AEM Accepts, published online ahead of print on 27 October 2006
Appl. Environ. Microbiol. doi:10.1128/AEM.02220-06

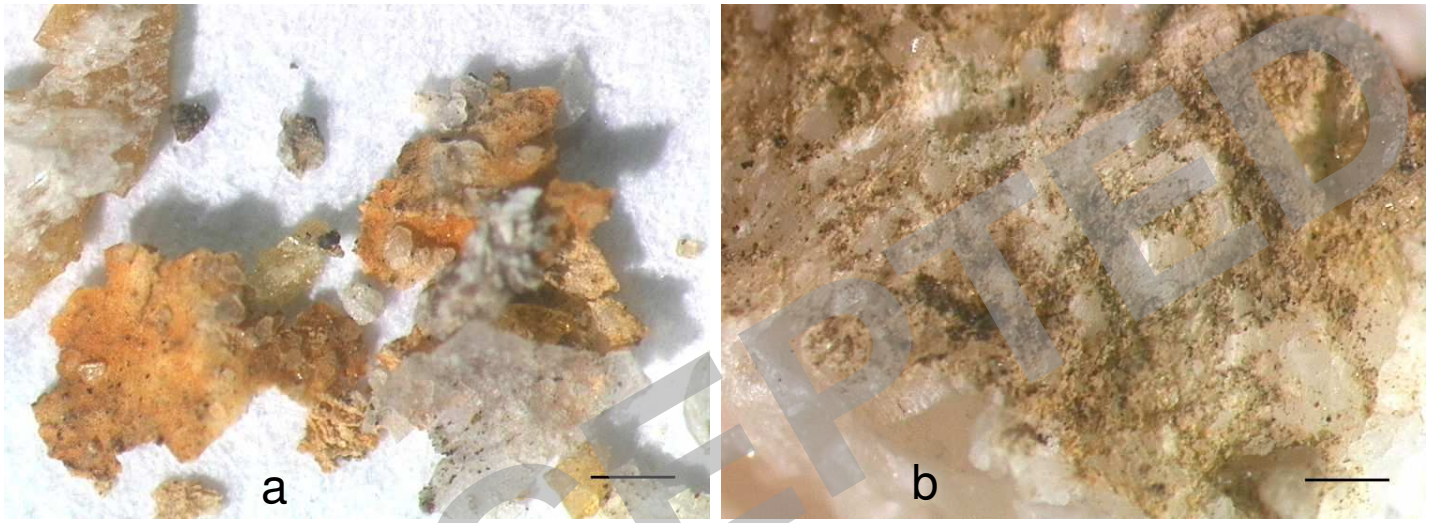

Fig. 1, CAPPITELLI ET AL. Synthetic consolidants attacked by melanin-producing fungi: the study case of the biodeterioration of the Milan Cathedral (Italy) marble treated with acrylics

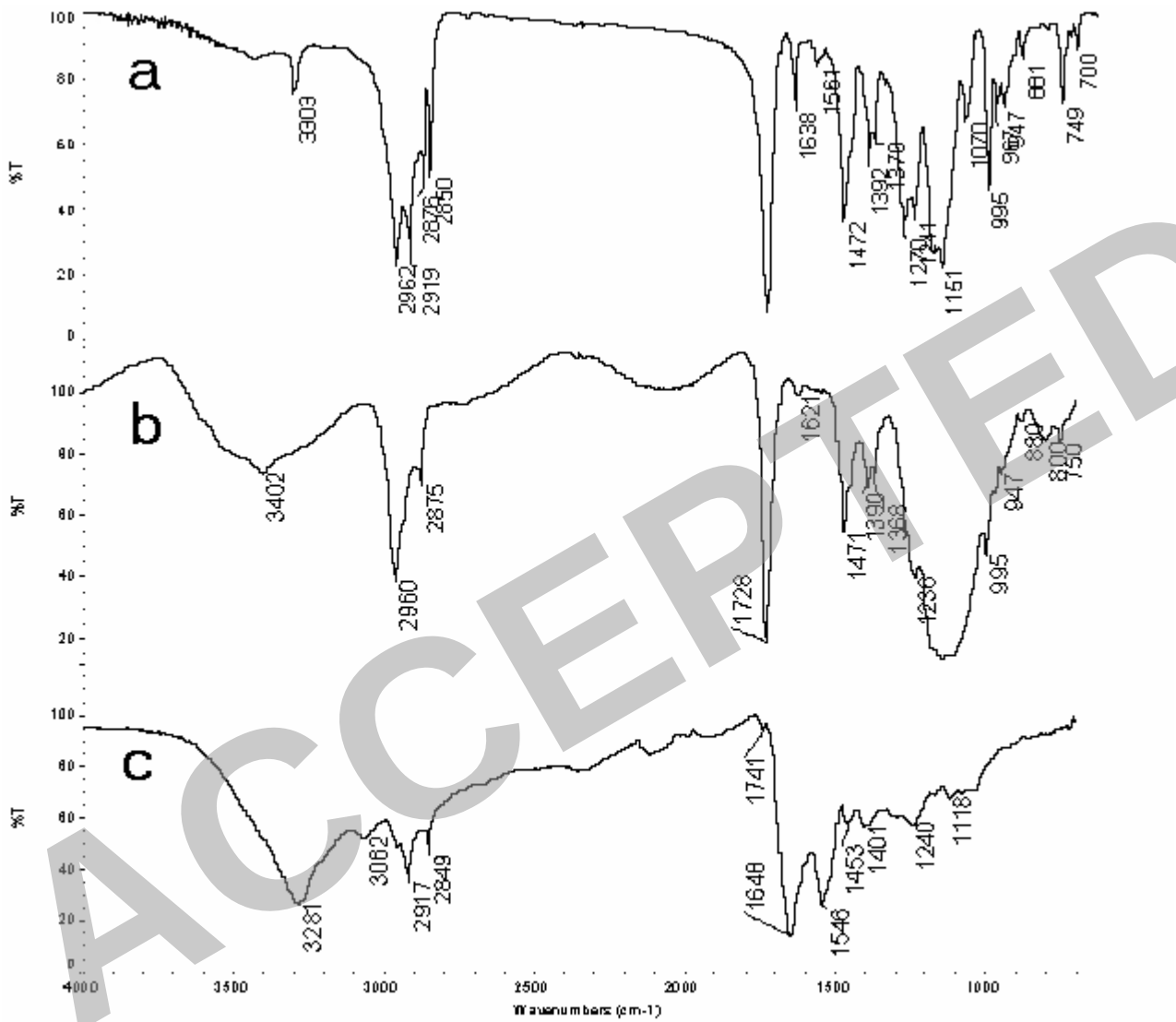

Fig. 2, CAPPITELLI ET AL. Synthetic consolidants attacked by melanin-producing fungi: the study case of the biodeterioration of the Milan Cathedral (Italy) marble treated with acrylics

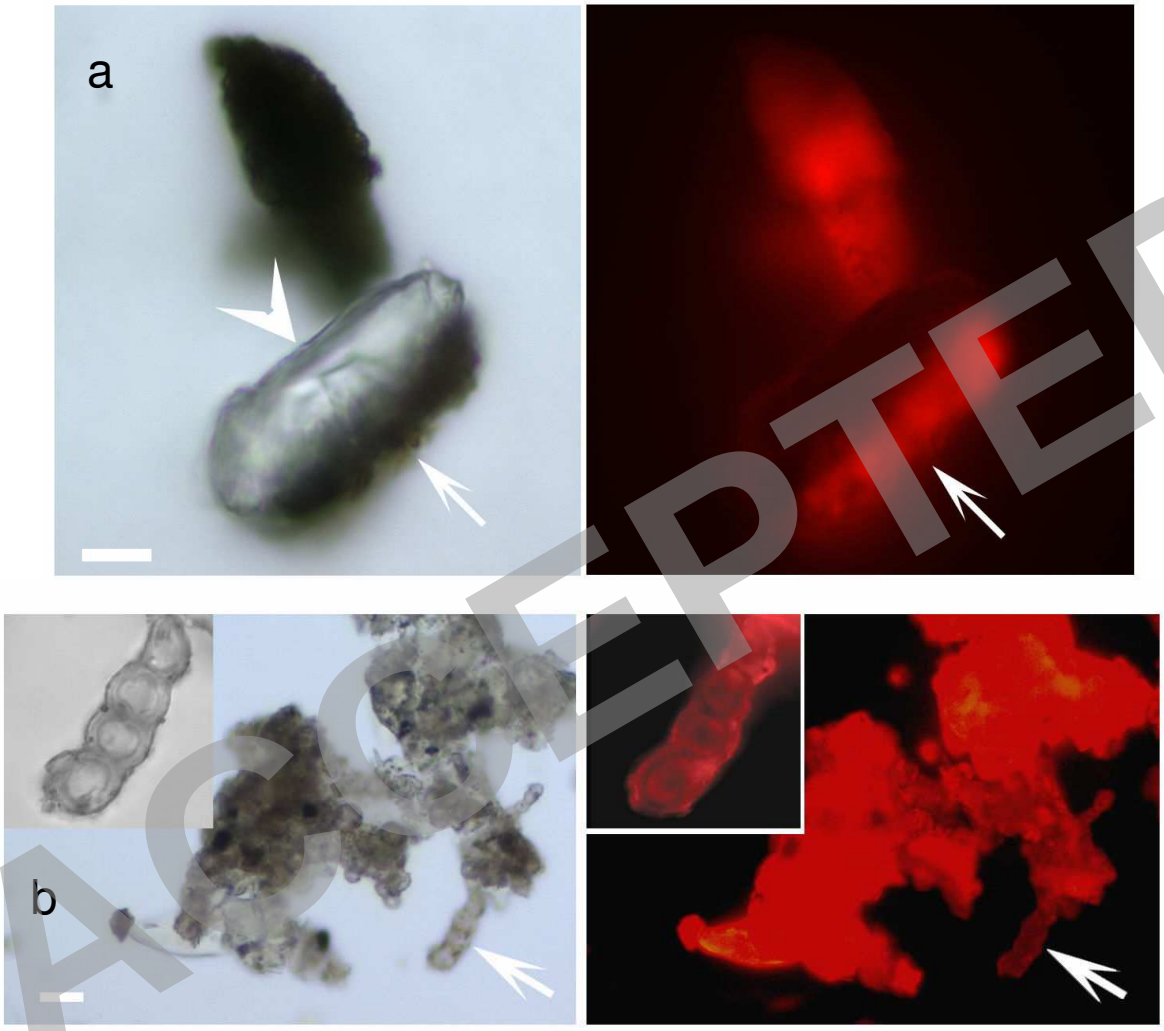

Fig. 3, CAPPITELLI ET AL. Synthetic consolidants attacked by melanin-producing fungi: the study case of the biodeterioration of the Milan Cathedral (Italy) marble treated with acrylics

Synthetic consolidants attacked by melanin-producing fungi: the study case of the biodeterioration of the Milan Cathedral (Italy) marble treated with acrylics

Francesca Cappitelli[1]*, Joshua D. Nosanchuk[2], Arturo Casadevall[2], Lucia Toniolo[3], Lorenzo Brusetti[1], Sofia Florio[4], Pamela Principi[1], Sara Borin[1] and Claudia Sorlini[1]

Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università degli Studi di Milano, Milan, Italy,[1] Division of Infectious Diseases, Department of Medicine, Albert Einstein College of Medicine, Golding 701, 1300 Morris Park Avenue, Bronx, NY 10461, USA,[2] Dipartimento di Chimica, Materiali e Ingegneria Chimica "Giulio Natta" Politecnico di Milano, Via Mancinelli 7, 20133 Milan, Italy,[3] and Dipartimento di Ecologia del Territorio e degli Ambienti Terrestri, University of Pavia, via S. Epifanio 14, 27100 Pavia, Italy[4]

Running title: Melanin-producing fungi growth on aged synthetic resins

* Corresponding author Dr. Francesca Cappitelli
Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università degli Studi di Milano, Via Celoria 2, Italy
phone no. +39-0250316721
fax no. +39-0250316694
e-mail francesca.cappitelli@unimi.it

Monuments and artistic stone surface are often consolidated and protected with synthtetic polymers, in particular acrylics. Although it is generally thought that acrylic polymers are resistant to biodeterioration, we report for the first time on the systematic occurrence of dematiaceous meristematic fungi on many marble samples of the Cathedral in Milan (Italy) previously treated with this material. Fourier transform infrared (FTIR) spectroscopy applied to the Milan cathedral stone samples revealed characteristic features of biodeteriorated synthetic resins that differentiated them from the aged but non-biodeteriorated samples. Samples showing biological colonisation were analysed for the presence of fungi. Cultivation and morphological characterisation and methods independent from cultivation, like Denaturing Gradient Gel Electrophoresis (DGGE) coupled with partial 18S rRNA gene sequencing and immunofluorescence staining with melanin-binding antibodies, showed that melanin-producing species are heavily present on the stone surfaces protected with acrylic resins. This observation raises the question of the effectiveness of acrylics in protecting stone artworks.

Keywords: synthetic resin biodeterioration; black fungi; cultural heritage conservation

INTRODUCTION

The protection and consolidation of stone materials is a critical step for the conservation of outdoor architectural monuments. Over the past decades a range of synthetic adhesives, consolidants and protectives have been applied to monuments in attempt to enhance their long-term preservation. Polyacrylates and polymethacrylates are among those more frequently used. Superficial treatments made with them are meant to have both protective and consolidating properties (19). In this respect, the Milan Cathedral is not an exception: since an intervention in the 1972, its marble surfaces have been protected with acrylic resins (poly-isobutylmethacrylate). Before their application, of crucial importance in the area of conservation is to know the durability of the treatments in outdoor conditions. Over the past forty years the chemical and physical stability of acrylic homo- and co-polymers has been extensively investigated and acrylics appeared a suitable solution for the application in the cultural heritage conservation (18). If natural polymers are easily subjected to biodeterioration, synthetic resins vary on their susceptibility to fungal attack depending on their chemical nature, the environmental conditions and the way they are applied (5,7). Freshly dried acrylic resins are among the most resistant resins to biological damage (6). However, little is known about the susceptibility to biological degradation of naturally aged acrylic resins, the only exception being the façade of Tempio Malatestiano (Rimini, Italy) treated with acrylic resins that presented black fungal growth in cracks and fissures (22). The Milan cathedral is currently under conservation treatment as it appears to be seriously damaged by surface erosion, micro-fractures, detachments and thick crusts as well as biological growth. In particular, at the first inspection, the Milan Cathedral presented an extensive blackening in the areas previously consolidated/protected with synthetic products. The blackening of stone surfaces may be caused by a variety of mechanisms, including air pollution, fly ash, oxidation of metal and biological pigments such as melanin (11, 15, 32). Numerous studies have established that the most of the blackening on artistic marbles and limestones exposed to outdoor environments is caused by dematiaceous fungi, and in particular those manifesting meristematic and sometimes yeast-like growth patterns (29). The pigmentation of

these fungi is largely due to deposition of melanins in the cell wall (reviewed in 20). Meristematic fungi form black clump-like cauliflower-like colonies consisting of isodiametrically dividing cells that colonize the rock surface and penetrate into the rock. It is well known that meristematic fungi, many of which have their natural ecological niche on rocks, physically attack the rock and cause aesthetic and structural damage on artistic stone. The cause of damage is not acid formation and dissolution of the mineral compounds but rather intercrystalline growth physical disruption of the weakest structural components of the crystals resulting in biopitting and formation of cracks and fissures (11, 26, 38). In addition, the growth of black fungi on white or light colored rocks causes selective absorption of solar radiation that can lead to local extension of crystals and as a consequence crystal decohesion (12). Differences in solar radiation adsorption by non-affected rock and rock affected by black fungi can result in temperature differences and thermal stresses that may promote rock cracking and degradation.

The aim of this work was to study the fungal microflora present on the synthetic resins used to consolidate the Milan Cathedral in order to inform conservators on the possible detrimental effects of the use of synthetic polymers for the consolidation and protection of stone surfaces.

MATERIALS AND METHODS

Sampling. Marble fragments were generally collected from the façade of the Cathedral of Milan where biological patinas were visually evinced on consolidated/protected marble. These patinas were always blackish in colour. Sample 14F033 was taken in an apparently non-biodeteriorated area. Sampling was performed using a sterile lancet and scalpel, and fragments were stored in sterile tubes at room temperature.

Sample codes are connected to the identification of the area of the façade where they are coming from. XXY00Z: first 2 digits indicate height from the ground; the central letter indicates the longitudinal area, last 3 digits are the progressive number of sample.

Mycological analyses of marble samples. Preliminary identification was based on the macroscopic features of colonies growing on agar plates and the micromorphology of the reproductive structures.

1) culture techniques

The presence of fungal colonisation on the biodeteriorated Candoglia marble of the Milan Cathedral was evaluated using the 2% malt extract agar (MEA) and dichloran rose bengal (DRBC) medium by Fluka (32, 38). Marble chips were incubated in the cultural medium at 25°C for one month to allow for the detection of slowly growing fungi.

2) microscopic observations

Touch preparations with adhesive tape (Fungi Tape, DID s.p.a., Milan, Italy) were used for direct microscopic observation according to the methodology described by Urzì and De Leo (36). The samples were analyzed using a digital epifluorescence microscope (Leica DM4000B), equipped with CoolSnap CF camera (Photometrics, Roper Scientific). Digital images were acquired by RS Image Ver. 1.7.3 (Roper Scientific, Inc.).

Fourier Transform Infrared (FTIR) Spectroscopy. Fourier transform infrared analyses, used to detect the acrylic resin in the samples, were carried out by a Nicolet Nexus spectrophotometer coupled with a Nicolet Continuum FTIR microscope equipped with a HgCdTe detector cooled with liquid N_2; spectra were recorded by a Graseby-Specac diamond cell accessory in transmission mode between 4000 and 700 cm^{-1}. To avoid contamination by the carbonatic substrate, the samples were carefully collected under an optical microscope by means of a needle-sampler.

Immunostaining technique. The immunostaining was employed to detect melanin -and therefore melanin-producing fungi- on the samples. Marble chips were immersed for 30 – 40 min in phosphate buffered saline solution (pH 7.0). The debris of the marble surface was attached to a freshly coated poly-L-lysine slide (Sigma Chemical Corporation, St. Louis, MO). Slides were incubated in Superblock (Pierce, Rockford, IL) blocking buffer for 4 h followed by incubation with 10 µg/ml of the melanin-binding monoclonal antibody (MAb) immunoglobulin M (IgM) 6D2 (µκ) (23) overnight at 4°C. After a wash, the slides were incubated with a 1:100 dilution of fluorescein

isothiocyanate (FITC)-conjugated goat anti-mouse (GAM) IgM (Southern Biotechnologies Associates, Inc., Birmingham, AL) for 1 h at 37°C. The slides were washed, mounted using a 50% glycerol-50% PBS-0.1 M *N*-propyl gallate solution, and viewed with an Olympus (Melville, NY) AX70 microscope equipped with an FITC filter. Negative controls consisted of slides incubated with the MAb 5C11 (μκ), which binds mycobacterial lipoarabinomannan (14), as the primary antibody or FITC-labeled antibody alone.

DGGE to study fungal community on marble samples and sequencing and phylogenetic analysis of DGGE bands. Fungi growing on marble façade of the Cathedral of Milan were characterized using the Denaturing Gradient Gel Electrophoresis (DGGE), a method independent from cultivation. Total DNA was extracted from samples pulverized in a mortar following a method previously described (24). DGGE fingerprint on the 18S rRNA gene was performed as described by Kowalchuk et al. (17) with the primers NS1-GC and NS2, except for the primer annealing temperature of thermal protocol reduced to 50°C to improve fragment amplification. PCR amplicons were separated in a 7% polyacrylamide gel with a denaturing gradient of urea and formamide of 40% (top) -60% (bottom), where 100% denaturation is considered urea 7M and formamide 40%. The electrophoresis was run at 110 V for 14 hours at 58°C in a D-Code apparatus (Bio-Rad). The gel was stained in a solution 1X of SybrGreen (Molecular Probes, Leiden, The Netherlands) for 30 min and its image captured in UV transillumination with a digital camera supported by a Gel Doc 2000 apparatus (Bio-Rad). Bands of interest were cut from the gel with a sterile scalpel; the DNA was extracted by incubating the gel fragments for 12 h in 100 μl of sterile distilled water at 37°C under agitation; 10 μl of the solution were then used as template to reamplify the fragment using the same DGGE primers without the GC-clamp and the same PCR conditions applied to the original stone DNA. The obtained amplicons were then purified using a QIAquick PCR Purification Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. Purified products were then sequenced with the NS1 primer using DYEnamic ET Terminator Cycle Sequencing Kit (Pharmacia) and an ABI 310 automated sequencer (Applied Biosystems). The

resulting sequences were compared with the sequence database at the National Center for Biotechnology Information (NCBI) using BLASTN facilities (1). Alignment with the corresponding 18S rRNA genes was performed by using software available at the Ribosomal Database Project website (9); secondary structure was taken into account when this was done. Phylogenetic analyses were performed by using Jukes and Cantor distance estimation with TREECON 1.3b package (37). A 50% majority rule bootstrap consensus tree (1000 replicates) was generated. Gaps were treated as a fifth base.

Nucleotide sequence accession number. The nucleotide sequence of 18S rRNA genes were deposited in the EMBL nucleotide sequence database (GenBank/EMBL/DDBJ) under the accession numbers AM236865 to AM236873.

RESULTS

The investigation of synthetic resins. The acrylic resin applied on the Milan cathedral façade in the '70s is a formulated product called "Surface Clear Preserving Opaco®" (supplied by ARD Raccanello, Padova (PD), Italy); it is poly-isobutylmethacrylate, charged with an additive that gives a mat aspect to the final coating. Figure 1 shows the surface deterioration of sample 14F033, an orange film (Fig. 1a), and sample 14F034, a pink-beige incoherent patina (Fig. 1b). The orange film is an aged residue of the acrylic protective coating, still present in some areas, and the pink-beige patina with blackish biological growth, observed in adjacent surface areas, which was definitely treated in the '70s with the same polymeric coating.

As FTIR spectroscopy is commonly used to identify synthetic polymeric products, this technique was employed also on our samples (see Fig.2). Figure 2 shows the freshly casted acrylic "Surface Clear Preserving Opaco®" (Fig. 2a) and the FTIR spectra corresponding to samples 14F033 and 14F034 (see Figs. 2b and 2c respectively). In Fig. 2, the peaks at 3303, 2919, 2850, 1638, 1561, 700 cm^{-1} should be ascribed to the additive. In the case of the orange thin film of the protective

171 coating on 14F033, FTIR analysis identified the acrylic polymer, together with large amount of
172 gypsum (see peaks at 1621, 1147 cm^{-1}) as shown in Fig. 2b. In contrast, the acrylic polymer was not
173 evidenced any more on the fragment 14F034 showing the biological patina (Fig. 2c). Indeed, the
174 995 cm^{-1} peak, related to the isobutyl group, and the 2962 e 1392 cm^{-1} peaks related to the methyl
175 groups, are drastically reduced and at the same time the 1730 cm^{-1} absorption band related to the
176 stretching vibration of carbonyl group is broadened towards higher frequencies. In addition, the
177 FTIR analysis of the sample 14F034 revealed the presence of a peptidic bond (see peaks at 1648,
178 1546 cm-1) that was ascribed to some proteinaceous material related to the fungal growth as
179 previously reported (6).
180 Thus the biodeterioration pattern of the acrylic resin in the presence of microorganisms is definitely
181 different from that obtained after environmental ageing.
182 **The phenotypic identification of fungi.** The list of cultivable fungi identified on the basis of the
183 macroscopic features of colonies and the micromorphology of the reproductive structures found on
184 the marble is listed in Table 1. Dematiaceuos species belonging to the genera *Alternaria*,
185 *Cladosporium* and *Epicoccum* were found together with species belonging to the genera
186 *Aspergillus*. Black and pink yeast cells were also found in some samples. All the microorganisms
187 isolated are reported in literature as common stone taxa (29, 35). On each sample at least one
188 organism that produces melanin was found. In all the samples, biological structures showing
189 meristematic growth were also observed directly on the surface using the adhesive tape procedure
190 without any cultivation step.
191 **DGGE and sequencing.** Traditional microbiological techniques are not always useful in
192 investigating multicellular and sporulating organisms as not all fungal species can be easily isolated
193 by the currently available methods, and, in addition, slow growing fungi are often overgrown by
194 fast growing ubiquitous species of minor ecological importance in culture (28). To better
195 characterize the fungal microflora, a DGGE-analysis coupled with the partial sequencing of 18S
196 rRNA gene fragments were performed. Although primer annealing temperature was reduced to

50°C to better address the amplification of fungal DNA, a successful amplification was possible for six samples out of ten. Even though the efficiency of amplification varied among samples, nine bands were clearly visible and were sequenced (Table 2). Fungi belonging to *Talaromyces flavus*, *Glyphium elatum*, *Cenococcum geophilum*, *Eladia saccula* and *Phoma herbarum* were identified. Percentages of similarity of the closest relative found in BLASTN search were between 96 and 100%. Less than 100% homology on the 18S rRNA gene could not be sufficient to identify a fungal species. However, for our purposes, it is sufficient information to evaluate on the investigated samples the presence of black fungi. Bands F2 and F5 attributed to *Talaromyces flavus* and *Glyphium elatum* were dominant in DGGE patterns and they could be originated by fungal species dominant in the population (data not shown). Three bands were attributed to algae belonging to *Trebouxia jamesii* of the order *Microthamniales*.

Immunostaining technique. In order to obtain rapid identification and localisation of melanin-producing fungi with a relatively easy to perform and accurate method, the immunostaining technique was applied to the ten stone specimens. The immunostaining procedure proved that the control (the intact freshly quarried Candoglia marble) was not fluorescent under the microscope while all the ten samples of the Milan cathedral showed fungal structures labelled by melanin-binding antibody as shown in Fig. 3. As expected, in Fig 3a the marble (indicated by an arrowhead) does not fluoresce whereas the fungal coat (indicated by an arrow) fluoresces intensely. In the magnified inset of Fig. 3b the typical meristematic growth is clearly visible.

DISCUSSION

Many tests on acrylics, polymers commonly used in conservation treatments, have been carried out to evaluate their chemical and physical stability. These tests proved that these materials are generally a good choice for the consolidation and protection of stone. In contrast, few studies have been carried out on the evaluation of the susceptibility of synthetic materials used in cultural heritage conservation to biological attack (5-7). In the last years, black fungi have been recognized

223 as the most conspicuous and probably the most damaging organisms attacking the surfaces of stone
224 monuments (11). We report for the first time on the systematic occurrence of dematiaceous
225 meristematic fungi on stone samples consolidated and protected with naturally aged acrylic resins.
226 Numerous studies have dealt with the analysis of meristematic fungi on rocks and historical
227 structures made of natural stone but none has taken into account the importance of the presence of
228 aged synthetic resins for this kind of microorganisms. It is worth mentioning that fungal growth on
229 synthetic polymers has been proven previously, even though freshly dried acrylic resins seemed to
230 be among the least susceptible compounds to fungal attack in laboratory conditions (5,6). Synthetic
231 resins on monuments show an advanced chemical and physical degradation after about 30 years of
232 environmental ageing, such as yellowing and cracking, which in turn is likely to facilitate biological
233 degradation. Under ultraviolet irradiation the main degradation pathway of acrylics is chain scission
234 (18). The oligomers produced by UV irradiation are surely more easily attacked by fungi than the
235 high molecular weight polymers that originated them. In particular, photoxidation of poly-
236 isobutylmethacrylate is quite efficient due to the branched isobutyl group of the polymer side chain
237 (8). In this paper, the decay process of the coating enhanced by biological growth was proved from
238 the recovery on the Milan cathedral marble surface of a material with proteinaceous features, which
239 forms an incoherent patina, replacing the polymeric film. Actually, FTIR spectra of samples
240 collected from adjacent areas indicate the presence of the partially decayed acrylic resin where
241 microbial colonization is not noticeable and the presence of a proteinaceous material where the
242 fungal growth was assessed.
243 A pioneer study on this topic was carried out by Pinna and Salvadori (22) who made optical and
244 electron microscopic observations of meristematic fungal growth in cracks and fissures treated with
245 acrylic resins on the façade of Tempio Malatestiano in Rimini (Italy). However, no further
246 investigation than documenting the presence of meristematic fungi has been reported from the
247 above mentioned Authors.

In our research, the combined used of light microscopy and cultural methods revealed meristematic fungi in all samples, including *Alternaria*, *Cladosporium* and *Epicoccum* and other genera showing the features of black fungi. In the case of meristematic fungi, the main drawback of culture is the length of time that is necessary for growth, at least one month and their morphological plasticity that greatly prevents direct microscopic identification. As a consequence, DNA-based methods have been successfully applied to study dematiaceuos fungi colonisation on different kind of sample-environment: restriction fragment length polymorphism (RFLP) (3, 10, 30, 31); random amplified polymorphic DNA (RAPD) (34) and partial or complete 18S rRNA gene sequencing (2, 16, 25). For this reason we employed sequencing of DGGE bands to detect fungi independently from cultivation. We could identify sequences which showed the 100% 18S rRNA gene similarity with both *Glyphium elatum* and *Coniosporium* sp. (Table 2). *Coniosporium spp.* have been isolated from ancient marbles in Turkey (Ac. N. AJ972863; H. Sert and K. Sterflinger, Microcolonial fungi from antique marbles in Perge / Side / Termessos (Antalya/Turkey), unpublished data) and Greece (26). The biodeteriorative potential of this genus has been investigated in detail for building stone of historical monuments (27, 30, 36). The dematiaceous fungi *Phoma* and *Alternaria* identified in two biodeteriorated samples are among the most conspicuous and probably the most damaging organisms identified for attacking and even penetrating the surfaces of stone monuments (33, 38). A number of experimental studies have shown the ability of some ectomycorrhizal fungi such as *Cenococcum geophilum* to dissolve Ca-bearing minerals (4, 13), present in the marble as calcium carbonate and calcium sulphate or gypsum. The alga *Trebouxia jamesii* has been detected by DGGE among the predominant species in some samples, since the primer annealing temperature was decreased to succeed in fungal 18S rRNA gene amplification. However, the finding of *T. jamesii* is interesting, because it is known that *T. jamesii* forms a lichen in association with the *Evernia mesomorpha*, another meristematic fungus (21).

DGGE results are important for providing information on the presence and genera of black fungi. From a conservation practice view, to prevent further damage is of great value to evaluate also the

spatial distribution of these fungi. As a consequence, we applied immunofluorescence that confirmed the presence of melanin-producing fungi and in addition provided their location and showed meristematic growth.

The results obtained in this paper clearly indicated that if acrylics are stable and play the role of protectives/consolidants from physical and chemical agents, this does not necessary mean that in the long term they will be the best choice for conservation. In conclusion, we have demonstrated that stones protected by aged synthetic acrylics can be heavily colonized by the black fungi thus acrylics instead of preventing damage could accelerate the decay process.

ACKNOWLEDGEMENTS

A.C. and J.D.N are supported in part by NIH AI52733. The Authors are very grateful to the "Veneranda Fabbrica del Duomo" for the assistance and support to the diagnostic work on the Milan Cathedral's façade.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403-410.
2. **Berbee, M. L., and J. W. Taylor.** 1992. Detecting morphological convergence in true fungi, using 18S rRNA gene sequencing data. Biosystems. **28**:117-125.
3. **Caligiorne, R. B., M. A. de Resende, E. Dias-Neto, S. C. Oliveira, and V. Azevedo.** 1999. Dematiaceous fungal pathogens: analysis of ribosomal DNA gene polymorphism by polymerase chain reaction-restriction fragment length polymorphism. Mycoses. **42**:609-614.
4. **Callot, G., D.Mousain, and C. Plassard.** 1985. Concentration of calcium carbonate on the walls of fungal hyphae. Agronomie. **5**:143-150.

5. **Cappitelli, F., E. Zanardini, and C. Sorlini.** 2004. The Biodeterioration of Synthetic Resins used in Conservation. Macromol. Biosci. **4**:399-406.
6. **Cappitelli, F., S. Vicini, P. Piaggio, P. Abbruscato, E. Princi, A. Casadevall, J. D. Nosanchuk, and E. Zanardini.** 2005. Investigation of fungal deterioration of synthetic paint binders using vibrational spectroscopic techniques. Macromol. Biosci. **5**:49-57.
7. **Cappitelli, F., C. Sorlini, E. Pedemonte, E. Princi, and S. Vicini**. 2006. Effectiveness of graft synthetic polymers in preventing biodeterioration of cellulose-based materials, Macromol. Symp. **238**:84-91.
8. **Chiantore, O., and M. Lazzari**. 2001. Photo-oxidative stability of paraloid acrylic protective polymers. Polymer **42**:17-27.
9. **Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, S. Chandra, D. M. McGarrell, T. M. Schmidt, G. M. Garrity, and J. M. Tiedje.** 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.
10. **deCock, A. W.** 1994. Population biology of *Hortaea werneckii* based on restriction patterns of mitochondrial DNA. Anton. Leeuw. Int. J. G. **65**:21–28.
11. **Diakumaku, E., A. A. Gorbushina, W. E. Krumbein, L. Panina, and S. Soukharjevski.** 1995. Black fungi in marble and limestones - an aesthetical, chemical and physical problem for the conservation of monuments. Sci. Total Environm. **167**:295-304.
12. **Dornieden, T., A. A. Gorbushina, and W. E. Krumbein.** 1997. Änderungen der physikalischen Eigenschaften von Marmor durch Pilzbewuchs. Int. Z. Bauinstandsetzen. **3**:441-454.
13. **Gharieb, M. M., and G. M. Gadd**. 1999. Influence of nitrogen source on the solubilization of natural gypsum. Mycol. Res. **103**:473-481.
14. **Glatman-Freedman, A., J. M. Martin, P. F. Riska, B. R. Bloom, and A. Casadevall.** 1996. Monoclonal antibodies to surface antigens of *Mycobacterium tuberculosis* and their

use in a modified enzyme-linked immunosorbent spot assay for detection of mycobacteria. J. Clin. Microbiol. **34**:2795-2802.

15. **Gorbushina, A., W. E. Krumbein, L. Panina, S. Soukharjevsky, and U. Wollenzien.** 1993. On the role of black fungi in colour change and biodeterioration of antique marbles. Geomicrobiol. J. **11**:205-221.

16. **Haase, G., L. Sonntag, Y. van de Peer, J. M. J. Uijthof, A. Podbielski, and B. Melzer-Krick.** 1995. Phylogenetic analysis of ten black yeast species using nuclear small subunit rRNA gene sequences. Anton. Leeuw. Int. J. G. **68**:9-33.

17. **Kowalchuk, G. A., G. Saskia, and J. W. Woldendorp.** 1997. Detection and Characterization of Fungal Infections of *Ammophila arenaria* (Marram Grass) Roots by Denaturing Gradient Gel Electrophoresis of Specifically Amplified 18S rDNA. Appl. Environ. Microbiol. **63**:3858–3865.

18. **Melo, M. J., S. Bracci, M. Camaiti, O. Chiantore and F. Piacenti**. 1999. Photodegradation of acrylic resins used in the conservation of stone. Polym. Degrad. Stabil. **66**:23-30

19. **Miliani, C., M. Ombelli, A. Morresi, and A. Romani.** 2002. Spectroscopic study of acrylic resins in solid matrices. Surface and Coatings Technology **151-152**:276-280.

20. **Nosanchuk, J. D., and A. Casadevall.** 2003. The contribution of melanin to microbial pathogenesis. Cell Microbiol. **5**:203-223.

21. **Piercey-Normore, M. D.** 2006. The lichen-forming ascomycete *Evernia mesomorpha* associates with multiple genotypes of *Trebouxia jamesii*. New Phytol. **169**:331-344.

22. **Pinna, D., and O. Salvadori.** 1999. Biological growth on Italian monuments restored with organic or carbonatic compounds, pp. 149-154. *In* Proceedings of international Conference on Microbiology and Conservation (ICMC 99), 16-19.6.1999, Florence.

23. **Rosas, A. L., J. D. Nosanchuk, M. Feldmesser, G. M. Cox, H. C. McDade, and A. Casadevall.** 2000. Synthesis of polymerized melanin by *Cryptococcus neoformans* in rodents. Infect. Immun. **68**:2845-2853.

24. **Schabereiter-Gurtner, C., G. Piñar, W. Lubitz, and S. Rölleke**. 2001. An advanced molecular strategy to identify bacterial communities on art objects. J. Microbiol. Methods **45**:77–87.

25. **Spatafora, J. W., T. G. Mitchell, and R. Vilgalys.** 1995. Analysis of genes coding for small-subunit rRNA sequences in studying phylogenetics of dematiaceous fungal pathogens. J. Clin. Microbiol. **33**:1322–1326.

26. **Sterflinger, K., and W. E. Krumbein.** 1997. Dematiaceous fungi as a major agent for biopitting on Mediterranean marbles and limestones. Geomicrobiol. J. 14: 219–231.

27. **Sterflinger, K., R. De Baere, G. S. de Hoog, R. De Wachter, W. E. Krumbein, and G. Haase.** 1997. *Coniosporium perforans* and *C. apollinis*, two new rock-inhabiting fungi isolated from marble in the Sanctuary of Delos (Cyclades, Greece). Anton. Leeuw. Int. J. G. **72**:349–363.

28. **Sterflinger, K., W. E. Krumbein, and A. Schwiertz.** 1998. A protocol for PCR in situ hybridization of hyphomycetes. Internatl. Microbiol. **1**:217–220.

29. **Sterflinger, K.** 2000. Fungi as geological agents. Geomicrobiol. J. **17**:97-124.

30. **Sterflinger, K., and H. Prillinger.** 2001. Molecular taxonomy and biodiversity of rock fungal communities in an urban environment (Vienna, Austria). Anton. Leeuw. Int. J. G. **80**:275-286.

31. **Uijthof, J. M. J., and G. S. de Hoog.** 1995. PCR-Ribotyping of isolates of currently accepted *Exophiala* and *Phaeococcomyces* species. Anton. Leeuw. Int. J. G. **68**: 35–42.

32. **Urzì, C., S. Lisi, G. Criseo, and M. Zagari.** 1992. Comparazione di terreni per l'enumerazione e l'isolamento di funghi deteriorigeni isolati da materiali naturali. Ann. Microbiol. Enzimol. **42**:185-193.

33. **Urzì, C., and M. Realini.** 1998. Colour changes of Noto's calcareous sandstone as related to its colonisation by microorganisms. Int. Biodeter. Biodegr. **31**:34-43.

34. **Urzì, C., F. De Leo, C. Lo Passo, and G. Criseo.** 1999. Intra-specific diversity of *Aureobasidium pullulans* strains isolated from rocks and other habitats assessed by physiological methods and by random amplified polymorphic DNA (RAPD). J. Microbiol. Meth. **36**:95–105.

35. **Urzì, C., F. De Leo, G. S. de Hoog, and K. Sterflinger.** 2000. Recent advances in the molecular biology and ecophysiology of meristematic stone-inhabiting fungi, pp. 3-19. *In* O. Ciferri, P. Tiano and G. Mastromei (ed.), Proccedings of the International Congress of Microbes and Art, Plenum Publishing Co. Ltd., New York.

36. **Urzì, C., and De Leo F.** 2001. Sampling with adhesive tape strips: an easy and rapid method to monitor microbial colonitation on monument surfaces. J. Microbiol. Meth. **44**:1-11.

37. **Van de Peer, Y., and R. De Wachter.** 1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput. Applic. Biosci. **10**:569-570.

38. **Wollenzien, U., G. S. de Hoog, W. E. Krumbein, and C. Urzí.** 1995. On the isolation of microcolonial fungi occurring on and in marble and other calcareous rocks. Sci. Total Environm. **167**:287-294.

394 Table 1. List of fungal taxa identified on the basis of the macroscopic features of colonies and the
395 micromorphology of the reproductive structures detected on the ten marble specimens of the Milan
396 Cathedral.
397

Taxa/sample	21HO25	I4F033	14F035	14F034	I3E036	20S028	I0M023	10T02I	20I026	19O029
Alternaria spp.				×				×		
Aspergillus sp.				×						
Cladosporium spp.				×		×		×	×	
Epicoccum nigrum						×				
pink yeast	×									
black yeast	×	×	×	×	×					
black microcolony			×	×			×		×	×
micelia sterilia					×			×		

398
399

400 Table 2. Identification of partial 18S rRNA gene sequences isolated from DGGE profiles; bold

401 crosses indicate DGGE bands sequenced; double crosses indicate a band of strong intensity.

402

Band	Closest relative	Accession Number	Taxon	%	21HO25	14F035	14F034	20S028	I0M023	10T02I
F1	*Trebouxia jamesii*	Z68700	Clorophyta	98				×		××
F2	*Talaromyces flavus*	M83262	Ascomycota	96				×		××
F3	*Trebouxia jamesii*	Z68705	Clorophyta	99	×			×	×	×
F4	*Trebouxia jamesii*	Z68700	Clorophyta	99	×				×	×
F5	*Glyphium elatum*	AF346419	Ascomycota	100	××				×	×
F7	*Cenococcum geophilum*	L76615	Ascomycota	98				×		
F8	*Glyphium elatum*	AF346419	Ascomycota	100				×		
F14	*Eladia saccula*	AB031391	Ascomycota	98			××			
F15	*Phoma herbarum*	AY293775	Ascomycota	98		××				

403 Fig. 1 a) sample 14F033: FTIR spectrum of a small quantity of orange film residue; b) sample
404 14F034: FTIR spectrum of the pink-beige patina over the calcite crystals. Scale bar 500 μm.
405
406 Fig. 2 FTIR spectrum of a) a film of "Surface Clear Preserving Opaco ®" freshly casted on NaCl
407 window; b) sample 14F033: marble microfragments with calcite crystals and residues of acrylic
408 polymer seen as an orange thin film; c) sample 14F034: biological patina on calcite crystals where
409 the polymeric film is almost completely deteriorated.
410
411 Fig. 3 a) A chip of the marble sample 19O029 with fungus coating part of the surface. The marble
412 (arrowhead) does not fluoresce whereas the fungal coat (arrow) fluoresces intensely. Scale bar 20
413 μm. b) A 20X power view of 13E036 with a magnified inset (100X). Scale bar 10 μm.
414