Microbial Community Analysis of Fresh and Old Microbial Biofilms on Bayon Temple Sandstone of Angkor Thom, Cambodia

Wensheng Lan · Hui Li · Wei-Dong Wang · Yoko Katayama · Ji-Dong Gu

Abstract The temples of Angkor monuments including Angkor Thom and Bayon in Cambodia and surrounding countries were exclusively constructed using sandstone. They are severely threatened by biodeterioration caused by active growth of different microorganisms on the sandstone surfaces, but knowledge on the microbial community and composition of the biofilms on the sandstone is not available from this region. This study investigated the microbial community diversity by examining the fresh and old biofilms of the biodeteriorated bas-relief wall surfaces of the Bayon Temple by analysis of 16S and 18S rRNA gene sequences. The results showed that the retrieved sequences were clustered in 11 bacterial, 11 eukaryotic and two archaeal divisions with disparate communities (Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Proteobacteria; Alveolata, Fungi, Metazoa, Viridiplantae; Crenarchaeote, and Euyarch-aeota). A comparison of the microbial communities between the fresh and old biofilms revealed that the bacterial community of old biofilm was very similar to the newly formed fresh biofilm in terms of bacterial composition, but the eukaryotic communities were distinctly different between these two. This information has important implications for understanding the formation process and development of the microbial diversity on the sandstone surfaces, and furthermore to the relationship between the extent of biodeterioration and succession of microbial communities on sandstone in tropic region.

Introduction

Khmer civilization is an important part of the world culture heritage, but unfortunately the only recorded history of this

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civilization and culture is mostly derived from the bas-relief on sandstone at different temples except one Chinese official's journal [10]. Angkor Thom and Bayon Temple have been under severe threats from both human and microbial processes in recent years [22]. Over the last 800–1,000 years, physical, chemical, and biological processes have made significant impact on these sandstone structures, resulting in serious deterioration of the sandstone and loss of many bas-relief and writing on sandstone surface. The deteriorating condition of the temple has brought great public awareness for action worldwide to preserve and protect the historical and cultural heritage. The causes of deterioration of Angkor Thom and Bayon Temple had been studied for chemical weathering, efflorescence [33], however little is known about the microbial flora living on the sandstone surfaces related to the biodeterioration on the wall of Angkor Thom and temples in this area. As the sandstone is porous and capable of permeation and trapping of moisture from natural rainfall, bioactivity on substratum material can achieve the highest levels under warm-humid climate where the environmental conditions are extremely favorable for the growth of diverse organisms, it is therefore necessary to evaluate the influence of the microbial impact on stone deterioration.

Recently, a diverse community of microorganisms of the epilithic and endolithic bacterial communities in limestone from a Maya archeological site was reported, results show that the endolithic bacterial community is distinctively different from the community on the limestone surface [24], which may be due to the influence of the physical and chemical properties of the calcareous stone materials. There are also distinctive bacterial and fungal populations on the surfaces of different mineral types, e.g., granite [12, 13]. Therefore, bacterial and fungal community on stones is common, depending on environmental conditions and the physicochemical properties of the material. In addition, pollution has a major impact on the microbial community and also the degradation of mineral materials [25]. The Angkor temples in Cambodia are mainly composed of sandstone and laterite [33], which are nutrient-poor substrate with high mineral content, but the combination of variable temperature and plentiful rainwater provide the basis for an active ecological niche containing highly specialized microorganisms to form biofilms on the sandstone. The biofilm can then interact with the substratum materials, dissolving minerals for nutrients. Once the autotrophic microorganisms including cyanobacteria and algae colonize on the surface of the wall and evolve into biofilm, other heterotrophic bacteria can initiate their involvement in the biofilm. The complex biofilm community can colonize the sandstone and develop into defined community, and their biochemical activity can result in degradation of the sandstone, especially where moisture is available.

Since biodeterioration of inorganic materials by microorganisms in open environment is rarely the activity of one or a few species of microorganisms, it is necessary to obtain information about the microbial community structure of the sandstone wall at Bayon Temple so that the possible biodeteriogens on the substratum can be identified. Given the fact that information on microbial communities on the sandstone wall of any temple in this area is not available, the objectives of this study were to investigate the characteristics of microbial community diversity and to compare two different biofilms to determine the microbial succession on sandstone in order to provide effective intervention to decrease the microbial deterioration processes.

Materials and Methods

Sampling Site and Samples

Angkor Thom and surrounding temples are subjected to high humidity and rainfall under tropical climate. A remarkable microbial colonization on surfaces of sandstone material has been observed on different sites of the roof and walls (Fig. S1). Experimental samples of this study were taken from a seriously deteriorated bas-relief in the gallery section of Bayon Temple, which does not receive direct sunlight, to study the microbial community on sandstone. Seven representative samples of two distinctive areas with different biofilms in appearance located at different heights from the deteriorated bas-relief walls at Bayon Temple (Fig. S1) were taken aseptically in October 2007. Description of this sampling site BYc is available elsewhere [22]. The Bayon Temples are mainly made of laterite and sandstone, the latter was used in sanctuaries and surfaces of buildings and platforms. The typical coloration spots were spread all over the wall surfaces on all roof and walls. There are many patches of biofilm with different colors on wall surfaces and developed in a very extensive carpet-like fashion. The biofilm, which was green and called "fresh biofilm" in this study while that, which was black and called "old biofilm" were gently scraped off from the wall. Samples byn1, byn2 were near the base of the bas-relief, and byn3 were near the base of a wall displayed black colors and showed surface deterioration in moist conditions. Samples byn4, byn5, byn7, and byn8 were green biofilm located above the black biofilm.

DNA Extraction

Standard method for total community DNA extraction was used to the seven samples from different sites involving the lysis of the cells with sodium dodecyl sulphate and N-lauryl sarcosine (Sarcosyl) in a buffer containing proteinase K.

This was followed by standard phenol/chloroform extractions to remove proteins and precipitation of the nucleic acids with ethanol. The samples from the cultural heritage sites may contain fungal spores. A CTAB/NaCl extraction method, normally used to extract DNA from plant cell, was used in the experiment [3]. Nucleic acids were purified with a DNA purification kit (V-Gene, China) and the quality of DNA preparations was verified by measuring the absorbance ratio at 260/280 nm.

PCR Amplification of Ribosomal DNA

The bacterial, archaeal and eukaryotic rRNA gene primers (Table 1) used in polymerase chain reaction (PCR) were 27BF and 1492BR [36], 109AF and 943AR [15], 82EF and 1391ER, 378EF, and 1492ER [7]. All PCR amplifications were performed using a GeneAmp PCR System 9600 thermocycler (Applied Biosystems, CA, USA). The bacterial rRNA gene PCR mixture comprised 50 µl, containing 25.7 µl sterile MilliQ water, 10 µl Colorless GoTaq® Flexi buffer (5×), 1 µl deoxynucleoside triphosphates (10 mM each), 5 µl MgCl$_2$ solution (25 mM), 2 µl forward primer (20 µM), 2 µl reverse primer (20 µM), 0.3 µl GoTaq® DNA polymerase (5 U/µl) (Promega Corporation, USA), and 4.0 µl template DNA (0.02 µg/µl). The PCR program was as follows: denaturing step of 95°C for 2 min, followed by 33 cycles of denaturing for 1 min at 95°C, annealing for 1 min at 55°C, and extension for 1.5 min at 72°C, followed by a final extension at 72°C for 7 min. The archaeal and eukaryotic rRNA gene PCR mixture was the same as those described above except the primers. The archaeal rRNA gene PCR program was as follows: denaturing step of 95°C for 2 min, followed by 33 cycles of denaturing for 45 s at 95°C, annealing for 45 s at 52°C, and extension for 1 min at 72°C, followed by a final extension at 72°C for 7 min. The eukaryotic rRNA gene PCR program used a temperature gradient PCR approach to provide a maximum coverage of eukaryotic rRNA genes in PCR amplifications. The cycling protocol was as follows: denaturing step of 94°C for 2 min, followed by 35 cycles of denaturing at 94°C for 1 min, annealing temperature gradient of 52–65°C for 1 min and

extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. Positive PCR products were selected and confirmed to be the expected size by gel electrophoresis. Amplification was performed three times with each primer set, and the products from respective primer sets were pooled to minimize PCR bias.

DNA Libraries Construction

All of the above resultant PCR products were separated by electrophoresis in 1.2% agarose gels. The bands with expected size were excised and purified with a QIAquick gel extraction kit (Qiagen USA). The purified DNA from respective primer sets were then cloned one by one with a pMD18 TA cloning kit (Takara, Dalian, China), in accordance with the manufacturer's instructions. The recombinant vectors were transformed into *Escherichia coli* JM109 competent cells.

Amplified Ribosomal DNA Restriction Analysis

Amplified ribosomal DNA restriction analysis (ARDRA) was performed to analyze the diversity of positive clones [34]. One set of universal primers M13uni and M13rev was used by PCR for identifying the clones of libraries. The templates of PCR were recombinant *E. coli* clones from libraries. PCR products were digested at 37°C for 4 h using restriction enzymes *Rsa*I and *Msp*I. The restriction fragments were separated by electrophoresis using 3% agarose gel. According to ARDRA patterns, clones with identical restriction patterns were grouped into one operational taxonomic units (OTUs).

Sequencing and Phylogenetic Analysis

Clones representing each distinct ARDRA pattern were chosen for sequencing. The plasmid DNA was isolated from selected clones with an AxyPrep-96 Plasmid Kit (Axygen, USA). The rRNA gene inserts were sequenced on automated ABI 3700 sequencer (Dye-Terminator Cycle Sequencing Ready Reaction FS Kit; PE Applied Biosys-

Table 1 PCR primer sets used in this study

Primer set	Sequence (5′→3′)	Target	Reference
27BF	AGAGTTTGATC(A/C)TGGCTCAG	Bacteria, 16S rRNA gene	[36]
1492BR	TACGG(C/T)TACCTTGTTACGACTT		
109AF	AC(G/T)GCTCAGTAACACGT	Archaea, 16S rRNA gene	[15]
912AR	CTCCCCCGCCAATTCCTTTA		
82EF	GAA(G/A/T)CTG(C/T)GAA(C/T)GGCTC	Eukaryote, 18S rRNA gene	[7]
1391ER	GGGCGGTGTGTACAA(A/G)G(A/G)G		
378EF	CGGAGA(A/G)GG(A/C)GC(A/C)TGAGA	Eukaryote, 18S rRNA gene	[7]
1492ER	ACCTTGTTACG(A/G)CTT		

Table 2 No. of clones in fresh- and old-biofilm clone libraries

Kingdom	Division	No. of clones in old biofilm	No. of clones in fresh biofilm
Archaeal	Crenarchaeote	14	18
	Euyarchaeota	16	22
Bacteria	Proteobacteria	104	64
	Cyanobacteria	36	67
	Bacteroidetes	14	39
	Acidobacteria	9	28
	Actinobacteria	14	8
	Chloroflexi	4	11
	Gemmatimonadetes	0	3
	Deinococcus-Thermus	0	3
Eukaryotes	Alveolata	12	28
	Viridiplantae	102	80
	Fungi	26	84
	Metazoa	2	6
	Stramenopiles	9	2

tems) using M13 universal sequencing primers. The resulting sequences were examined for possible chimeric artifacts using the programs CHIMERA-CHECK [5] in the Ribosomal Database Project II (http://rdp.cme.msu.edu). Unaligned sequences were compared with the National Center for Biotechnology Information database using the BLAST search program to find closely related sequences [1]. Sequences were aligned to their nearest neighbor using CLUSTAL×34. Phylogenetic trees were constructed based on the Kimura two-parameter model [20] and the neighbor-joining algorithm [31] using the PHYLIP package [9]. Bootstrap analysis with 1,000 replicates was applied to assign confidence levels to the nodes of trees. Groupings that occurred in less than 50% of replicates were excluded.

Statistical Analysis

A rarefaction analysis [17] and coverage [14] were applied to estimate the representation of the phylotypes and to characterize the microbial diversity of these samples. The rarefaction curves were produced with the software Analytic Rarefaction 1.3 (http://www.uga.edu/~strata/software/Software.html). The coverage of clone libraries was calculated from the equation $C = [1 - (n_1/N)] \times 100$, described by Good, where C is the homologous coverage, n_1 is the number of phylotypes appearing only once in the library, and N is the total number of clones examined. Composition of the communities of "old biofilm" and "fresh biofilm" was compared by using the similarity measurement of the Renkonen index [21] calculated as $P = \sum \text{minimum} (p_{1i}, p_{2i})$, where P is the similarity between community one and two, p_{1i} is the proportion of group i in community 1, p_{2i} is the proportion of group i in community two.

Nucleotide Sequences Accession Number

The GenBank accession numbers for bacterial 16S rRNA gene sequences are GQ395248 to GQ395289, for archaeal 16S rRNA gene sequences are GQ395290 to GQ395293; and for eukaryotes 18S rRNA gene sequences are GQ395294 to GQ395312 in this study.

Results

A total of 180 and 280 bacterial, 150 and 200 eukaryotic, 30 and 40 archaeal clones were screened from the old- and fresh-biofilm libraries, respectively (Table 2). A total of 41 bacterial, four archaeal, and 19 eukaryotic OTUs were
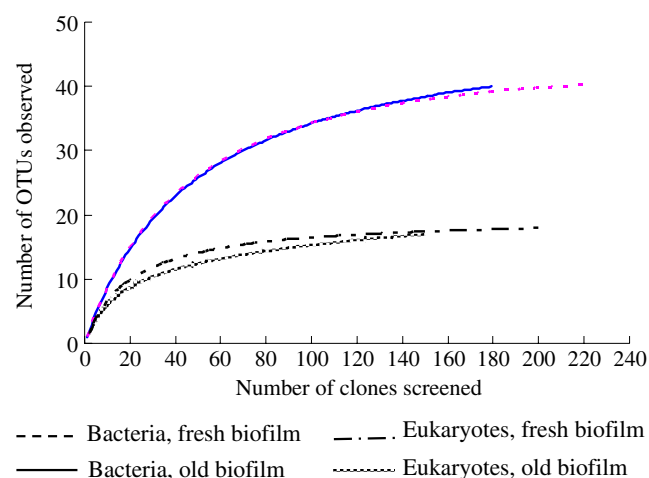
Figure 1 Rarefaction curve for the bacterial and eukaryotic communities. Clones were grouped by phylum and subphylum using BLAST results

Figure 2 Phylogenetic relationships based on partial ribosomal small subunit gene sequences of fresh and old biofilm clones (shown in *boldface*) isolated from bas-relief of Bayon Temple with sequences from members of the **a** Alphaproteobacteria; **b** Beta, Gamma, and Deltaproteobacteria; **c** Actinobacteria, Acidobacteria, Bacteroidetes, Cyanobacteria, Deinococcus-Thermus, and Gemmatimonadetes; **d** Eukaryote; and **e** Archaea. The number in parentheses followed the OTUs name was the clone number obtained in this study. Neighbor-joining trees; bootstrap values based on 1,000 replicates are indicated for branches supported by 50% of trees. *Scale bar* represents 0.2 nucleotide changes per position
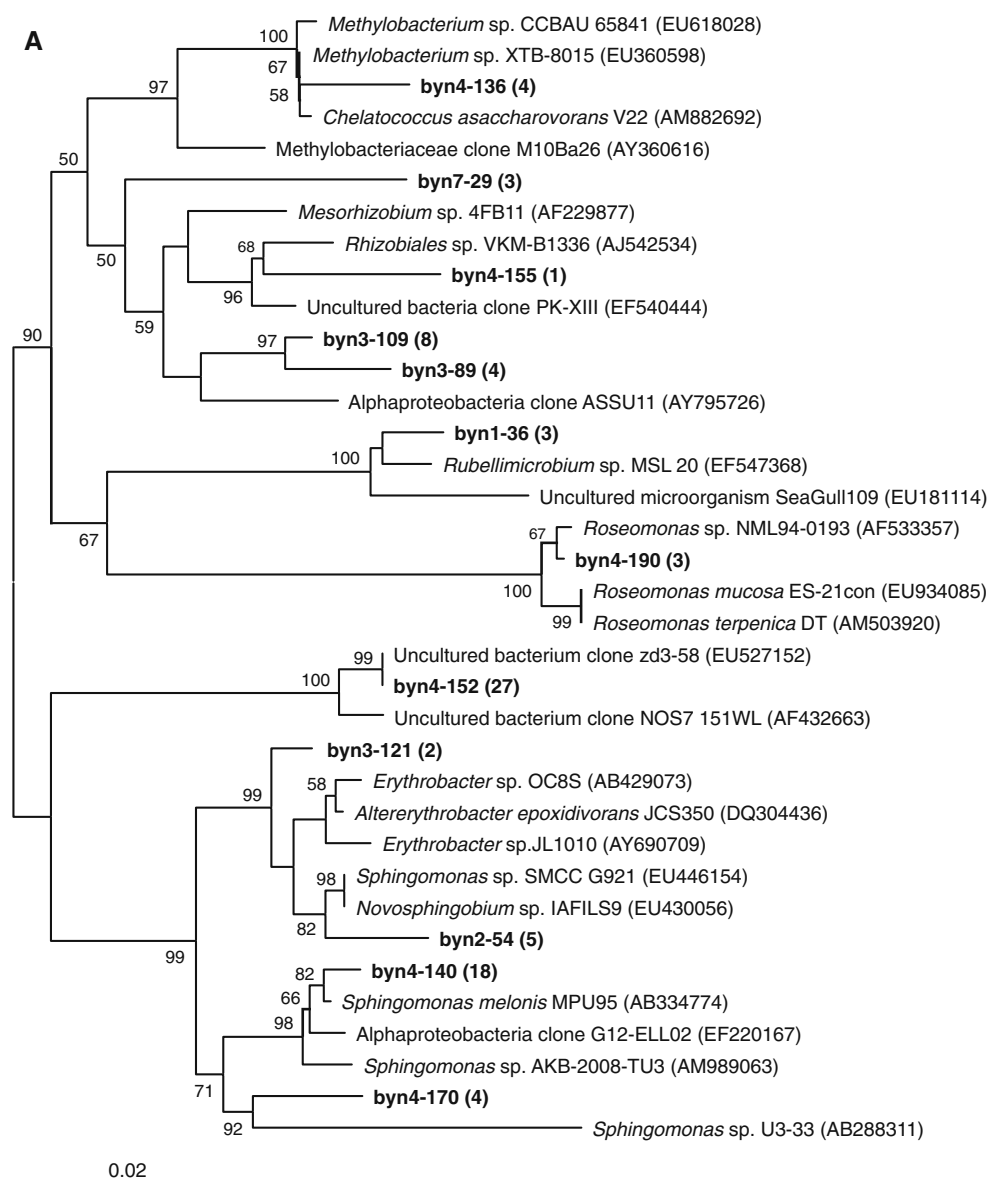

obtained by sequences alignment. The coverage of bacterial, eukaryotic, and archaeal clone libraries was all above 96%, indicating that the major of the biofilm diversity in the clone libraries was detected. In addition, the rarefaction curves generated from our clones reached the asymptote (Fig. 1), indicating that the diversity in the libraries was representative of the community and there was no need for further sampling of more clones. Phylogenetic analysis showed that at least eight bacterial, five eukaryotes, and two archaeal division existed in the niche on the sandstone walls at Bayon Temple. Among the bacterial clones, the majority of sequences were affiliated with proteobacteria (Fig. 2a, b), which made up 30% for old biofilm and 12% for fresh biofilm (Fig. S2), with Alphaproteobacteria being the most abundant (Fig. 2a). Within this cluster, byn4-152 and byn4-140 were the two most common sequences

(Fig. 2). The closest BLAST match to clone byn4-152 was uncultured bacterium clone zd3-58 isolated from forest cut-block surface organic matter from the British Columbia Ministry of Forests Long-Term Soil Productivity installation near Williams Lake, BC, Canada [4], whereas the closest BLAST match to clone byn4-140 was *Sphingomonas melonis* MPU95 isolated from a sea urchin (Fig. 2a).

A large number of clones from the bacterial communities were closely related to some photosynthetic bacteria that belong to Cyanobacteria and Chloroflexi division (Fig. 2c), which appeared to be very common in these samples (20% for old biofilm and 24% for fresh biofilm bacterial clone libraries). A small number of clones from the bacterial communities were closely related to the Acidobacteria and Bacteroidetes (Fig. 2c) as well as the Deinococcus-Thermus, Gemmatimonadetes, and Actinobacteria
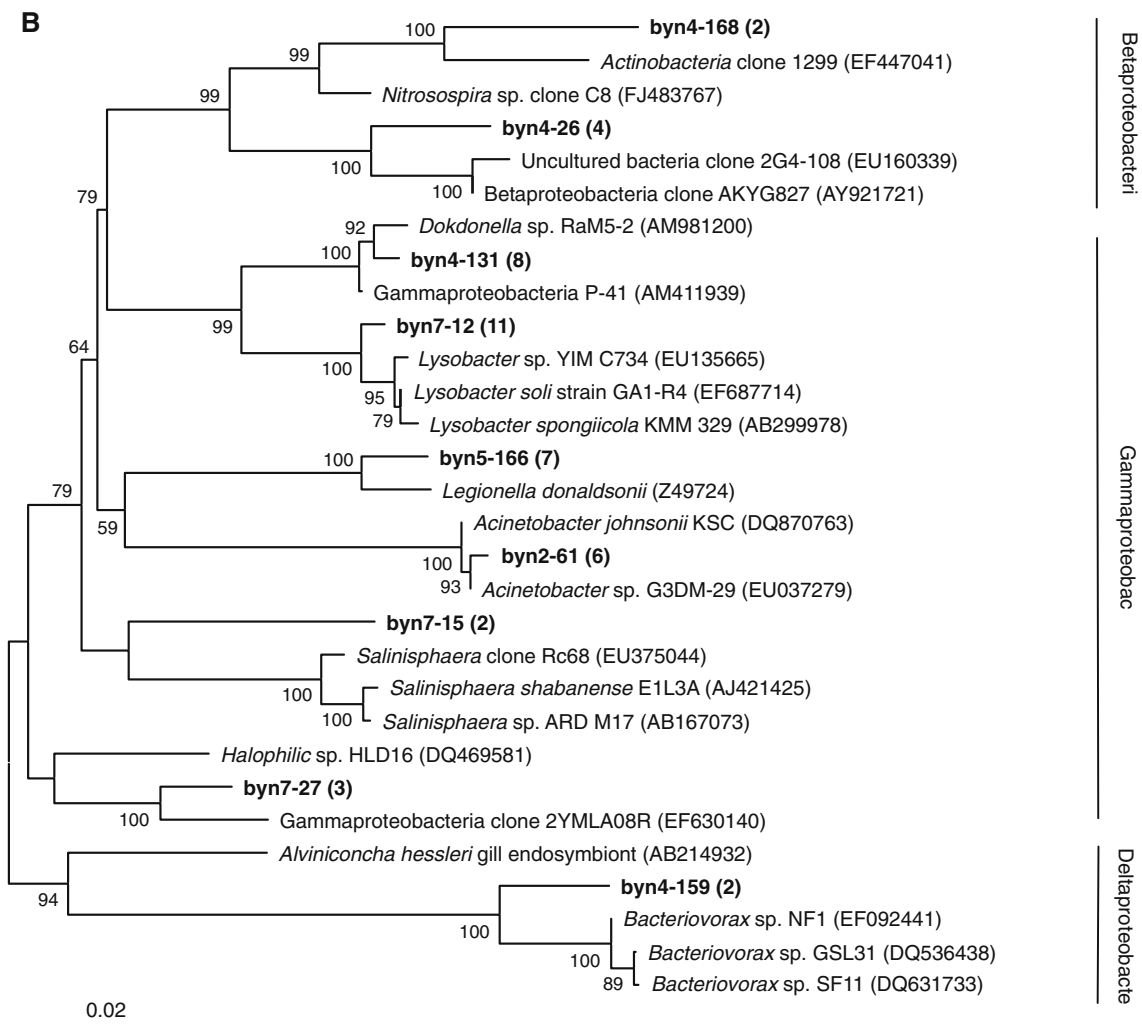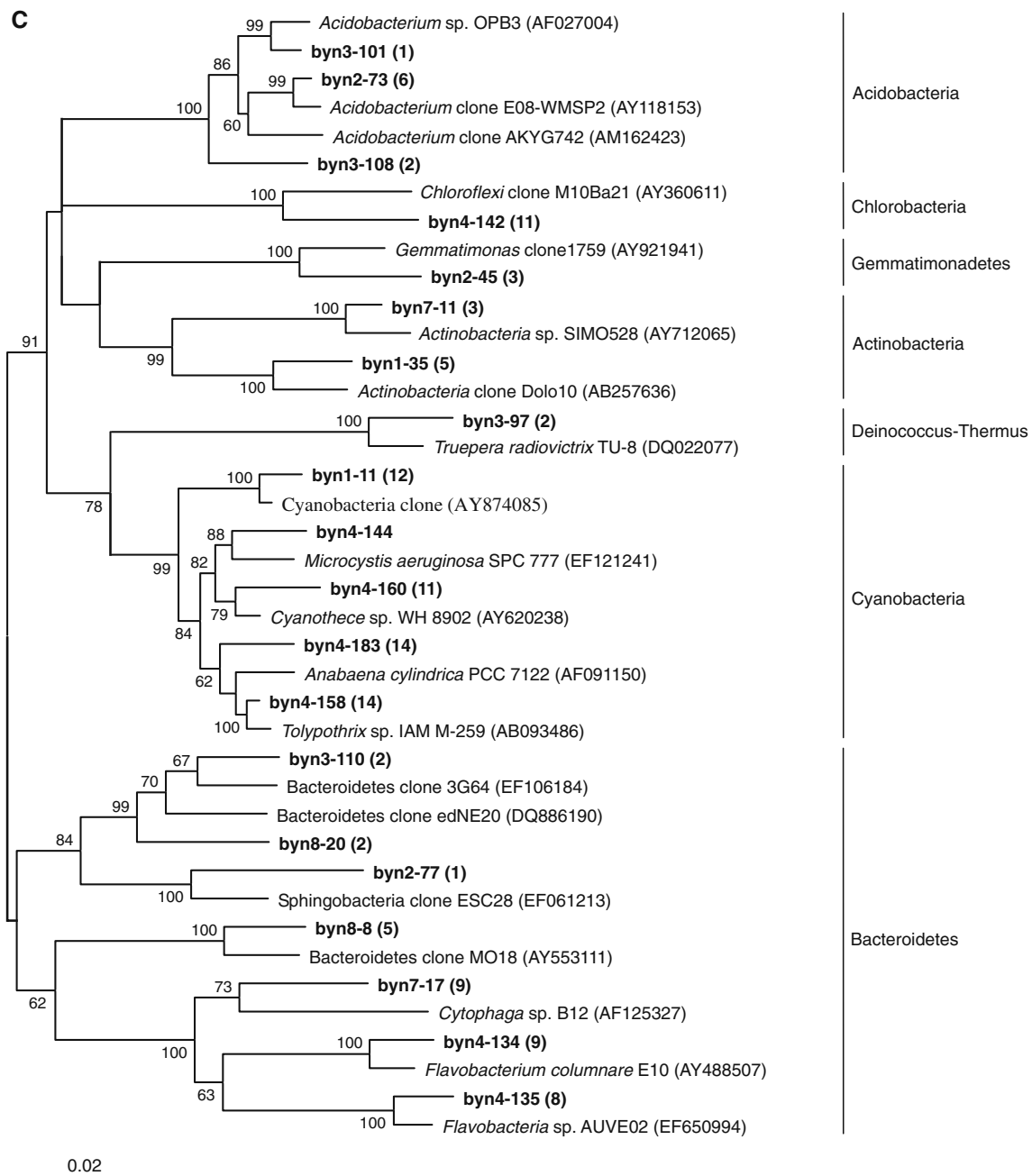
Figure 2 (continued)

(Fig. 2c). Most of the eukaryotic clones were grouped in fungi and algae which were also frequently detected on the external walls [11] whereas only a few clones belonged to protozoa and metozoa acting as the predators in the sandstone biotope. The total clones of fungi accounted for 7% of old biofilm and 18% of fresh biofilm (Fig. S2), among them the clones related to Dikarya and Chytridiomycota were detected in all samples. Within the Dikarya subdivision, there were two groups of sequences primarily related to the Basidiomycota and Ascomycota accounting for 3%, 4% of old biofilm and 10%, 16% of fresh biofilm eukaryotic clone libraries, respectively.

Among the algal clones, the majority of sequences were related to Chlorophyta accounting for 52% of old biofilm and 30% of fresh biofilm eukaryotic clone libraries. All of the samples from the different sites contain Chlorophyta. These samples were taken from bottom of the wall. There was a cluster of sequences within the Chlorophyta primarily consisting of clones from epilithic samples. Within this

cluster, three of the sequences were closely related to the sequences previously obtained from dolomite stone materials. The closest BLAST matches to clones byn4-45, byn5-111 and byn8-143 (Fig. 2d) were Chlorophyte clone DA-15 (AB257666), Chlorophyte clone DA-12 (AB257663) and Chlorophyte clone DA-14 (AB257665), respectively. These clones were isolated from the rock in Central Alps, Switzerland [18]. And a number of clones contained sequences similar to the Streptophyta accounting for 16% of old biofilm and 7% of fresh biofilm eukaryotic clone libraries. A cluster of sequence byn3-38 (Fig. 2d) and related within the Streptophyta contained clones primarily belonging to green algae [30].

In addition, all the archaeal sequences found fell into Crenarchaeota and Euryarchaeota division (Fig. 2e), which were the most commonly encountered and abundant environmental archaeal sequences. Within the Euryarchaeota, there was a cluster of sequence phylotypes previously obtained from stone cultural heritage materials. The

C

Clade	Bootstrap/Taxa

Acidobacteria

99 *Acidobacterium* sp. OPB3 (AF027004)
byn3-101 (1)
86
99 **byn2-73 (6)**
Acidobacterium clone E08-WMSP2 (AY118153)
100
60
Acidobacterium clone AKYG742 (AM162423)
byn3-108 (2)

Chlorobacteria
100 *Chloroflexi* clone M10Ba21 (AY360611)
byn4-142 (11)

Gemmatimonadetes
100 *Gemmatimonas* clone1759 (AY921941)
byn2-45 (3)

Actinobacteria
100 **byn7-11 (3)**
Actinobacteria sp. SIMO528 (AY712065)
99
byn1-35 (5)
100 *Actinobacteria* clone Dolo10 (AB257636)
91

Deinococcus-Thermus
100 **byn3-97 (2)**
Truepera radiovictrix TU-8 (DQ022077)

Cyanobacteria
100 **byn1-11 (12)**
Cyanobacteria clone (AY874085)
78
88 **byn4-144**
Microcystis aeruginosa SPC 777 (EF121241)
99 82
byn4-160 (11)
79 *Cyanothece* sp. WH 8902 (AY620238)
84
byn4-183 (14)
Anabaena cylindrica PCC 7122 (AF091150)
62
byn4-158 (14)
100 *Tolypothrix* sp. IAM M-259 (AB093486)

Bacteroidetes
67 **byn3-110 (2)**
70 Bacteroidetes clone 3G64 (EF106184)
99
Bacteroidetes clone edNE20 (DQ886190)
84
byn8-20 (2)
byn2-77 (1)
100 Sphingobacteria clone ESC28 (EF061213)
100 **byn8-8 (5)**
Bacteroidetes clone MO18 (AY553111)
73 **byn7-17 (9)**
Cytophaga sp. B12 (AF125327)
100 100 **byn4-134 (9)**
Flavobacterium columnare E10 (AY488507)
63 **byn4-135 (8)**
100 *Flavobacteria* sp. AUVE02 (EF650994)
62

0.02

Figure 2 (continued)

closest match to clone byn3-12 (Fig. 2e) was *Halobacterium* clone K14 (AM159641) isolated from the mural paintings on the deteriorated ancient wall surface [28], whereas the closest match to clone byn5-30 (Fig. 2e) was Uncultured archaeon clone 371 (EF188566) isolated from the "white colonizations" on the paleolithic paintings, Altamira Cave, Spain [29]. The sequence phylotypes clustered in the Crenarhaeota were found in all of the samples, in which the closest match to clone byn2-22 (Fig. 2e) was uncultured archaeon clone ARC 10SAF2-82

(DQ782341) isolated from spacecraft assembly clean rooms [27]. Taxonomic composition of the old biofilm and fresh biofilm communities appeared to be different (Fig. S2). Similarity of the communities determined using the Renkonen index (RI) was 0.78, 0.63 and 0.98 for the bacterial, eukaryotic and archaeal clone libraries between the old and fresh biofilms, respectively. The similarity of bacterial communities was higher than eukaryotic communities, whereas the similarity of archaeal communities was almost identical between the two types of biofilm samples.
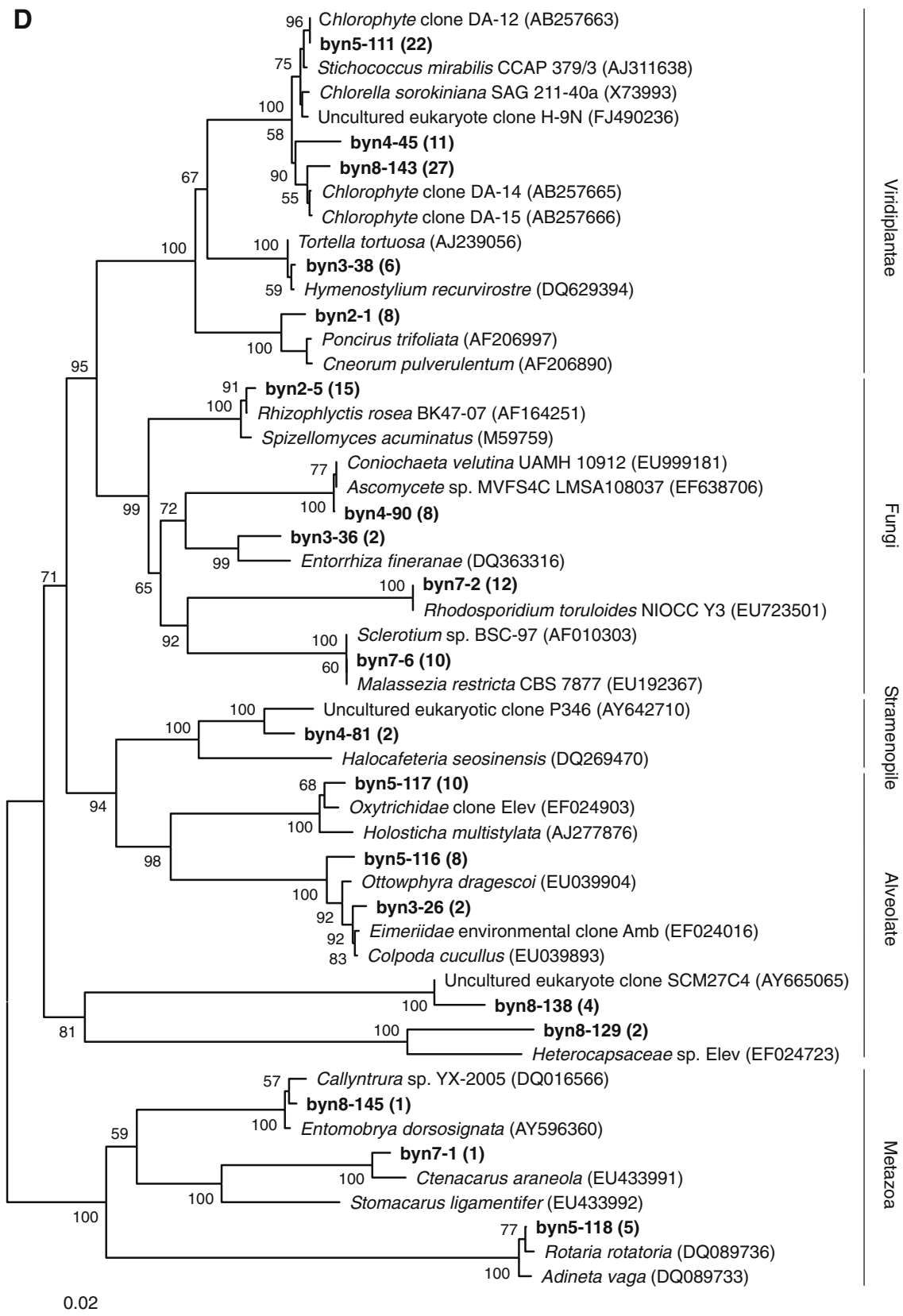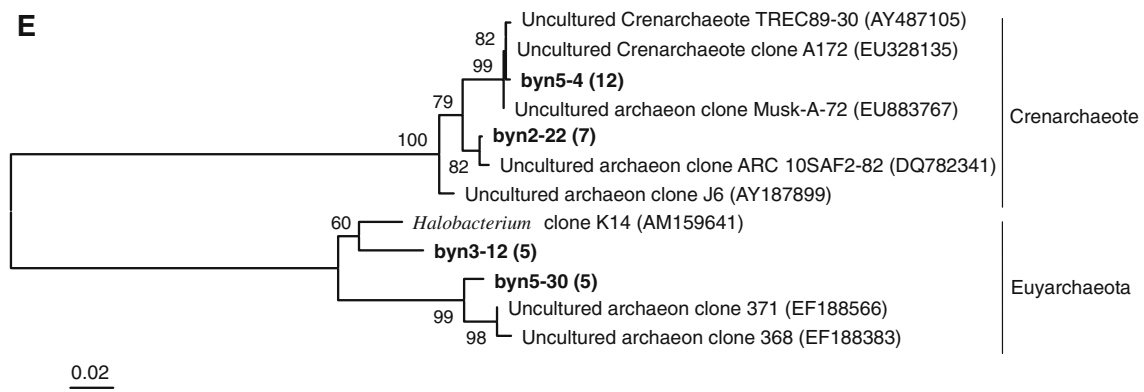
D

Figure 2 (continued)

E

Figure 2 (continued)

Discussion

Microbial colonization of stones depends on environmental factors, such as water availability, pH, climate, nutrient sources, and on petrologic parameters, such as mineral composition, as well as porosity and permeability of the material [2]. Angkor Thom and the surrounding temples made of sandstone are particularly susceptible to the colonization by microorganisms due to porosity of sandstone and water retention capability. Autotrophic microorganisms, both photoautotroph and chemoautotroph, are the pioneering inhabitants on surfaces of building [23]. The identified photoautotrophic microorganisms include cyanobacteria and green microalgae widespread occurred on roof and wall of Angkor Thom and temples, which were found on historic buildings frequently [6]. Cyanobacteria posses a number of features which explain their widespread occurrence and success, among these tolerance to desiccation and water stress, ability to utilize low light intensity efficiently and tolerance to high levels of salts, and resistance to high temperatures. The main consequences after colonization by cyanobacteria and algae were improved retention of water, immobilization of carbon through photosynthesis, encouragement of colonization by fungi and macroorganisms to form a succession of different group of organisms [11].

Biodeterioration of inorganic materials is a process involving several types of organisms such as bacteria, fungi and algae in combination with lichens or mosses. The ability of the stone-colonizing microflora to cover and even penetrate material surface layers leads to the formation of complex biofilms in which the microbial cells are embedded [24]. The microbial colonization of stones commonly starts with phototrophic organisms building up a visible protective biofilm on the nutrient-poor stone surface [35]. Cyanobacteria are oxygenic phototrophic bacteria that frequently occur on stone surface, causing damage due to the initial organic carbon immobilized on surface of stone providing opportunities for subsequent microorganisms to establish their community. Moreover, filamentous bacterial populations are able to penetrate deeper into the sandstone inducing more damage and fungal can play their role in a similar way [16]. Algae can form thick biofilm on surfaces together with heterotrophic bacteria, clone sequences representative of algae comprised a substantial portion of the sandstone surface biofilm communities (>40% of clones).

At Angkor Thom and Bayon Temple, the color of the exterior stone surfaces of the structure are black while surface of the same material is green if shading is available or direct sunlight is not possible. This difference is mainly due to the growth of cyanobacteria and algae. The cyanobacteria are a morphologically diverse group of phototrophic prokaryotes possessing the ability to synthesize chlorophyll α and phycobilin pigments. All of the cyanobacteria and algae can produce pigments during their life cycle. The dark green color of bny1 and bny2 samples could be due to cyanobacteria and algae. The cyanobacterial clones detected from samples showed low similarity with the ones from GenBank and they could be species specific for the wall of Bayon Temple. The microbial community of bny3 samples was with little cyanobacteria and algae present. Thus bny3 site did not show intensive green color. The biofilms of byn4, byn5, byn7, and byn8 contained not only a large number of cyanobacteria and algae, but also Dematiaceous fungi. Dematiaceous fungi are also called black fungi, which belong to pigment-producing ascomycete fungi. Because this fungus produces a wide variety of pigments, including α-carotene, β-carotene and melanin etc., the wall surfaces inhabited by black fungi can be stained with different colors. The sequences bearing high similarity with that of known species include *Lecythophora* sp. for producing orange-pink pigmentation, *Cladosporium cladosporioides* for producing melanin dark brown pigmentation and *Coniosporium apollinis* for melanin black pigmentation [32]. They not only cause esthetically detrimental effect of the wall surface due to their pigments, but also can produce organic acids and enzymes that may have much greater harm to the substratum material of the temple wall.

Haloarchaea were detected in all of the collected samples. The deteriorated bas-relief walls of Bayon contain hygroscopic salts, such as carbonates, chlorides, nitrates, sulfates, etc. [33], which may form microniches with sufficiently high salt concentrations for halophilic microorganisms to thrive, especially in drying period, e.g., winter months. The haloarchaea constitute a well-defined group of Euryarchaeota with several peculiarities, many of them related to their specialization for life in halophilic conditions [19]. Crenarchaeota sequences are related to known sequences on an average level of 99%, associated with two species and all are uncultured environmental samples (Fig. 2e). Heterotrophic bacteria and fungi are commonly involved in secondary colonization at the expense of metabolites and cellular debris of the formers, and other environmental sources of organic matter. Chemoorganotrophic bacteria are most diverse and abundance both on sandstone and in biofilm (>50% clones), as a mediating factor in the microflora [23]. Fungi are important components of rock surface microbial communities [8]. In this study, the similarity of fungi sequences to those deposited in databases are very high (Fig. 2d). Four sequences show similarities of 99% and two are identical. Ascomycota are common worldwide distributed phylum occurring chiefly as soil related species. Fungal community structure is driven by the chemical composition of mineral substrates. Distinct fungal populations exist on the surfaces of different mineral types [12], and its abundance shows great difference in community structure between the old biofilm and the fresh biofilm. Moreover, some protozoa and metozoa were also detected unintentionally in the course of eukaryotic detection. These might be the predators in the biotope because this community on surface is very dynamics in light of the humid and hot tropic climate conditions. This indicated that the biofilms on sandstone are a balanced community whose members are co-evolved to enable recycling of essential elements for activity and growth. Likely advantages of this biofilm lifestyle are the higher availability of nutrients on surfaces as well as the possibility of optimal long-term positioning in relation to other microorganisms or physico-chemical gradients. However, biodeterioration is the result of complex microbial interactions through the biofilm community and not the consequence of the action of a particular group of microorganisms [35].

The chemoorganotrophic fungi, bacteria and actinomycetes can grow on accumulated organic matter from dead cells and trapped debris. Fungal growth may result in the liberation of enzymes and organic acids, which can damage the materials through hyphal penetration into the surface, leading to mechanical destruction through shrinking and swelling [26]. Mineral nutrients dissolved by biochemical process can in turn provide the nutrient needed for further growth of the established community. Biodete-

rioration of cultural heritage materials occurs primarily through the formation and growth of biofilms [35]. The sandstones beneath the biofilms can be decomposed to clay minerals resulting in loss of surface materials significantly. At the same time, mineral salts leached out from substratum can form crystal when the surface is dried, resulting in significant stress on the surface mineral and exfoliation.

In conclusion, the microbial community detected in the biofilm on sandstone surface in tropic Asia included cyanobacteria, algae, fungi, archaea and protozoa. The prokaryotic community of old biofilm is similar with fresh biofilm, but eukaryotic communities between the different biofilms are distinctly different. More studies are necessary to further elucidate the driving force of biofilm formation and mechanisms of interaction between commensal microbiota and sandstone surface to understand the biodeterioration of bas-relief wall surfaces of Bayon Temple.

References

1. Altschul SF, Thomas LM, Alejandro AS, Zhang JH, Zhang Zh, Webb M, David JL (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402
2. Arino X, Saiz-Jimenez C (1996) Factors affecting the colonization and distribution of cyanobactera, algae and lichens in ancient mortars. In: Riederer J (ed) Proceedings of the eighth international congress on deterioration and conservation of stone. Rathgen-Forschungslabor, Berlin, pp 725–731
3. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1995) Short protocols in molecular biology, 3rd edn. Wiley, New York
4. Axelrood PE, Chow ML, Radomski CC, McDermott JM, Davies J (2002) Molecular characterization of bacterial diversity from British Columbia forest soils subjected to disturbance. Can J Microbiol 48(7):655–674
5. Cole JR, Chai B, Marsh TL, Farris RJ, Wang Q, Kulam SA, Chandra S, McGarrell DM, Schmidt TM, Garrity GM, Tiedje JM (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res 31:442–443
6. Crispim CA, Gaylarde CC, Gaylarde PM, Copp J, Neilan BA (2003) Molecular biology for investigation of cyanobacterial populations on historic buildings in Brazil. In: Saiz-Jimenez C (ed) Molecular biology and cultural heritage. Zwets & Zeitlinger, Lisse, pp 141–143

7. Dawson SC, Pace NR (2002) Novel kingdom-level eukaryotic diversity in anoxic environments. Proc Natl Acad Sci USA 99:8324–8329

8. Etienne S, Dupont J (2002) Fungal weathering of basaltic rocks in a cold oceanic environment (Iceland): comparison between experimental and field observations. Earth Surf Process Land 27:737–748

9. Felsenstein J (2005) PHYLIP: Phylogeny inference package, Version 3.65. University of Washington, Seattle

10. Freeman M, Jaques C (2003) Ancient Angkor. Amarin Co., Thailand

11. Gaylarde PM, Gaylarde CC (2000) Algae and cyanobacteria on painted buildings in Latin America. Int Biodeterior Biodegrad 46:93–97

12. Gleeson DB, Clipson N, Melville K, Gadd GM, McDermott FP (2005) Characterization of fungal community structure on a weathered pegmatitic granite. Microb Ecol 50:360–368

13. Gleeson DB, Kennedy NM, Clipson N, Melville K, Gadd GM, McDermott FP (2006) Characterization of bacterial community structure on a weathered pegmatitic granite. Microb Ecol 51:526–534

14. Good IJ (1953) The population frequencies of species and the estimation of population parameters. Biometrika 40:237–264

15. Grosskopf R, Janssen PH, Liesack W (1998) Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. Appl Environ Microbiol 64:960–969

16. Gu J-D, Ford TE, Mitchell R (1996) Fungal degradation of concrete. In: Sand W (ed) DECHEMA monographs. Biodeterioration and biodegradation. VCH, Frankfurt, pp 135–142

17. Heck KL Jr, van Belle G, Simberloff D (1975) Explicit calculation of the rarefaction diversity measurement and the determination of sufficient sample size. Ecology 56:1459–1461

18. Horath T, Bachofen R (2009) Molecular characterization of an endolithic microbial community in dolomite rock in the Central Alps (Switzerland). Microb Ecol 58:290–306

19. Kates M (1978) The phytanyl ether-linked polar lipids and isoprenoid neutral lipids of extremely halophilic bacteria. Prog Chem Fats Other Lipids 15:301–342

20. Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

21. Krebs CJ (1999) Ecological methodology, 2nd edn. Benjamin-Cummings, Menlo Park, CA

22. Li XS, Arai H, Shimoda I, Kuraishi H, Katayama Y (2008) Enumeration of sulfur-oxidizing microorganisms on deteriorating stone of the Angkor monuments, Cambodia. Microb Environ 23:293–298

23. May E, Papida S, Abdulla H, Tayler S, Dewedar A (2000) Comparative studies of microbial communities on stone monuments in temperate and semi-arid climates. In: Ciferri O, Tiano P, Mastromei G (eds) Of microbes and art: the role of microbial communities in the degradation and protection of cultural heritage. Kluwer, Dordrecht, pp 49–62

24. McNamara CJ, Perry TD, Bearce KA, Hernandez-Duque G, Mitchell R (2006) Epilithic and endolithic bacterial communities in limestone from a Maya archaeological site. Microb Ecol 51:51–64

25. Mitchell R, Gu J-D (2000) Changes in the biofilm microflora of limestone caused by atmospheric pollutants. Int Biodeterior Biodegrad 46:299–303

26. Mohammadi P, Krumbein WE (2008) Biodeterioration of ancient stone materials from the Persepolis monuments (Iran). Aerobiologia 24:27–33

27. Moissl C, Bruckner JC, Venkateswaran K (2008) Archaeal diversity analysis of spacecraft assembly clean rooms. ISME J 2:115–119

28. Piñar G, Saiz-Jimenez C, Schabereiter-Gurtner C, Blanco-Varela MT, Lubitz W, Rölleke S (2001) Archaeal communities in two disparate deteriorated ancient wall paintings: detection, identification and temporal monitoring by denaturing gradient gel electrophoresis. FEMS Microbiol Ecol 37:45–54

29. Portillo MC, Saiz-Jimenez C, Gonzalez JM (2009) Molecular characterization of total and metabolically active bacterial communities of "white colonizations" in the Altamira Cave, Spain. Res Microbiol 160:41–47

30. Qiu YL, Li L, Wang B, Chen Z, Knoop V, Groth-Malonek M, Dombrovska O, Lee J, Kent L, Rest J, Estabrook GF, Hendry TA, Taylor DW, Testa CM, Ambros M, Crandall-Stotler B, Duff RJ, Stech M, Frey W, Quandt D, Davis CC (2006) The deepest divergences in land plants inferred from phylogenomic evidence. Proc Natl Acad Sci USA 103:15511–15516

31. Saitou N, Nei M (1987) The neighbor-joining method: a method for constructing phylogenetic trees. Mol Biol Evol 4:406–425

32. Sterflinger K (2000) Fungi as geologic agents. Geomicrobiol J 17:97–124

33. Uchida E, Ogawa Y, Maeda N, Nakagawa T (2000) Deterioration of stone materials in the Angkor monuments, Cambodia. Eng Geol 55:101–112

34. Vaneechoutte M, Beenhouwer HD, Claeys G, Verschraegen G, Rouck AD, Paepe N, Elaichouni A, Portaels F (1993) Identification of mycobacterium species by using amplified ribosomal DNA restriction analysis. J Clin Microbiol 31:2061–2065

35. Warscheid T, Braams J (2000) Biodeterioration of stone: a review. Int Biodeterior Biodegrad 46:343–368

36. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogeny study. J Bacteriol 173:697–703