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MOLECULAR CHARACTERIZATION OF THE MICROFLORA DWELLING ON STAINED AREAS OF THE NATIONAL MUSEUM OF THE AMERICAN INDIAN BUILDING

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Introduction

The study aimed to assess the black staining found on the facing stone of the National Museum of the American Indian (NMAI) building through a molecular characterization of the microbial communities of three samples. The samples were: #2 from the low wall by the loading dock at the north-west corner of the NMAI building; #5 in correspondence with the runoff from the scupper on the east wall of the roof near the Senator Daniel K. Inouye Terrace, and #7 from the same east wall but from the lowest ashlar. Bacteria, cyanobacteria, eukaryotic algae and fungi were studied by several techniques, such as Denaturing Gradient Gel Electrophoresis (DGGE), principal component analysis applied to the DGGE profiles, and gene sequencing.

Materials and Methods

DNA extraction and amplification

Total genomic DNA was extracted in a laminar flow hood as reported by Ausubel et al. (1994) with the follow modifications: no lysozyme was added and four freeze and thaw cycles -80°C/70°C 5 min each were performed before the addition of the proteinase K.

Partial 16S rRNA gene amplification for subsequent denaturing gradient gel electrophoresis (DGGE) analysis was performed for the bacteria with the primer set 357F-GC (CCTACGGGAGGCAGCAG) and 907R (CCGTCAATTCCTTTGAGTTT). GC clamp was as follows: *5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGCACGGGGGG-3'. The thermal cycles used for amplification were as follows: 4 min preincubation at 94°C, 10 cycles of 94°C, 61°C, 72°C for 1 min each step, and 20 cycles of 94°C for 30 s, 56°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min (Thermohybaid PCR P×2 Thermal Cycler).

For fungi the internal transcribed spacer 1 (ITS1) region , 5.8S rDNA and ITS2 (Chen et al., 2001) were amplified in two steps in a semi-nested procedure with the following primers: ITS4 (TCCTCCGCTTATTGATATGC) and NS5 (AACTTCCCGGAATTGACGGAAG) for the first amplification (White et al., 1990) and ITS1f-GC (CTTGGTCATTTAGAGGAAGTAA) and ITS4 for the second amplification step. GC clamp was as follows: 5'-CGCCCGCCGCGCGGCGGGCGGGGCGGGGCACGGGGGG-3' (Gardes and Bruns, 1993).

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For algae partial 18S rRNA gene amplification was performed with the primer set NS1-GC and NS2 as reported by Oros-Sichler et al. (2006) with the annealing temperature modified to 63°C.

Primers CYA359-GC and CYA781R (equimolar mixture between primers CYA781Ra and CYA781Rb) were used for PCR amplification of cyanobacterial 16S rRNA genes as described in Nübel et al. (1997). The thermal cycles used for amplification were as follows: 5 min at 94°C, 35 cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 2 min, and a final extension step at 72°C for 10 min (Thermohybaid PCR P×2 Thermal Cycler)

Aliquots of amplicons were loaded in 1.2% agarose gel in 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA) to verify specificity.

Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed using polyacrylamide gel (8% of a 37:1 acrylamide–bisacrylamide mixture in a Tris acetate EDTA (TAE) 1X buffer, 0.75 mm thick, 16×10 cm) with a 30–50% denaturant gradient for Fungi and Algae and 40–60% denaturant gradient for Bacteria and Cyanobacteria. Gels were run overnight at 60 V in TAE 1X buffer at 60°C in DCode apparatus (Bio-Rad, Milan, Italy). Gene fragments were visualized after SYBR® Green (Sigma-Aldrich, Milan, Italy) staining by UV transillumination (Gel Doc 2000 apparatus, Bio-Rad, Milan, Italy). Clearly defined bands were cut out from the gel and sequenced at an external facility (Primm, Milan, Italy) with the ABI Prism 3900 Genetic Analyser (Applied Biosystems)

The sequences retrieved were blasted against the NCBI database (http://www.ncbi.nlm.nih.gov) and the most probable identification organism was given.

DGGE profile analyses

Using DGGE profiles the line plots were generated with ImageJ software (Rasband, 2008), and then imported into an Excel® file as x/y values.

To examine the relative similarities among samples, the matrix of x/y values of DGGE line profiles was analyzed by principal component analysis (PCA) using the first two factors, which described most of the variation in the data set. Multivariate investigations were conducted with XLSTAT version 2009.4.07 (Addinsoft, NY) software using the Pearson correlation as similarity index. The significance of the PCA model was tested by a cross-validation procedure.

Community profiles were subjected to peak fitting analyses (PeakFit, SPSS, Inc.) to quantify the DNA band position (peaks representing individual taxonomic units, differentiated by a reference fragment value, Rf) and intensities (peak area representing the abundance of each taxonomic unit*)*. Baselines were subtracted from each line profile using the AutoFit 2nd Deriv Zero routine with the best fit option. After baseline correction, the peaks were resolved with a deconvolution curve fit, which defines a visible peak as one that

produces a local maximum in the input data. A standard peak width is assigned to all peaks using the default parameter `full width at half maximum' that is utilized for fitting Gaussian curves to the peaks.

Results

The obtained DGGE profiles were analyzed to evaluate the similarities among the communities of the three samples. Figure 1 illustrates the result for DGGE profile analysis in the case of the bacterial communities. From the species richness analysis it could be established that #7 is the least rich in species while #5 contains the highest number of different species.

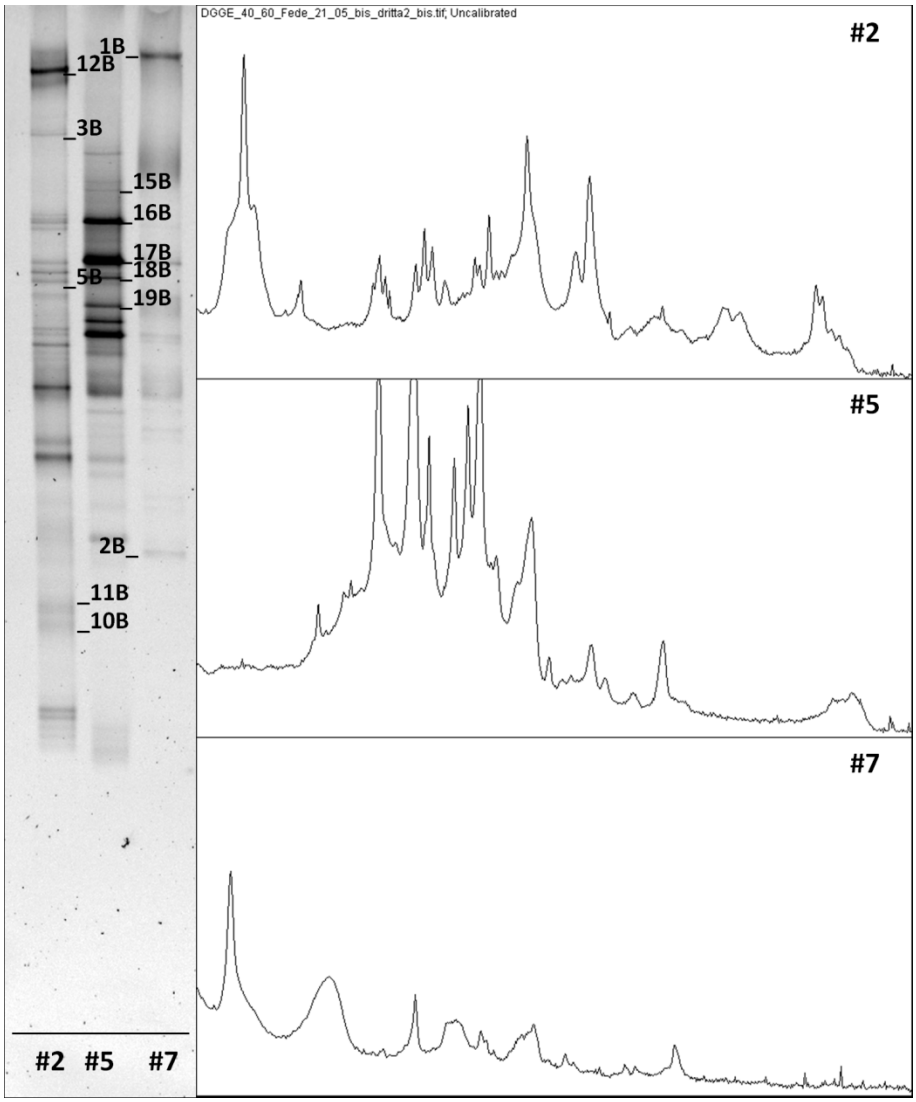

Figure 1. 16S rRNA gene DGGE profiles of the samples (photograph) with the corresponding line plot profiles (graphs)

An exploratory examination of the PCR-DGGE fingerprints was performed using Principal Component Analysis (PCA), a technique to extract, rationalize and visualize all the

useful information from a complex matrix of data with N rows (observations) and K columns (variables). In this technique, variables constitute a homogeneous group and information is simplified into a new reduced number of variables, called principal components (PC). These new variables, those which better explain the original data, are built as a linear combination of the original ones.

When two PC have been derived they together define a plane, a window into the K-dimensional variable space. By projecting all the observations (collected samples) onto this low-dimensional sub-space and plotting the results, it is possible to visualize the structure of the investigated data set.

The plot in Figure 2 is a map of the three investigated samples. Samples close to each other have similar microbial community composition, whereas those far from each other are dissimilar with respect to DGGE profiles. Principal component analysis showed that both in the case of the total community—including bacteria, fungi and eukaryotic algae—, the case of the bacterial community, and the case of cyanobacterial community taken by itself (Figure 2), the communities of the samples were different, i.e., no sample community groups with that of another sample.

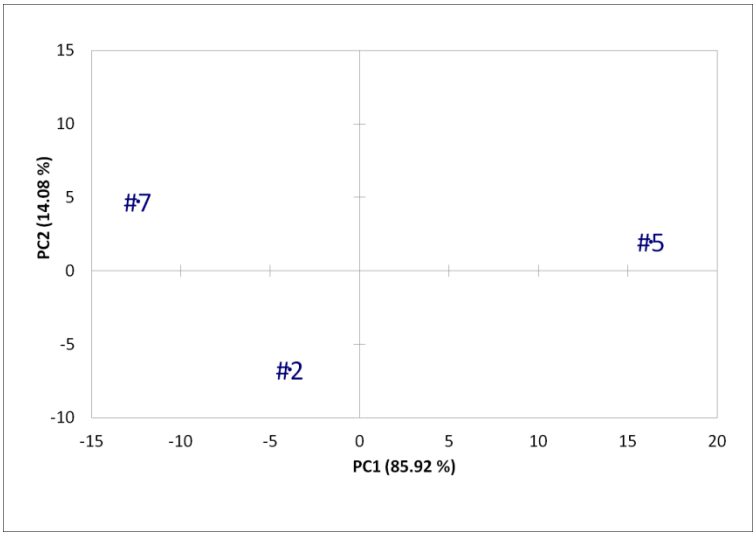

Figure 2. Principal component analysis results for the cyanobacterial communities of the three samples. The plot provides a map of how the collected samples relate to each other with respect to DGGE profiles. The first Principal Component (PC1) is a new axis representing the direction of maximum variation through the PCR-DGGE data sets. The second Principal Component (PC2) is another axis in the space, orthogonal to the first and positioned to represent the next highest variation through the PCR-DGGE data sets. The plot of the two-dimensional scores, defined by PC1 and PC2, accounted for 100% of the input data variability for cyanobacteria and therefore is adequate to describe the system under investigation.

Finally, the samples were characterized by sequencing the DGGE bands for bacteria, cyanobacteria, fungi, and algae. In Table 1 the identification of the DNA sequences is reported.

Table 1. Identification of gene sequences isolated from DGGE profiles. A cross indicates that the band was present in the sample.

Band	Closest relative	Type of microorganism	# 2	# 5	# 7	% identity
1C	Uncultured Oscillatoriales cyanobacterium	Cyanobacteria		x		98%
6C	Uncultured cyanobacterium	Cyanobacteria		x		96%
7C	Uncultured chlorophyte	Chlorophyta	x			98%
4C	Uncultured cyanobacterium	Cyanobacteria		x		98%
3C	Uncultured cyanobacterium	Cyanobacteria	x	x		98%
2C	Uncultured cyanobacterium	Cyanobacteria		x		98%
4A	*Myrmecia* sp. H1VF1	Chlorophyta	x	x	x	98%
5A	uncultured Trebouxiophyceae	Chlorophyta	x			97%
5F	*Epicoccum nigrum*	Fungi (Ascomycota)		x		99%
12F	Ascomycete sp. MA 4698	Fungi (Ascomycota)		x		99%
13F	*Capnobotryella* sp. MA 4775	Fungi (Ascomycota)		x		99%
16F	*Pleopsidium flavum*	Fungi (Ascomycota)	x			95%
21F	Cladonia	Fungi (Ascomycota)	x			94%
22F		Good sequence but no match		x		
23F		Good sequence but no match		x		
1B	*Calothrix desertica* PCC 7102	Cyanobacteria			x	99%
2B	Uncultured *Candidatus Solibacter* sp.	Bacteria (Acidobacteria)			x	92%
3B	Uncultured Acidobacteria bacterium	Bacteria (Acidobacteria)	x			94%
5B	Uncultured cyanobacterium	Cyanobacteria	x	x		98%
10B	Uncultured endolithic bacterium	Bacteria	x			98%
11B	Uncultured *Deinococcus* sp.	Bacteria (Deinococcales)	x			99%
12B	Uncultured chlorophyte	Chlorophyta	x			98%
15B	Uncultured bacterium	Bacteria		x		99%
16B	*Spirosoma rigui* strain WPCB118	Bacteria (Cytophagales)	x	x		90%
17B	Uncultured Oscillatoriales cyanobacterium	Cyanobacteria	x	x		98%
18B	*Synechococcus* sp. HH-1	Cyanobacteria		x		98%
19B	Uncultured *Chroococcidiopsis* sp.	Cyanobacteria		x		98%

Conclusions

From our study it can be seen that cyanobacteria and green algae (chlorophyta) are present in all samples. Fungi are only found in samples #2 and #5, and black fungi only in #5. Acidobacteria and Deinococcus, which were identified on other monuments, e.g., temples of Angkor Thom and Bayon in Cambodia (Lan et al., 2010), were found in samples #2 and #7, for the former, and #2, for the latter. Finally, note that endolithic microorganisms —inhabiting pore spaces within the rock— habitats—were also included, e.g., band 10B is an endolithic bacterium.

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APPENDIX

Location of the samples taken from the NMAI building for analyses

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The samples were collected in April 2010, upon the occasion of the visit by the four invited scientists for their further analysis. Four samples were collected and subdivided into the specimens that the scientist subsequently analyzed.

Sample #2, a fairly large spalling flake with overall black surfaces on both front and back, was readily detached from the lowest course of the loading dock wall on the west side of the building. The sample area receives rainwater from sloping capstones without any overhang; some rainwater also splashes up from the black granite ledge below (Figure 1).

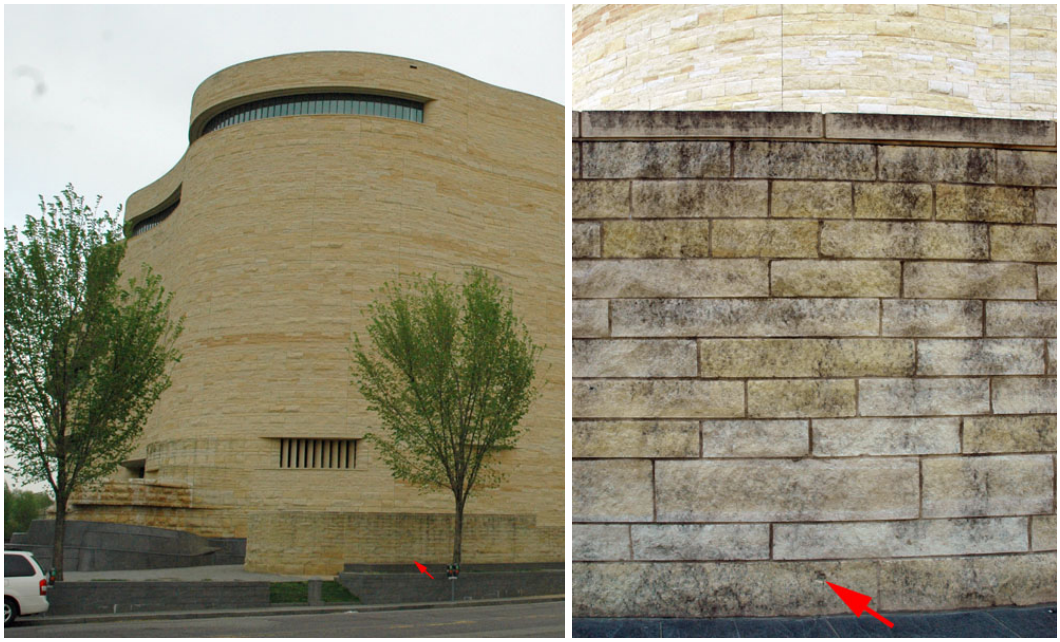

Figure 1. Sample #2 location indicated by arrows: left, general view of the exterior loading dock wall; right, detail (April 2010).

Samples #5, #7, and #9 were taken from areas of heavy biocolonization on the fifth floor terrace east wall below scuppers that drain the roofs above (Figure 2). Samples #5 and #7 were spalling flakes easily detached from below the same scupper: #5 from an area with a uniform black deposit and sample #7 from a slightly recessed area on an adjacent block with spotty surface biocolonization (Figure 3L). Sample #9 was scraped from dense black material on a membrane below a second scupper (Figure 3R).

Figure 2. Left, view of the east face of the 5th floor terrace showing locations of samples #5, #7, and #9 from darkened areas below two scuppers, which drain water from the small terrace above and, in turn, the main roof terrace with the dome (April 2010).

Figure 3. Details of sample locations shown in the previous figure. Left, samples #5 and #7 were flakes detached from areas indicated by the arrows. Note the uniform black colonization where sample #5 was taken, compared to the spotty area to its right where sample #7 was removed; right, sample #9 was scraped from the membrane at the bottom of the wall (April 2010).

The larger samples studied by May and Warscheid were pieces cut from extra blocks left over from the construction of the building.