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Phenotypic and Molecular Biological Characterization of Cyanobacteria from Marble Surfaces of Treated and Untreated Sites of Propylaea (Acropolis, Athens)

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Cyanobacteria cause aesthetic damage to marble surfaces and in particular their endolithic mode of life contributes to the breakdown of rock crystalline structures. The aim of this work was to estimate, with both phenotypical and molecular approach, the composition of cyanobacterial communities on the Propylaea marbles of the Acropolis Monuments. The two selected sampling sites were treated and untreated with a commercial biocide in order to estimate its effect on the cyanobacterial diversity. Our study revealed that in both sampling sites were present 13 phenotypes and 10 phylotypes and that the cyanobacterial taxa were considerably lower in the treated site.

Keywords: Acropolis, ancient marble, biocide, cyanobacteria, phenotype, phylotype

Introduction

The Acropolis monuments are regarded as some of the most impressive cultural and artistic monuments of mankind. Acropolis was built in the 3rd century BC from pentelic marble and is situated in the center of Athens. Many of the Acropolis monuments are suffering deterioration caused by environmental and biological factors. Despite the cultural value of the Acropolis monuments, few references are available on literature concerning the photosynthetic microbial communities, including cyanobacteria, eukaryotic algae and lichens, thriving on the calcareous building material (Anagnostidis and Roussomoustakaki 1983; Anagnostidis et al. 1992).

However, it is well known that cyanobacteria biodeterioration of outdoor stone/marble monuments has received considerable attention for the last 20 years (Krumbein and Urzi 1992; Macedo et al. 2009; Urzi and Albertano 2001; Zurita

et al. 2005). The exposed stone monuments are susceptible to weathering agents, which can cause the deterioration of the stone (Daffonchio et al. 2000). These agents induce physical and chemical weathering processes and prepare the stone surfaces for rapid microbial colonization (Warscheid and Braams 2000). Stone works of art can be colonized by different groups of microorganisms, including bacteria, cyanobacteria, algae and fungi.

Cyanobacteria are considered the pioneers in the process of biodeterioration. It has been shown that the environmental conditions, climate, microclimate and other site-specific characteristics are crucial for the development of cyanobacterial flora and affect any type of stone. Cyanobacterial diversity and its abundance were clearly depended on the availability of water, allowing microorganisms to form subaerial biofilms (Crispim and Gaylarde 2005; Lamenti et al. 2000; Macedo et al. 2009; Ortega-Calvo et al. 1991; Tiano et al. 1995; Zurita et al. 2005).

Cyanobacteria can develop different strategies in order to survive under unfavorable conditions. These include the formation of extracellular polymeric substances (**EPS**), the pigmentation as a protection against desiccation, direct sunlight and the endolithic mode of life.

Traditionally, the identity of deteriogenic cyanobacteria has been investigated by microscopical methods based on morphological characters (fresh and cultured material). The modern advances in microbial ecology call into question the validity of mere phenotypical approaches to the study of cyanobacterial communities (Chacón et al. 2006). However,

the danger of relying only on DNA analysis for evaluation of the cyanobacterial diversity cannot be neglected (Gaylarde and Gaylarde 2005). Without any doubt, to assess the true nature of epilithic and endolithic cyanobacterial communities which is essential for monitoring their impact on stone by applying the suitable treatment, a polyphasic approach ecological, phenotypical and molecular biological elements are needed.

In order to control biological growth on stone monuments, biocidal treatments were occasionally applied. It has to be mentioned that the approach must evaluate both the history and condition of the artifact, as well as the physical and chemical damaging factors (Scheerer et al. 2009). The aim of this study was to characterize the cyanobacterial diversity (from treated and untreated surfaces) dwelling on the Propylaea marbles of the Acropolis with phenotypical and molecular approach.

Materials and Methods

Samples Site and Sampling Procedure

The Propylaea is a part of the Acropolis monuments which is located in Athens, Attiki, Greece ($37°58'18.20''$N $23°43'30.50''$E). The main building material is pentelic marble which consists of calcium carbonate. Up on the marble there are areas with colored surface layers which are distinguished in the "skin", an orange-brown layer, composed from calcium oxalate and calcium phosphate, and the whiting "coating" that covers the "skin" and consists only of calcium carbonate. Restoration work is in progress since 1980 by conservators from the Acropolis Restoration Service.

The area of sampling -shaded most of the day, exposed to the open air, under the influence of the climatic fluctuations-had been divided into two equal parts, P1 and P2 (Figure 1). The sampling site P1 had been left untreated while the sampling site P2 had been treated with the Architectural Biocide D/2). The biocide was applied in September 2009 and specimens were collected under sterile condition one year after (September 2010). The selection of the sampling site, as well as the treatment with the biocide and the collection of specimens was under the supervision of the Acropolis Restoration Service. The sampling procedure followed a two-dimensional ma-trix, the total sampling site was 1 m² and five samples were collected within it. Each individual sample of every sampling

site was mixed to provide a representative sample for each sampling site (Savvides et al. 2011). Specimens were subdivided into three parts for a) microscopic analysis and culture purposes, b) SEM analysis and c) DNA analysis and cloning.

Isolation and Characterization of Cyanobacterial Isolates

The samples before any use were homogenized by transferring into sterile tubes mixed with 10 ml sterile Ringer's solution (0.25 strength) and shaken on an orbital shaker (Stuart Scientific Co.Ltd., Great Britain) at maximum speed (500 rev min^{-1}) for 1h (Wellington et al. 1990). Then they were used for microscopic analysis and for isolation of cyanobacteria using batch cultures and selective media, Enrichment cultures were obtained in flasks and petri-dishes with BG11 (Stanier et al. 1971). Cultures were maintained in a large incubator (SANYO, Gallenkamp) under stable conditions with daylight at room temperature.

Microscopy (LM, SEM)

Collected material was partly fixed with formaldehyde solution at a final concentration 2.5% and partly kept alive for culturing. Both natural (fixed with formaldehyde) and cultured material was observed on glass slides under a high resolution microscope (Photomicroscope III, Zeiss, Germany).

For scanning electron microscopy (SEM, JEOL Ltd, Tokyo, Japan), specimens were dehydrated in an alcohol series (30–100%), critical point dried and spray coated in gold-palladium. They were observed under a JEOL JSM 35 scanning microscope.

Cloning and DNA Analysis

DNA was extracted from enrichment cultures using the Ultra Clean^{TM} Water DNA Isolation Kit (MoBio Laboratories Inc., USA) following the manufacturer's protocol and dissolved in 1 ml of PCR water. DNA from sample P1 was diluted 1:10 with PCR water before PCR amplification, to overcome persistent PCR inhibition problems, whereas sample P2 was amplified undiluted. For cyanobacterial 16S rDNA amplification semi-nested PCR had to be applied. Both PCR reactions were performed using the cyanobacterial primers CYA- 359F and CYA-781R (Nübel et al. 1997). An initial denaturation step at 94°C for 1 min was followed by 35 PCR cycles (94°C denaturation for 1min; primer annealing at 59°C for 1 min; and primer

Fig. 1. The sampling site at the west side of the east wall of the Propylaea. (a) Collection of surface samples consisting of flakes, (b) Magnified view of the surface of the marble from which samples were collected (color figure available online).

extension at 72°C for 2 min), and a final 10 min elongation step at 72°C. PCR products were visualized on a 1,2% agarose gel under UV light, bands were excised, and PCR products were extracted with the Nucleospin Extract II PCR Clean-up kit (Macherey-Nagel GmbH & Co. KG, Germany) following the manufacturer's protocol.

The PCR products were cloned using the TOPO XL PCR cloning kit (Invitrogen Corporation, Hercules, CA, USA) and chemical transformation, according to the manufacturer's specifications. For each clone library clones containing an insert of ca. 400 bp were grown in liquid LB medium with kanamycin. Plasmid DNA was extracted using the Nucle-ospin Plasmid QuickPure kit (Macherey-Nagel GmbH & Co. KG, Germany) for DNA sequencing and screened for ap-propriate sized inserts with the vector-specific primers, M13F and M13R. Purified plasmids (with the primers M13F and M13R) were sent for sequencing to Macrogen Inc. (Seoul, Ko-rea). Each sequence read was approximately 850 bp and was checked for chimeras, using the CHIMERA-CHECK func-tion of the Ribosomal Database Project II. For the detection of closest relatives, all sequences were compared with the BLAST function (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence data were compiled using the MEGA4 software (Tamura et al. 2007) and aligned with sequences obtained from the GenBank (www.ncbi.nlm.nih.gov) databases using the ClustalX aligning utility. Phylogenetic analyses were performed using neighbor-joining methods implemented in MEGA4 and bootstrap with 1000 comparisons. Cyanobacterial clone library coverage was evaluated with Good's coverage estimator (Kemp and Aller 2004).

Biocidal Treatments

Architectural Biocide D/2 (CATHEDRAL STONE) (active ingredients: octyldecyl dimethyl ammonium chloride, dioctyl dimethyl ammonium chloride, didecyl dimethyl ammonium chloride, alcyl (C14 50%, C12 40%, C16 10%) dimethyl benzyl ammonium chloride) was used at a concentration of 100% (v/v). The application was performed with the use of a large brush following vertical, horizontal and circular movements during two successive days.

Results and Discussion

Microscopical analysis revealed 13 cyanobacterial pheno-types, 12 of which were detected at the untreated site and only 4 were detected at the treated site (Table 1). The repre-sentatives belong to 3 orders (Oscillatoriales, Chroococcales, Nostocales). No representatives from the true-branched fila-mentous cyanobacteria (Stigonematales) were detected. It is worth noticing that a great number of those taxa were previ-ously reported in Acropolis monuments (Anagnostidis et al. 1983, 1992, Anagnostidis and Pantazidou 1991).

Comparing our findings with similar studies it can be con-cluded that the cyanobacteria found in this study belonged to the common species on Mediterranean stone monuments. Among others, the genera *Chroococcidiopsis*, *Cyanosarcina*,

Table 1. Cyanobacteria taxa found at the untreated and treated sampling sites

Cyanobacteria	Untreated (P1)	Treated (P2)
Aphanothece sp.	+	−
Chroococcidiopsis kashaii	+	−
Chroococcus cohaerens	+	−
Cyanosarcina parthenonensis	+	−
Cyanosarcina sp.	+	−
Gloecapsa kuetzingiana	+	−
Leptolyngbya gracillima	+	−
Leptolyngbya sp.	+	+
Leptolyngbya schimidlei	+	−
Nostoc cf. *commune*	−	+
Nostoc punctiforme	+	+
Pseudocapsa dubia	+	−
Pseudophormidium hollerbachianum	+	+

Gloeocapsa and *Leptolynbgya* found in this study (Figures 2, 3), have representatives previously reported from marble monuments and statues (Anagnostidis et al. 1983, 1992; Anagnostidis and Pantazidou 1991; Bolivar and Sanchez-Castillo 1997; Caneva et al. 1992; Dupuy et al. 1976; Pantazidou and Theoulakis 1997).

Ortega-Calvo et al. (1995) stated that the genera *Gloeo-capsa, Chroococcus, Phormidium* and *Microcoleus* are consid-ered to be the most common on monuments located in Europe, America and Asia and also as cosmopolitan genera and there-fore their presence was not clearly dependent on the nature of lithic substrate or the climate.

The lithic substrate (pentelic marble) is characterized of low porosity, a condition which is unfavorable for installation and proliferation of endolithic microbial forms; which our findings confirmed. In fact, it is not only the substrate but also the environmental parameters, the orientation (shadow
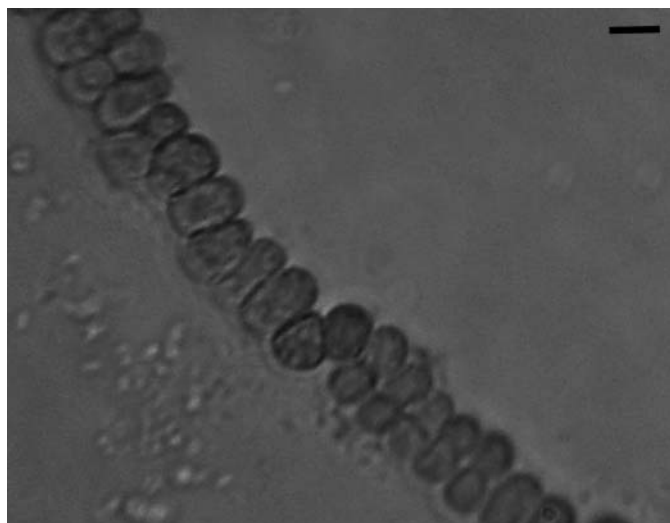

Fig. 2. The tolerant to the applied biocide Nostoc punctiforme under LM. Scale bar = 5 μm.
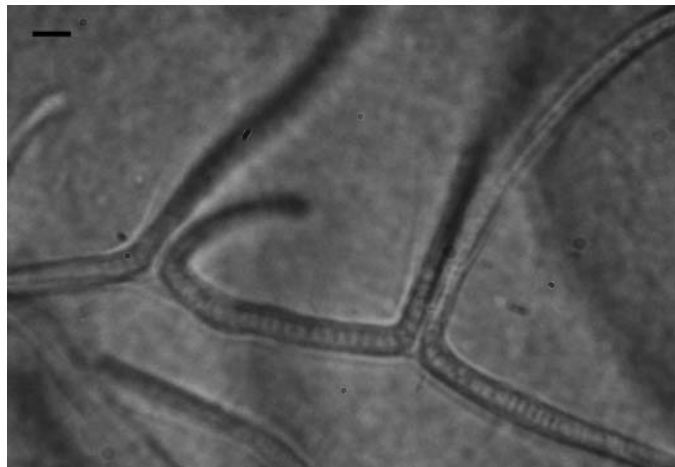
Fig. 3. The filamentous cyanobacterium *Pseudophormidium hollerbachianum* under LM. Scale bar = 3 μm.

or light) and the humidity that play a crucial role for their establishment and proliferation (Macedo et al. 2009).

It has been reported that under extreme conditions such as direct light, high temperature and lack of humidity, endolithic mode of cyanobacterial life occurs. They commonly inhabit the outer millimetres to inner centimetres of rocks exposed to such environments (Walker et al. 2005). In this study cryptoendolithic growth of *Leptolyngbya gracillima* and *Chroococcidiopsis* sp was observed.

It is worth noticing that the cyanobacteria, which are commonly found on stone monuments, have a gelatinous sheath that protects them from dehydration allowing them to colonize stone and marble surfaces even under drought (Macedo et al. 2009; Ortega-Calvo et al. 1991). The firm and gelatinous and/or mucilaginous, occasionally colored sheaths of the phenotypes found i.e. *Nostoc punctiforme* and *Pseudophormidium hollerbachianum* (Figures 2, 3) offers desiccation, UV protection and additional advantage against the biocidal treatment. Especially the heterocystous forms (Nostocales) are reported to be more hardy and tolerant to biocides (Tiwari et al. 2001).

From our observations it seemed that the biocidal action of Architectural Biocide D/2 was still maintained 12 months after its application (Figure 4). Architectural Biocide D/2 belongs to the Quartenary Ammonium Compounds (QACs), which impair the membranes. It may infect the membrane active transport system or disrupt the membrane integrity (Denyer 1995).

Microscopic analysis of fresh and culture material was implied for the characterization at genus level of chroococcoid and filamentous heterocystous cyanobacteria. To identify not only the different phenetic diversity but also the genotypic diversity of culturable and unculturable cyanobacterial isolates on the selected marble surfaces, 16S rDNA analysis were undertaken.

Based on a ≥98% similarity cut-off limit, clone library derived from site P1 from the untreated site yielded 6 phylotypes (Table 2), whereas clone library derived from site P2 from the treated site yielded 4 phylotypes (Table 3). Cyanobacterial
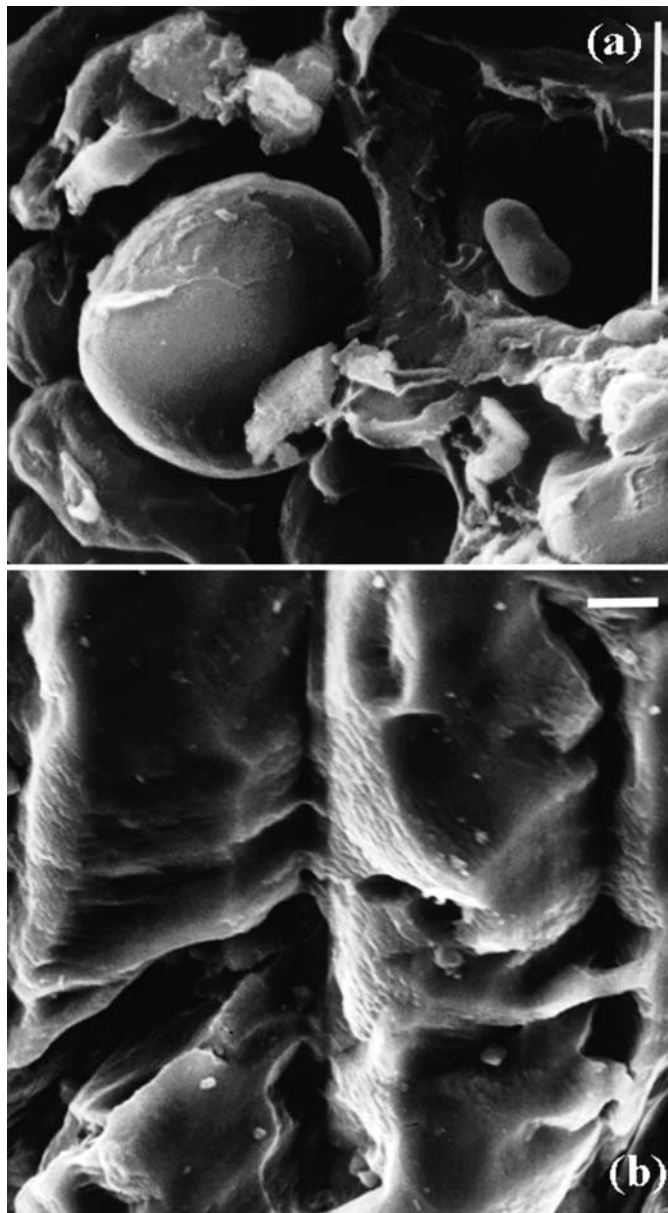

Fig. 4. (a) Presence of coccoid cyanobacterial cells at the untreated P1 sampling site under SEM, (b) Absence of cyanobacterial cells after treatment with the biocide under SEM. Scale bar = 10 μm.

phylotypes *Nostoc* sp. and *Leptolyngbya* sp. were dominant in both libraries. These strains are reported to be widely present in broad variations in many similar ecosystems (Norris and Castenholz 2006; Portillo et al. 2009; Walker and Pace 2007). The clone library coverage for the cyanobacterial 16S rRNA gene was satisfactory for both libraries, according to Good's C estimator (Kemp and Aller 2004) as all curves reached a plateau above 0.80 with the number of clones analyzed. Two singletons (phylotypes that appear only one time in the library) were retrieved from each one of the two libraries.

Among the 10 different phylotypes, 6 phylotypes were within the species level, showing more than 97% sequence identity (Stackenbrandt and Ebers 2006). On the other hand,

Table 2. Clone library from the untreated site P1

Clone (Frequency)[1]	Phylogenetic affiliation	Closest phylogenetic relative (GenBank Accession Number)	% Identity[2]	GenBank Accession Number
CYAP01 (6/20)	Oscillatoriales	*Leptolyngbya* sp. FI5–2HA4 (HM018683.1)	98.0	JQ690661
CYAP07 (6/20)	Nostocales	*Nostoc* sp. 9E-03 (FR798938.1)	99.0	JQ690662
CYAP13 (3/20)	Nostocales	*Nostoc* sp. 9E-03 (FR798938.1)	96.0	JQ690663
CYAP16 (3/20)	Pleurocapsales	Uncultured *Chroococcidiopsis* sp. clone Thd_c8 (DQ390517.1)	99.0	JQ690664
CYAP19 (1/20)	Cyanobacteria	Uncultured cyanobacterium clone 12–31 (JN814156.1)	84.0	JQ690665
CYAP20 (1/20)	Oscillatoriales	*Leptolyngbya* sp. FI5–2HA3–2 (HM018684.1)	86.0	JQ690666

[1]Clones that have ≥98% similar nucleic acid sequences within each sampling site are represented by a single sequence, with the number of clones out of the total in parentheses.
[2]The percentage identity with the 16 rDNA sequence of the closest phylogenetic relative.

3 phylotypes were less than 95% identical to the closest 16S rRNA gene in the nucleotide sequence database (Tables 2, 3). It has to be mentioned that identities below 97% have little meaning even at the genus level, and reflect the large gaps in the database at the present time between cultivated strains and collected environmental samples (Horath and Bachofen 2009; Norris and Castenholz 2006).

Clone library P2 from the treated site showed lower cyanobacterial diversity compared to the one from the untreated site P1. This assumption could be attributed to the antimicrobial action of the biocide applied, as mentioned previously. On the other hand, in the untreated site P1, comparing the results of phenotypical and molecular approaches for the detection of cyanobacteria, clone analysis failed to detect coccoid cyanobacteria, with the exception of *Chroococcidiopsis* sp. Wacklin (2006) mentioned that cyanobacterial diversity may be overestimated as far as heterocystous cyanobacteria are concerned, as strains of the order Chroococcales carry only one or two copies of the 16S rRNA gene. Moreover, this inconsistency could just reflect the large gaps in the sequence databases (Crispim et al. 2006; Horath and Bachofen 2009). When considered the treated site P2, both approaches reflect almost identical images of the cyanobacterial community of the marble surface.

These phylotypes together with 24 sequences from GenBank were used for the phylogenetic analysis. Four well-

supported clusters were found after the construction of the phylogenetic tree (Figure 5). The cluster I gathered strains of the heterocystous cyanobacterium *Nostoc*. There are three closely related to our findings strains, *Nostoc* sp. 9E-03, Nostoc sp. VP2–08 and an uncultured cyanobacterium isolate (referred to as uncultured cyanobacterium isolate 2 in Figure 5). The first two strains had been isolated from monumental fountains (Cuzman et al. 2010), and the third one was referred to as endolithic isolation (Singler et al. 2003). The phylotypes CYAP07, CYAP19, CYAP20, CYAP13, CYAE12, CYAE01 were also related to a *Leptolyngbya* sp. reported from similar ecosystems (Norris and Castenholz 2006; Portillo et al. 2009; Walker and Pace 2007).

Cluster II consisted of the phylotypes CYAP01, CYAE06 (both identified as *Leptolyngbya* sp. F15–2HA4 with 98% sequence identity, see Table 2 and 3), the strain *Leptolyngbya crispate* SEV4–3-C6 and an uncultured cyanobacterium isolate derived from endolithic isolates (Singler et al. 2003). Clusters I and II are closely related and clearly separated from the others (Figure 5).

Cluster III included the phylotype CVAE13, which is related to strains commonly found in similar ecosystems and to one uncultured cyanobacterium isolate from frescoes (Cappitelli et al. 2009).

Cluster IV was represented by the phylotype CYAP16 identified as *Chroococcidiopsis* sp. Clone Thd_c8 with 99%

Table 3. Clone library from the treated site P2

Clone (Frequency)[1]	Phylogenetic affiliation	Closest phylogenetic relative (GenBank Accession Number)	% Identity[2]	GenBank Accession Number
CYAE01 (5/13)	Nostocales	*Nostoc* sp. SAG 39.87 (DQ185252.1)	99,0	JQ690667
CYAE06 (6/13)	Oscillatoriales	*Leptolyngbya* sp. FI5–2HA4 (HM018683.1)	98,0	JQ690668
CYAE12 (1/13)	Nostocales	*Nostoc* sp. 9E-03 (FR798938.1)	97,0	JQ690669
CYAE13 (1/13)	Nostocales	*Cylindrospermum* sp. A1345 (DQ897365.1)	85,0	JQ690670

[1]Clones that have ≥98% similar nucleic acid sequences within each sampling site are represented by a single sequence, with the number of clones out of the total in parentheses.
[2]The percentage identity with the 16 rDNA sequence of the closest phylogenetic relative.
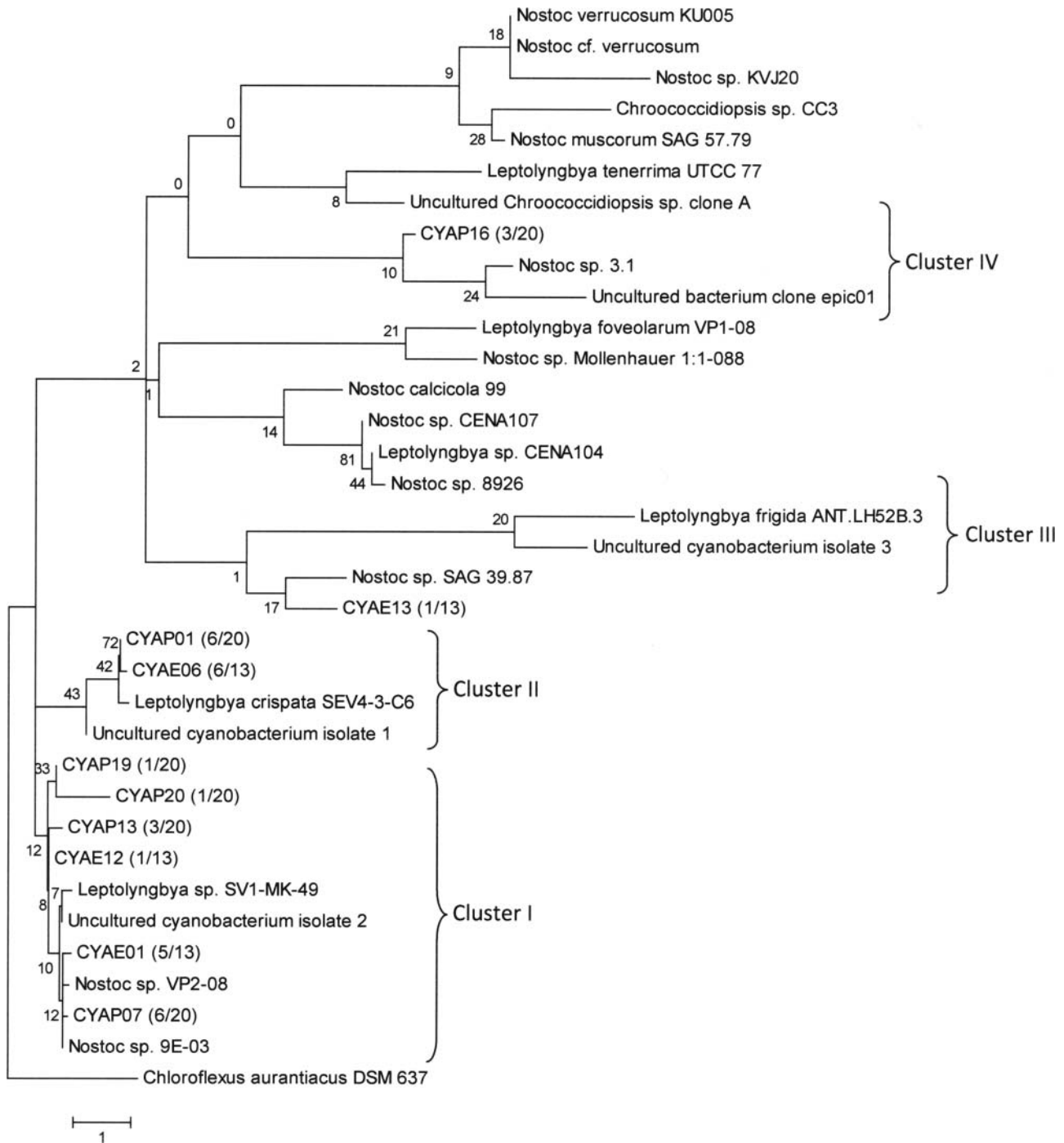
Fig. 5. Phylogenetic tree of the cyanobacterial 16S rDNA from P1, P2 sampling sites, based on neighbor-joining method. One thousand bootstrap analyses (distance) were conducted. The tree was rooted with *Chloroflexus aurantiacus*. Clones that have 98% similar nucleic acid sequences within each sampling site are represented by a single sequence, with the number of clones out of the total in parentheses.

sequence identity. This phylotype was closely related to an un-cultured bacterium isolate (refered as uncultured bacterium clone epic01 in Figure 5) derived from epilithic and endolithic bacterial communities from a Maya archaeological site (McNamara et al. 2006).

In conclusion, if a direct comparison between the two ap-proaches could be allowed— estimation of cyanobacterial di-versity by phenotypic and/or molecular biological methods— data obtained by both approaches reflected similar images of the cyanobacterial community for the treated site P2 on genus

level (Tables 1 and 3), while for the untreated site P1 genera like *Aphanothece* and *Gloeocapsa* were absent from molecular analysis results (Tables 1 and 2).

Summarizing, the antimicrobial action of the QAC, Architectural Biocide D/2 was maintained active 12 months after its application. The observed inconsistencies between cultivation and molecular approaches highlighted the need for a polyphasic methodology to be applied. Polyphasic studies that incorporate both microscopical and molecular techniques are vital in order to increase our knowledge of cyanobacterial diversity and to assess the efficiency of different strategies to control deteriogenic biofilm communities in cultural artwork.

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