatula and placed in sterile containers. Thermal water for cyanobacterial cultures was collected at the sampling sites in sterile glass vials and tubes. Samples used for molecular analysis were collected in the same way from enrichments in solid culture media or natural mats, and placed on ice during transportation to the laboratory for DNA purification.

### Physico-chemical analysis

Spring water temperature, pH, conductivity, total dissolved solids, dissolved oxygen and total alkalinity by titration (as  $\text{HCO}_3^-$ ) were measured in the field using a WTW probe and a conductivimeter (Mettler Toledo Mate 90 England). Potassium ( $\text{K}^+$ ), Calcium ( $\text{Ca}^{2+}$ ), sodium ( $\text{Na}^+$ ), Magnesium ( $\text{Mg}^{2+}$ ), Lithium ( $\text{Li}^+$ ), nitrate ( $\text{NO}_3^-$ ), sulfate ( $\text{SO}_4^{2-}$ ), chloride ( $\text{Cl}^-$ ), fluorine ( $\text{F}^-$ ) and bromine ( $\text{Br}^-$ ) contents were determined by ion chromatography (IC, DionexDX-120). The total silica in water was determined by a colorimetric method. The content of metals, namely, aluminum, arsenic, iron, manganese and zinc was analyzed using a Perkin Elmer Optima Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, 7000 DV). The analytical error for IC and ICP-OES was  $\leq 5$  %. Values are provided in Table 2.

**Table 1** General characteristics of the Algerian hot springs and samples analyzed in this study

Sampling site	Sample name	Nature of sample	Sample temperature (°C)	Spring name (region)	Coordinates	Altitude (m above sea level)	Source temperature (°C)	Flow rate (l/s)	pH	Conductivity (mS/cm)	TDS (g/l)	Dissolved oxygen (mg/l)
St.1	St1P	Thermal water	70	El Bibanes (Bordj Bou Arreridj)	36°11'47.68''N 4°23'23.64''E	554	75	6	6.42	23.6	14.60	1.5
St.3	St3S	Thermal sediment	50	Essalihine (Khenchela)	35°26'16.95''N 7°05'09.92''E	1.118	50	50	7.10	3.41	1.72	4.7
St.4	St4P	Thermal water	50	Knif (Khenchela)	35°29'18.77''N 7°15'11.90''E	1.075	50	60	6.70	3.6	1.82	3.3
St.5	St5	Thermal water	39	Tassa (Souk Ahras)	36°13'55.89''N 8°02'42.38''E	1.075	39	4	6.07	3.03	1.56	0.2
St.7	St7S St7C1	Thermal sediment Calcite concretion (Fig. 1a)	70 45–60	Meskoutine (Guelma)	36°27'35.16''N 7°16'09.98''E	351	93	80	6.27	2.01	1.12	0.5
St.7L	St7L	Calcite concretion (Fig. 1b)	45–60									
St.7T	St7T	Microbial mat (Fig. 1c)	60									
St.7–70	St7–70	Calcite concretion (Fig. 1d)	70									
St.8	St8	Calcite concretion (Fig. 1e)	54	Beni Guéchat (Mila)	36°25'09.84''N 5°59'17.92''E	556	54	0.5	6.02	24.4	15.50	0.4
St.10	St10	Thermal sediment	53	Ibainen (Bordj Bou Arreridj)	36°22'11.17''N 4°46'55.95''E	472	53	6	6.69	18.5	11.55	3.3
St.13	St13	Thermal sediment	70	Bouhadjar	35°21'57.69''N 0°57'55.36''W	161	70	10	6.80	5.5	2.75	5.0
St.16	St16	Thermal sediment	52	Sidi Yahia (Béjaia, Grande Kabylie)	36°24'55.38''N 4°36'12.57''E	226	52	30	6.45	28.1	17.56	5.0

TDS total dissolved solids

**Table 2** Major and minor mineral water components (mg/l) of analyzed Algerian hot springs

Hot spring	Ca <sup>2+</sup>	Mg <sup>2+</sup>	Na <sup>+</sup>	K <sup>+</sup>	Cl <sup>-</sup>	SO <sub>4</sub> <sup>2-</sup>	NO <sub>3</sub> <sup>-</sup>	HCO <sub>3</sub> <sup>-</sup>	SiO <sub>2</sub>	Sr <sup>2+</sup>	Li <sup>+</sup>	F <sup>-</sup>	Br <sup>-</sup>	Fe	Zn <sup>2+</sup>	Al <sup>3+</sup>	Mn
St.1	439	54	5,316	207	8,484	1,025	0.032	569	52.8	4	6	3	4	1.5	0.151	0	0
St.3	64	23	602	18	678	296	4.4	297	36.8	2.1	2,055	0.52	2.17	0.01	0	0	0
St.4	110	18	650	12	541	452	8.9	342	38	2.6	2,061	1.17	2.15	0.002	0.150	0	0.014
St.5	286	34	417	17	670	13	0.66	921	19.2	8.4	1.63	2.6	2.4	0.142	0.152	0.27	0
St.7	220	33	229	25	308	368	0.006	366	54.9	3.3	1,165	2.7	2.1	0.135	0	0	0.030
St.8	901	86	5,405	100	9,906	1,414	0.036	531	41.4	8	4	2.5	2.5	6	0.01	0	0
St.10	520	99	3,573	62	5,494	1,628	0.592	529	42	4.5	2	3	4	1.921	0	0	0.023
St.13	159	40	1,048	50	1,660	50	0.1	715	50.6	5.15	1.28	1.94	3.06	0	0	1,349	0
St.16	605	131	6,568	93	9,928	1,689	0.188	657	39.9	3	3	1.5	1	1.98	4,459	0	0.196

Analytical error ≤5 %

### Morphological classification of cyanobacteria and enrichment cultures

Morphological classification of natural and cultivated cyanobacteria was based on characters observable under a light Zeiss microscope (400–1000×) and photographed with a super HAD/CCD-Sony-DSC-S930 camera. Morphotypes were identified down to the genus level on the basis of the identification systems proposed by Komárek and Anagnostidis (1989, 1999) and Castenholz (2001). Cyanobacterial cells were counted from each sample three times using a Nageotte counting chamber on 50 µl of a homogenized suspension obtained from 1 g of mat, sediment or concretion, or from 1 ml of water per liter. We took into account the cell number of filaments and colonies. Species diversity was calculated using the Shannon–Weaver index (Shannon and Weaver 1963). Cultivation and isolation attempts were conducted in liquid BG-11 medium and on agar plates of BG-11 medium, using incubation temperatures of 40, 45 and 55 °C. Fluorescent tubes were used for illumination (2500 lux). When cyanobacterial growth was observed, one single filament or colony was transferred to 20 ml of fresh medium and incubated until growth occurred.

### DNA purification, PCR amplification, cloning and sequencing

DNA was purified from subsamples of approximately 200 µl of homogenized microbial mats or cell pellets of enrichment cultures using the PowerBiofilm DNA isolation kit (MoBio, Carlsbad, CA USA) following manufacturer's instructions. DNA was eluted in 100 µl of Tris–HCl, pH 8 and conserved at –20 °C. Cyanobacterial 16S rRNA genes plus the adjacent ITS were amplified by polymerase chain reaction (PCR) using the specific primers CYA106F (5'-CGGACGGGTGAGTAACGCGTGA) and 23S0R (5'-CTTCGCCTCTGTGTGCCTAGGT). PCR reactions were carried out in 25 µl of reaction buffer, containing 1 µl of the eluted DNA, 1.5 mM MgCl<sub>2</sub>, dNTPs (10 nmol each), 20 pmol of each primer, and 0.2 U Taq platinum DNA polymerase (Invitrogen). PCR reactions were performed under the following conditions: 35 cycles (denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, extension at 72 °C for 2 min) preceded by 2-min denaturation at 94 °C, and followed by 7-min extension at 72 °C. 16 rRNA gene libraries were constructed for all positive amplifications using the TopoTA cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Clone inserts were PCR-amplified using flanking vector primers, and inserts of expected size were partially sequenced (Beckman Coulter Genomics, Takeley, UK) with the specific cyanobacterial reverse primer CYA-1380R, located

towards the end of the 16S rRNA gene (5'- TA-ACGACTTCGGGCGTGACC). A total 13 gene libraries were constructed and 292 gene sequences determined. Sequences were deposited in GenBank with accession numbers KJ659374–KJ659422.

### Sequence analysis

16S rRNA gene sequences retrieved from our samples were compared with sequences in the database GenBank (<http://www.ncbi.nlm.nih.gov/>) and in the curated SILVA database (Pruesse et al. 2007) by BLAST (Altschul et al. 1997). Our sequences were considered to belong to the same operational taxonomic unit (OTU) when they shared more than 97 % identity. We retrieved the closest sequences found in databases and included them in an alignment containing also sequences from the closest cultivated members and some representative sequences of major cyanobacterial taxa. Sequences were aligned using MUSCLE (Edgar 2004). Ambiguously aligned positions and gaps were eliminated using Gblocks (Castresana 2000). A total of 761 conserved positions were retained for the subsequent analysis. The resulting sequence alignment was used as input to build a phylogenetic tree by maximum likelihood using Treefinder (Jobb et al. 2004) with a General Time Reversible (GTR) model of sequence evolution, and taking among-site rate variation into account using a four-category discrete approximation of a  $\Gamma$  distribution. ML bootstrap proportions were inferred using 1,000 replicates. Trees were visualized with FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>).

## Results and discussion

### Physico-chemical parameters and mineral composition of hot spring waters

The physico-chemical parameters measured *in situ* at the different hot spring samples are reported in Table 1. The temperatures of thermal waters vary between 39 °C measured at Tassa (St5) and 93 °C at Meskoutine spring (St7), which is the hottest spring in Algeria. The pH of the analyzed water samples is in a narrow range (6.02–7.10), neutral and slightly acid. These waters have high gas content and gas emission was observed during sampling. CO<sub>2</sub> is the most abundant of those gases, constituting up to 59 % of total emissions, followed by nitrogen (Issaadi 1992). Conductivity values vary from 2 to 28 mS/cm, reflecting a high mineral content due to deep subsurface mineral dissolution by thermal waters. Accordingly, the total dissolved solids vary from 1.1 to 17.5 g/l. The important amount of mineral salts in springs of Sidi Yahia

(St16), Béni Guéchat (St8), Ibâinen (St10) and El Bibanes (St1) indicate leaching of subsurface evaporite deposits. Our samples can be classed in two groups according to the conductivity-based classification proposed by Issaadi (1992). Class 2 includes thermal springs with conductivities between 2 and 7.5 mS/cm. Five springs St3, St4, St5, St7 and St13 belong to this class. They are either enriched in bicarbonate sodium chloride or in calcium sulfate waters rich in sodium chloride. Class 4 includes springs with conductivities higher than 15 mS/cm, where the mineral content is essentially of evaporite origin. They are rich in sodium chloride and calcium sulfate. Four springs belong to this class, St1, St8, St10 and St16.

The results of major and minor components are listed in Table 2. The thermal springs, St1, St7, St8, St10, St13 and St16, share several physico-chemical characteristics, including high temperature, slightly acid pH, high concentration of sodium chloride, calcium sulfate and silica, high mineral and gas content (Issaadi 1992). They are also quite enriched in minor elements, notably strontium, lithium, fluorine, bromine and iron, due to the leaching of reservoir rocks, especially the Béni Guéchat spring (St8). At Tassa spring (St5), with the lowest temperature among the studied sites, the water contains reduced gases and a characteristic odor of H<sub>2</sub>S due to sulfate reduction. This makes these waters slightly acidic (pH 6.07). They are also the most strontium rich.

Because iron is an important limiting factor for cyanobacteria, we also analyzed the iron content in the sediments in comparison with the thermal waters (Table S1). Cyanobacteria assimilate dissolved Fe<sup>3+</sup> and, in environments where dissolved Fe<sup>3+</sup> is too low, many cyanobacteria possess siderophores favoring its uptake or its mobilization from chelates (Hopkinson and Morel 2009). The Algerian hot springs have very different levels of iron in water, ranging from 0 to 6 mg/l (Table 2), which might have an influence on the cyanobacterial diversity associated to the springs. However, many of the cyanobacteria in these springs were benthic and, therefore, associated to the mineral substrate. Although in general there was a good agreement between iron concentrations in the sediment and in the water (Table S1), in some cases there were substantial differences. For instance, samples St4 and St7C1 had high sediment iron content (82.5 and 96 g/kg, respectively), but very low water iron concentrations (Table S1). It would be interesting, though it is challenging, to ascertain the real amount of bioavailable iron for these cyanobacteria to establish potential correlations between iron availability and cyanobacterial diversity. Likewise, it would be interesting to see whether different physico-chemical parameters interfere mutually and may influence the cyanobacterial composition. This would require the inclusion of the same type of metadata

**Table 3** Distribution, mean abundance ( $10^3$  cells l<sup>-1</sup> or g<sup>-1</sup>) and biodiversity index values of cyanobacterial morphospecies in nine Algerian hot springs

Morphotype	Culture temperature (°C)	St1P (70 °C)	St3S (50 °C)	St4P (50 °C)	St5 (39 °C)	St7S (70 °C)	St7C1 (45–60 °C)	St7T (60 °C)	St7–70 (70 °C)	St8 (54 °C)	St10 (53 °C)	St13 (70 °C)	St16 (52 °C)
<i>Leptolyngbya</i> sp.			120,523 ± 24,104	4 ± 0.8			17,229 ± 3,445	13,642 ± 2,728	25,573 ± 5,114	452 ± 90.4			
<i>Leptolyngbya foveolarum</i>	45–55		+	+			+	+	+	+			
<i>Leptolyngbya laminosa</i>	45–55	60 ± 12	51,756 ± 10,351	5 ± 1								716 ± 142	
		+	+	+								+	
<i>Leptolyngbya amphiveginata</i>	40–45					2,800 ± 560	5,743 ± 1,148	16,996 ± 3,399					
<i>Gloeocapsa</i> sp.	45–55			7 ± 1.4		+	+	+					18 ± 3
<i>Gloeocapsa gelatinosa</i>	45–55			+			7,657 ± 1,531		5,056 ± 1,011				+
<i>Gloeocapsopsis crepidinum</i>	45–55						+		+				
<i>Stigonema</i> sp.	45–55		700 ± 140				552 ± 110						
		+	+				+						
<i>Fischerella</i> sp.	45–55		621 ± 124										
		+	+										
<i>Fischerella thermalis</i>	45–55												27 ± 5
<i>Synechocystis thermalis</i>	45–55											88 ± 17.6	+
												+	
<i>Synechococcus elongatus</i>	45–55											428 ± 85	10 ± 2
<i>Synechococcus nidulans</i>	40				288 ± 58							+	+
				+	+								
<i>Cyanobacterium</i> sp.	45–55										14,784 ± 2,956		
											+		
<i>Microcoleus</i> sp.	40–55									132 ± 26			
										+			
<i>Chroococcus minutus</i>	40–45									136 ± 27			
										+			
<i>Geitlerinema</i> sp.	40											184 ± 37	89 ± 18
												+	+
<i>Cyanodictyon</i> sp.	40											134 ± 27	
												+	
<i>Anabaenopsis</i> sp.	40												
		100 ± 20											
		+											



**Table 3** continued

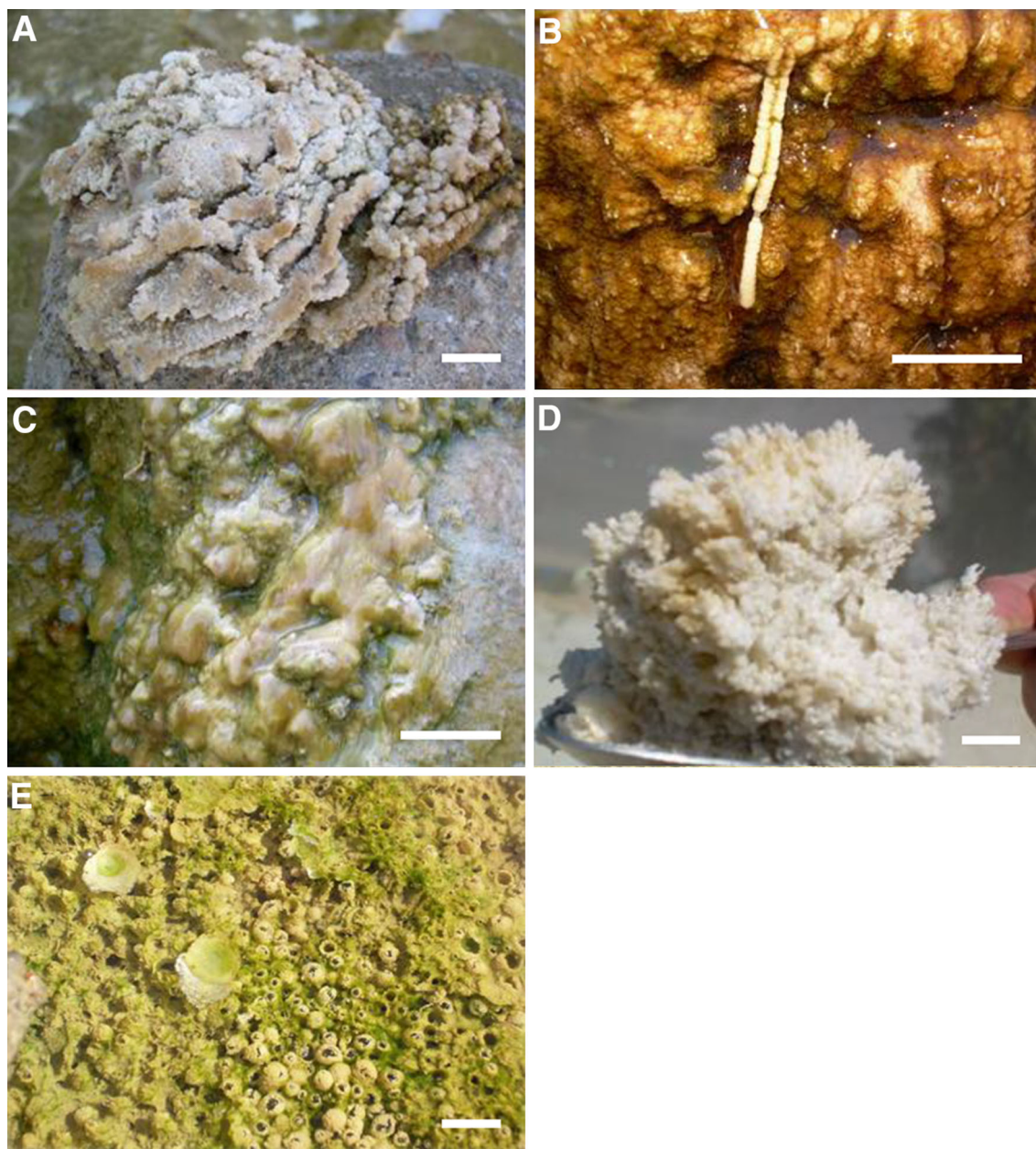
Morphotype	Culture temperature (°C)	St1P (70 °C)	St3S (50 °C)	St4P (50 °C)	St5 (39 °C)	St7S (70 °C)	St7C1 (45–60 °C)	St7L (45–60 °C)	St7T (60 °C)	St7–70 (70 °C)	St8 (54 °C)	St10 (53 °C)	St13 (70 °C)	St16 (52 °C)
Total number of cyanobacterial cells in field samples		160 ± 32	173,600 ± 34,720	16 ± 3.2	288 ± 58	30,629 ± 6,129					720 ± 144	14,784 ± 2,956	1,150 ± 310	144 ± 29
Biodiversity index (bits, cell <sup>-1</sup> ) per hot spring site		0.954	0.947	1.546	0	2.716					1.32	0	1.416	1.52

The original temperature or temperature gradient of collected samples is indicated in brackets under sample names. The distribution of cyanobacterial morphospecies enriched in culture from the studied Algerian hot springs is indicated by a 'plus' sign. Shadowed cells correspond to species that have been also unequivocally identified by 16S rRNA gene sequence

associated to cyanobacterial diversity studies from many more hot springs across the world to carry out meaningful multivariate statistical analyses.

#### Identification, quantification and enrichment of cyanobacterial morphospecies

Using optical microscopy, we identified 19 distinct morphospecies of cyanobacteria in the different Algerian hot springs. We counted cell numbers in the different samples and made biodiversity estimates for cyanobacteria in the different hot springs based on these values (Table 3). However, these estimates should be taken with caution due to the high heterogeneity of the studied systems. This is exemplified by the variation in counts and diversity of morphospecies identified in the subsamples collected from Meskoutine spring (St7 samples; Table 3). Remarkably, we were able to enrich all the morphospecies identified in field samples at temperatures ranging from 40 to 55 °C (Table 3; Figure S2, S3). All the enrichments were done in the same culture medium (BG11) suggesting that, despite considerable differences in water and substrate chemistry, these cyanobacteria can adapt to different environmental conditions. The samples from Bouhadjar (St13), Essalihine Khenchela (St3S), Sidi Yahia (St16) were slightly more diverse, with 4–5 morphotypes identified, followed by Meskoutine hot spring (St7S, St7C1, St7T, St7L and St7–70), Béni Guéchat (St8) and Knif (St4P), with 3 morphotypes identified (Table 3). The most abundant morphotypes corresponded to filamentous cyanobacteria of the genus *Leptolyngbya* with four species, *Leptolyngbya* sp., *L. foveolarum*, *L. laminosa* and *L. amplivaginata* (Figure S2), followed by *Synechococcus* and *Gloeocapsa* within the unicellular cyanobacteria (Figure S3). These genera, and most particularly *Leptolyngbya*, were present in the hottest sampling sites at 70 °C and also in the 45–60 °C range (Table 1). Accordingly, they dominated the isolates obtained with the temperature incubation range of 45–55 °C, and were isolated in majority from mats and concretions. *Leptolyngbya* sp. was detected in six of the thirteen spring samples studied. Together with *L. laminosa*, they dominated the solid thermal samples, whereas *L. foveolarum* dominated thermal water samples. In the temperature range of 40–45 °C, *Chroococcus minutus* and *L. amplivaginata* were, respectively, isolated from the sites St8 and St16. At the lower temperature of 40 °C, we could isolate *Synechococcus nidulans*, and enrich *Geitlerinema* sp., *Cyanodictyon* sp and *Anabaenopsis* sp. The concretion St7C1 from Meskoutine spring and the sediment from St10 Ibainen spring contained only one morphotype which were, respectively, the coccoid colonial *Gloeocapsopsis crepidinum* and *Cyanobacterium* sp. Members of the genus *Fischerella* (Stigonematales) were characterized by



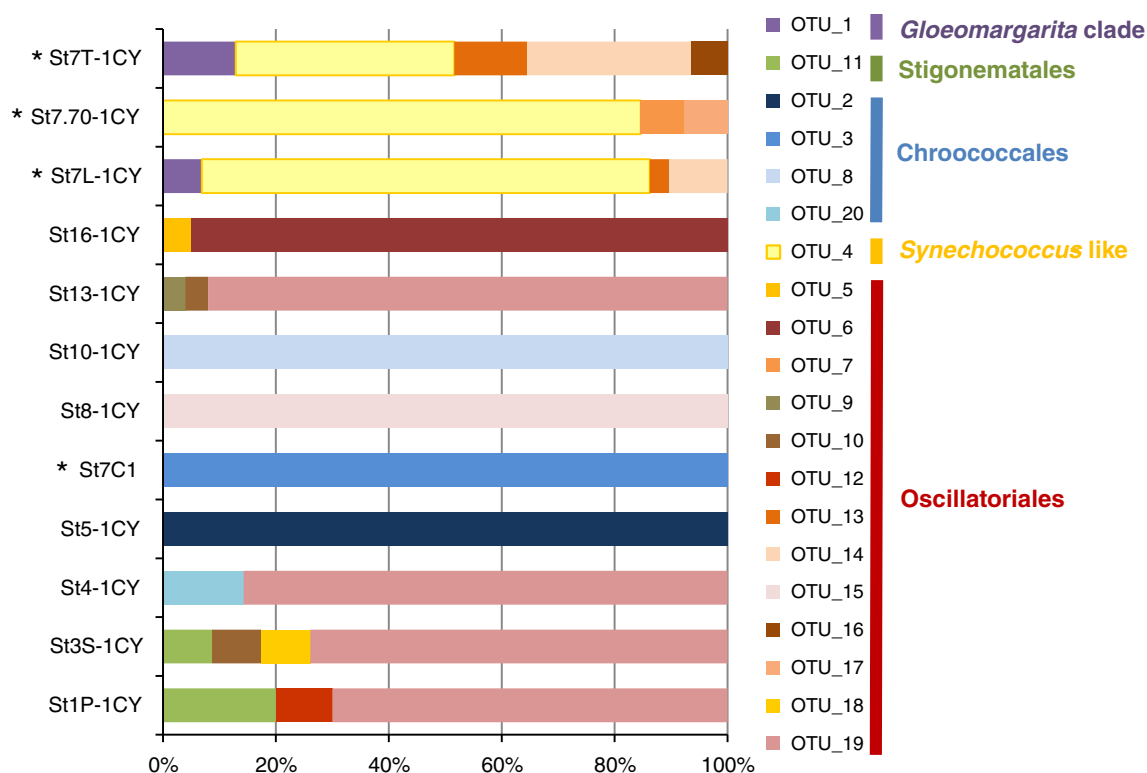
**Fig. 1** Selection of thermal spring samples analyzed. Calcite concretions and bacterial mats found in St7 Meskoutine spring: **a** St7C1, **b** St7L, **c** St7T, **d** St7-70; and from St8 Beni Guéchat spring (**e**). Scale bar, 1 cm

filamentous trichomes with true branching. Interestingly, the two *Fischerella* strains enriched from, respectively, St.3 and St16 (Table 3) were able to form heterocysts at 50 °C in culture, indicating that nitrogen fixation is possible at that temperature. Since the strains grow well in the range 45–55 °C in normal BG11 medium, it may well be that they are able to fix nitrogen in the same temperature range. Heterocyst-containing cyanobacteria have been observed in natural samples at 45 °C (Coman et al. 2013) and up to 63 °C (Ionescu et al. 2010). Nitrogen fixation can occur not only in some thermophilic cyanobacteria, but

notably, at much higher temperatures in some thermophilic and hyperthermophilic archaea (Sen and Peters 2006; Mehta and Baross 2006).

The diversity of cyanobacterial morphospecies identified in the Algerian hot springs is in general agreement with that observed by pioneers of thermophilic cyanobacterial research decades ago in the Yellowstone National Park (Castenholz 1969; 2008). One of the dominant Algerian morphotypes corresponded to the genus of filamentous cyanobacteria *Leptolyngbya*, which has been detected in hot springs worldwide (e.g., Ionescu et al. 2010;





**Fig. 2** Diversity and relative proportions of cyanobacterial 16S rRNA gene-based OTUs in gene libraries obtained from enrichment cultures and natural samples. Environmental samples are labeled with an *asterisk*

Coman et al. 2013; Dadheech et al. 2013; Mackenzie et al. 2013). Another important morphotype systematically associated with hot springs is that of *Synechococcus/Thermosynechococcus* spp. The occurrence of *Lepidolynghya*, *Synechococcus* and other cyanobacteria often found in most springs (e.g., *Fischerella/Mastigocladus*) might suggest that there is no geographical barrier to the dispersal of these thermophilic taxa. However, analysis at finer scales seems to suggest that, despite apparent cosmopolitan distribution under particular strong environmental selection, these organisms experience isolation by distance (Papke et al. 2003). Nonetheless, disentangling geographical distance from physico-chemical parameters is not easy and more in-depth studies will be required to properly test this hypothesis.

#### Diversity of cyanobacteria based on 16S rRNA gene sequences

We also studied the cyanobacterial diversity by amplification, cloning and sequencing 16S rRNA genes from the different enrichment cultures as well as from various natural mats and mineral crusts associated to the hottest St7 Meskoutine spring, i.e., samples St7T, St7C1, St7L and St7-70 (Table 1). This molecular analysis should serve to compare previously identified morphospecies with 16S

rRNA gene-based operational taxonomic units (OTUs) and, eventually, validate and/or refine morphological studies. We identified a total of 20 OTUs (Fig. 2; Table S2), which correlates well with the number of morphospecies (19) previously identified. These OTUs affiliated to five order-level cyanobacterial clades, namely the Oscillatoriales, Chroococcales, Stigonematales, one clade of *Synechococcus* and the recently identified *Candidatus* Gloeomargarita clade (Couradeau et al. 2012). Nonetheless, three groups were found to dominate the different enrichments or natural samples, the Oscillatoriales (St1P, St3S, St8, St13, St16, St7T), the Chroococcales (St5, St7c1, St10) and the *Synechococcus*-like, which were dominant or very abundant in several natural samples collected at Meskoutine (St7) (Fig. 2). It is interesting to note that the relative abundance of sequences in gene libraries from enrichments and the natural St7 samples was in relative good agreement with counts of cyanobacterial morphospecies from field samples, at least in terms of dominance of large cyanobacterial groups (Figure S4). There were only a few exceptions (St7-70 and St7L) where Chroococcales appeared to dominate in gene libraries whereas Oscillatoriales seemed to dominate in morphology-based counts. However, this can be easily explained by local spatial heterogeneity. Collectively, the diversity and relative abundance for all St7 samples observed

morphologically seem compatible with that found by molecular analysis.

The agreement of molecular and morphological observations was also good at finer level. As shown in Table 3, for 18 identified morphotypes, there was a perfect correlation between the morphospecies identification and the occurrence of the corresponding specific 16S rRNA gene in gene libraries (Table S2), the phylogenetic position of which was demonstrated by the reconstruction of a phylogenetic tree (Fig. 3). In the cases where only one morphotype was observed (the two enrichments St5 and St10 and the two natural samples St7C1 and St7S), all the clones analyzed yielded 16S rRNA gene sequences that matched well the observed morphospecies. These were *Synechococcus nidulans* for St5, *Cyanobacterium* sp. for St10 and *Gloeocapsopsis crepidinium* for the natural sample St7C1 (Table 3; Fig. 3; Table S2). In agreement with the nature of its substrate, *G. crepidinium* was described by Komárek and Anagnostidis (1999) as forming agglomerates of irregular packets surrounded by mucilaginous envelopes and inhabiting mainly stony substrates. In the case of St7S, the 16S rRNA gene sequence of *Leptolyngbya laminosa* was obtained after direct amplification and sequencing, without cloning steps, which further reinforces the idea that this species is largely dominant in this sediment sample.

In several cases (14 out of 32 occurrences), there was not a clear correspondence between the morphospecies identified and the sequences retrieved. There are several explanations to this. First, for some cyanobacterial species, especially for *Gloeocapsa gelatinosa*, which was not detected by sequence, it may be that the cellular lysis was prevented or limited by its thick mucilaginous capsule, biasing subsequent DNA purification and gene amplification steps. Second, in some cases, we identified 16S rRNA gene sequences that were very divergent from described morphospecies, so that their correct identification is not possible due to the lack of good reference morphospecies. This was the case, for instance, of OTUs 9 and 10, which had only 91–93 % identity with the closest sequences in the database (Table S2; Fig. 3). Finally, in most cases, the absence of a direct unequivocal correspondence between morphospecies and 16S rRNA gene markers occurred in case of morphospecies with little morphological distinctive features. Thus, we did not retrieve sequences belonging to *Synechococcus elongatus* or *S. thermalis*, but we detected other sequences that belong to other *Synechococcus* species or to *Synechococcus*-like cyanobacteria (Fig. 3). This illustrates well the fact that a single apparent morphospecies may hinder a large genetic diversity and explains why the genus *Synechococcus* is polyphyletic, encompassing at least 8 different clades distributed across the phylogenetic tree of cyanobacteria (Robertson et al. 2001). This also highlights the need of carrying out molecular phylogenetic

**Fig. 3** Maximum likelihood phylogenetic tree of cyanobacterial 16S rRNA gene sequences retrieved from Algerian hot springs. The different colors correspond to those used in Fig. 2. Bootstrap values higher than 50 % are shown at nodes. The scale bar indicates the number of substitutions per a unit branch length

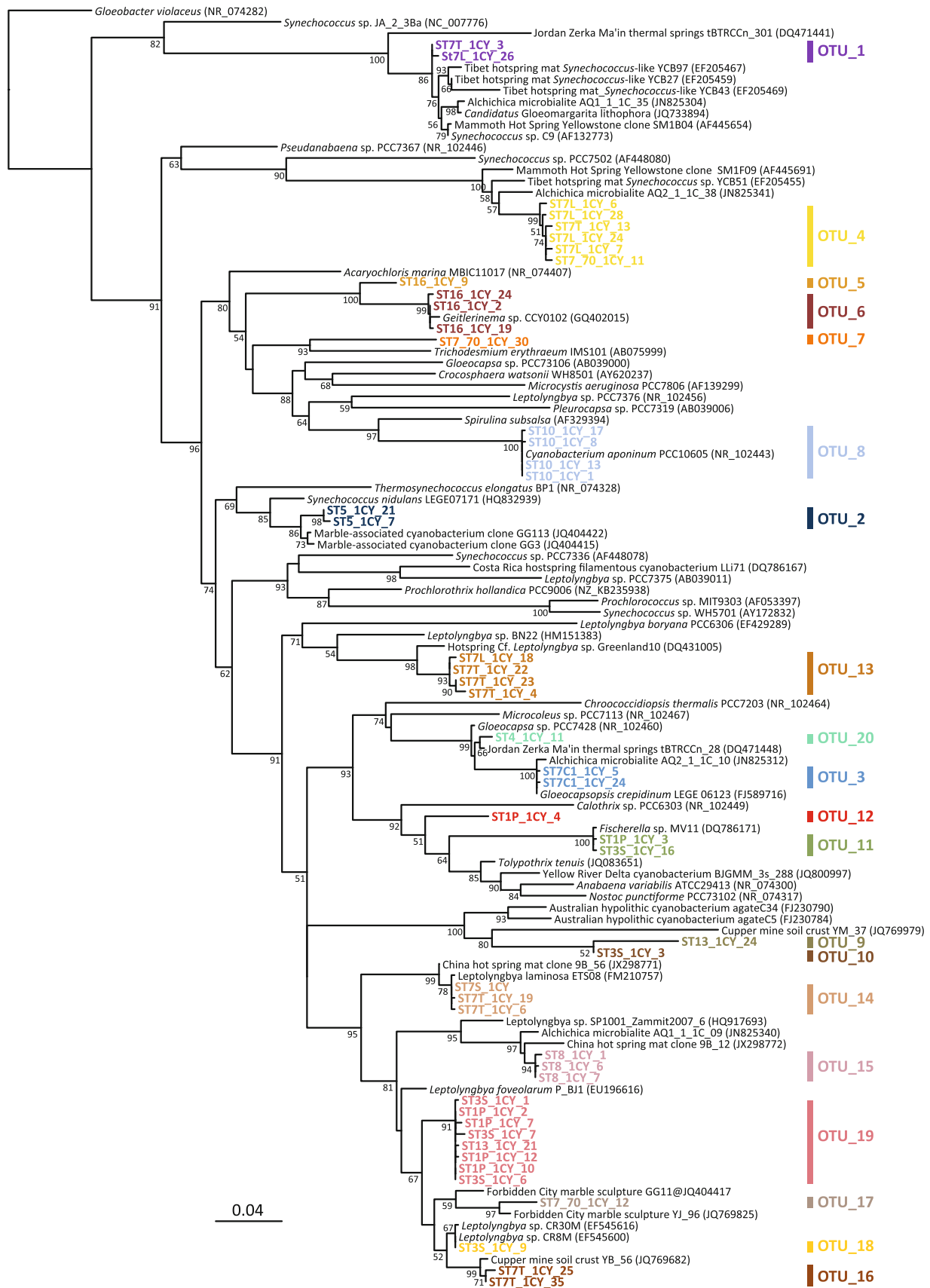
analyses to differentiate between the various *Synechococcus*-like lineages. This may lead to make a better link with their ecology. For instance, it has been noted for a long time that the most thermophilic cyanobacteria are *Synechococcus*-like (Dyer and Gafford 1961; Castenholz 1969; Miller and Castenholz 2000). However, *Synechococcus* is also typical of much colder environments, namely oceans, but molecular phylogenetic analyses show that oceanic *Synechococcus* form a distinct cluster (Robertson et al. 2001; Zwirgmaier et al. 2008).

Interestingly, we identified sequences related to *Candidatus Gloeomargarita lithophora* in concretions and microbial mats of the St7 Meskoutine spring, constituting up to 12 % of the clones in microbial mat libraries. Although *Ca. G. lithophora* was isolated from a freshwater microbialite and grows well at 20–25 °C (Couradeau et al. 2012), its closest relative sequences in databases correspond to sequences retrieved from thermophilic microbial mats in Yellowstone or Tibet hot springs (Fig. 3). The earliest branching sequence to the whole *Gloeomargarita* clade is also a sequence identified in thermal springs in Jordan (tBTRCCn\_301; Ionescu et al. 2010). This suggests that the ancestor of this clade was a thermophilic cyanobacterium thriving in microbial mats.

In addition to the presence of *Ca. G. lithophora* relatives, the hottest Algerian spring of Meskoutine was essentially dominated by one *Synechococcus*-like clade, OTU 4, which, similar to *Gloeomargarita*, has as closest relative sequences 16S rRNA genes amplified from Yellowstone and Tibet hot springs as well as from Alchichica microbialites, Mexico (Couradeau et al. 2011). This OTU made more than 80 % of the clones in the gene library from the hottest collected sample, St7-70 (70 °C), indicating that this phylotype corresponds to extreme thermophilic cyanobacteria. In addition to these two unicellular highly thermophilic OTUs (*Synechococcus* and *Gloeomargarita*-like), the other most thermophilic cyanobacteria were filamentous Oscillatoriales belonging to the genus *Leptolyngbya* (Table 3; Fig. 3). Again, the closest relatives to those sequences came from hot spring associated mat clones (Fig. 3). At lower temperature springs, cyanobacterial diversity was dominated by different species of Oscillatoriales and Chroococcales (Fig. 2).

#### Concluding remarks

The cyanobacteria identified in water, microbial mats and concretions associated to various Algerian hot springs



spanning temperatures of  $\sim 40$ – $70$  °C were relatively diverse, but depended on the site. Thus, species diversity for cyanobacteria assessed by the Shannon and Weaver index (Table 3) ranged from 0 (a single morphospecies) to 2,716. Notably, the highest value was recorded in Meskoutine (St.7), which is the hottest spring. This indicates that several cyanobacterial lineages have adapted to life at high temperature and are capable of making photosynthesis up to at least  $70$  °C in these Algerian systems. The diversity of cyanobacteria shifted from a variety of Oscillatoriales, Stigonematales and Chroococcales in the sources with the lower temperature range studied ( $40$ – $55$  °C) to a diversity dominated by members of the filamentous genus *Leptolyngbya* (Oscillatoriales) and *Synechococcus*, present in the hottest spring's mats (up to  $70$  °C). The dominance of *Synechococcus*-like cyanobacteria in geothermal springs has been known for a long time (Dyer and Gafford 1961; Castenholz 1969; Miller and Castenholz 2000). Many of these highly thermophilic cyanobacteria seem to be simultaneously associated to calcifying microbial mats, having as closest relatives in databases sequences from organisms thriving in hot spring mats or, in several cases, carbonate microbialites or other rocky substrates, e.g., marble (Fig. 3). This may be due to the fact that carbonate precipitation is thermodynamically favored at higher temperatures. Indeed, the St7 Meskoutine spring situated in the province of Guelma, occurs in an area bearing travertine deposits. However, whether carbonate formation is dominated by inorganic processes as has been proposed for some hot spring travertines in Yellowstone (Fouke et al. 2000) or whether biological processes have a determinant influence remains unknown. Nonetheless, the presence of close relatives to the intracellularly calcifying cyanobacterium *Candidatus Gloeomargarita lithophora* suggests that biology could have an influence in the process. At any rate, the presence of diverse cyanobacteria in these Algerian hot springs, some of which can fix nitrogen (e.g., *Fischerella* species), makes them key players as primary producers in these hot ecosystems. The isolation of several of these species in culture is an important step for an in-depth study of their biology under various laboratory conditions.

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