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Marvasi, Massimiliano, Donnarumma, F., Frandi, A., Mastromei, G., Sterflinger, K., Tiano, P. and Perito, B. (2012) Black microcolonial fungi as deteriogens of two famous marble statues in Florence, Italy. International Biodeterioration & Biodegradation, 68 (0, Jou). pp. 36-44. ISSN 0964-8305

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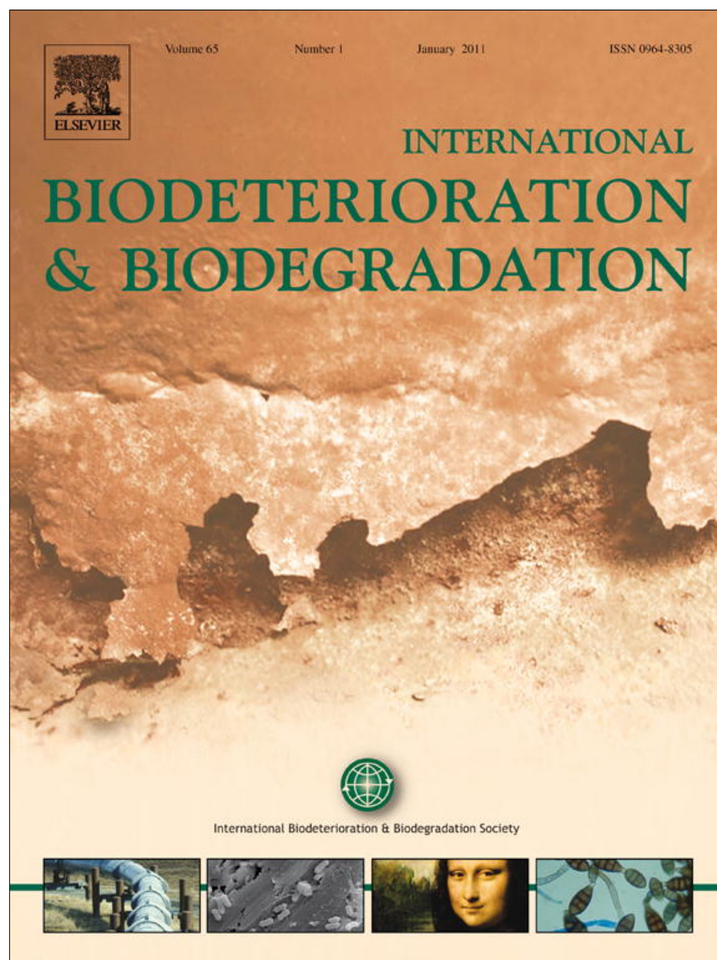

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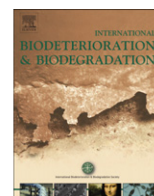

Black microcolonial fungi as deteriogens of two famous marble statues in Florence, Italy

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ARTICLE INFO

Article history:
Received 8 April 2011
Received in revised form
19 October 2011
Accepted 25 October 2011
Available online

Keywords:
Black MCF
Rock-inhabiting fungi
Marble
Biodeterioration
ITS rDNA
AFLP

ABSTRACT

Blackened areas on outdoor marble statues are a significant esthetic problem due to the presence of deteriorating agents. Microcolonial black fungi, which have their natural ecological niche on rocks, play an important role in deterioration of stones used in monuments, such as marble and limestone. Black fungi were isolated from two very valuable statues exposed to the outdoor environment in Florence, Italy, the "Ratto delle Sabine" and the "Copia del David," and these fungi were demonstrated to be responsible for the blackening areas on the statues. The black strains showed many features common to members of rock-inhabitants dematiaceous fungi. Morphological and molecular characterization, including phylogenetic analysis, indicated that the strains isolated from both statues and in different times belong to the same species and can be assigned to the rock-inhabitant genera *Sarcinomyces* and *Phaeococcomyces*. Red yeasts growing in close proximity to the black ones, with no visible effect on the statues, were also characterized on the morphological and molecular level and identified as *Sporobolomyces yunnanensis*.

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1. Introduction

One of the most common deterioration phenomena of marble in Mediterranean cities is the formation of black crusts and dark discolorations. Possible causes are: (a) deposition of ash and dirt on the surface and within fissures and cracks; (b) formation of calcium sulfate crusts – gypsum – due to the reaction of carbonate with acid rain; and (c) colonization by the so-called black or dematiaceous fungi, an artificial group of darkly pigmented fungi that includes black yeast-like fungi, meristematic fungi, and microcolonial fungi (MCF) (Sterflinger, 2006). Analysis and characterization of dark discolorations and crusts on marble are of primary concern for restorers as they choose the appropriate conservation treatment. Microcolonial fungi form cauliflower-like microcolonies on and in rocks, incrusting the surface with melanin and imparting a dark, blackish brown appearance to the rock surface (Sert et al., 2007). These organisms are slow-growing and natural inhabitants of rock surfaces, in contrast to many other darkly pigmented airborne fungi that are only deposited on rocks (Diakumaku et al., 1995). Microcolonial fungi are commonly isolated from the sun-exposed surfaces of natural rocks and stone monuments in the Mediterranean Basin (De Leo et al., 2003; Ruibal et al., 2005, 2008), but they also colonize rocks in dry cold and desert climates (Staley et al., 1982; Selbmann et al., 2005). All of these fungi have the same growth characteristics and morphological features. Many exhibit meristematic growth but their morphology is highly pleomorphic, depending on the environment. Although these fungi are morphologically similar, their phylogenetic origins are different (Urzí et al., 2000; Ruibal et al., 2009). The black MCF are polyphyletic and, within the *Ascomycota*, they occur among at least three orders: *Chaetothyriales*, *Dothideales*, and *Pleosporales* (Sterflinger and Prillinger, 2001; Sterflinger, 2006; Ruibal et al., 2009).

In a rock ecosystem, black MCF and yeast-like fungi, which are regular inhabitants, play an important role in its structural and esthetic alteration (Sert et al., 2007) and are considered among the most harmful microorganisms associated with monumental stone biodeterioration (Urzí et al., 2000). Marble damage seems not to be caused by acid formation and mineral dissolution (Diakumaku et al., 1995; Wollenzien et al., 1995), but it is hypothesized that these fungi may exert a mechanical destructive force on marble (Sterflinger and Krumbein, 1997).

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doi:10.1016/j.ibiod.2011.10.011

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Here we report on the characterization of black MCF and yeast-like fungi isolated from deteriorated areas of two marble statues of significant cultural value. Both are located in the Piazza della Signoria, Florence: the "Ratto delle Sabine" (RdS) sculpted in 1583 by Giambologna and the copy of the original statue of the "David" (CdD) of Michelangelo, sculpted in 1910 by Luigi Arighetti. Due to constant and extended exposure to outdoor weather, both statues have suffered significant esthetic spoilage. Black crusts, green biological patinas, and dark gray spots are widespread, mainly in some areas of the David; while the Ratto delle Sabine is less damaged, being partly sheltered by the Loggia dei Lanzi. During a monitoring campaign of RdS and a restoration intervention on CdD, we investigated the origin of the dark gray spots found in several areas of both statues. The aim of this work was to identify the possible biogenic origin of these spots and to characterize the organisms involved in this important alteration phenomenon.

2. Materials and methods

2.1. Artwork description, sampling, and isolation of fungi

The "Ratto delle Sabine" statue was sculpted in 1583 by Giambologna from a unique piece of Carrara marble, creating the first statue with multiple view points. The statue is 4.1 m high and is located inside the Loggia dei Lanzi in the Piazza della Signoria (Fig. 1A). In 2000 and 2001, a complete restoration intervention was undertaken. In 2003, monitoring was started of the behavior of two water repellents. During this time, gray dark spots were observed to progressively increase in several areas of the stone surface. Sampling was carried out in autumn 2006 and was repeated in autumn 2007 before (September) and after (October) a cleaning intervention with ammonium carbonate compresses. For the cleaning intervention, a 5% solution of ammonium carbonate in deionized water was mixed with Arbocell BW40. A poultice was applied, interposing a Japanese paper, on the stone surface to be cleaned for 1 h. The treatment was repeated twice at 10-min intervals. The site of the 2007 sampling is indicated in Fig. 1A1.

The "Copia del David" (Fig. 1B) has been located outside the main façade of "Palazzo Vecchio" in the Piazza della Signoria since 1911. During a restoration intervention areas with gray dark spots were observed, and sampling was carried out in autumn 2008 (Fig. 1B1).

On both statues, a sterile scalpel was used to gently scrape a small amount of superficial particulates from some of the blackish areas. Samples were put in sterile tubes and stored at −20 °C. They were resuspended in sterile physiological solution and plated on 2% malt extract agar (MEA, OXOID). Plates were incubated at 27 °C for 1–2 wk. Fungal colonies were isolated and re-isolated several times to obtain pure cultures on MEA. Pure cultures were stored in sterile glass tubes with MEA with 6 μg ml^{-1} tetracycline at 6 °C.

2.2. Morphological characterization of fungi and microscopic analysis

Fungal isolates were morphologically characterized from cultures grown on MEA. Isolated colonies were examined with a stereomicroscope (Olympus SZX9) to characterize their shape. Cell morphology was observed in fresh samples with an optical phase contrast microscope (LEICA DM LB at 200, 400, and 1000×) and by electron microscopy (ESEM Quanta-200 FEI) without fixation.

2.3. Molecular characterization of fungi

2.3.1. DNA extraction, rDNA amplification, and sequencing

Pieces of fungal colonies (0.14 g of each) grown on MEA were excised with a scalpel, and DNA was extracted using a Fast DNA Kit (QBIOgen) following the manufacturer's directions. A small amount of all DNAs was analyzed by electrophoresis on 0.8% agarose gel in TBE buffer. DNA concentrations were measured with a biophotometer (Eppendorf). 18S genes and internal transcribed spacer (ITS) fragments (ITS I/5.8S/ITS II) of ribosomal DNA (rDNA) were amplified from genomic DNA using primers NS1 and NS8 (amplified fragment length about 1700 bp; Noda et al., 1995) and primers EF3RCNL and ITS4 (amplified fragment length about 650 bp; Lord et al., 2002), respectively. As a positive control, genomic DNA of *Candida albicans* ATCC 10231 was used. The negative control was sterile H_2O in order to detect contamination of reagents.

The 18S rDNA was amplified by an initial denaturing step of 2 min at 96 °C followed by 30 cycles at 96 °C for 30 s, 55 °C for 30 s,

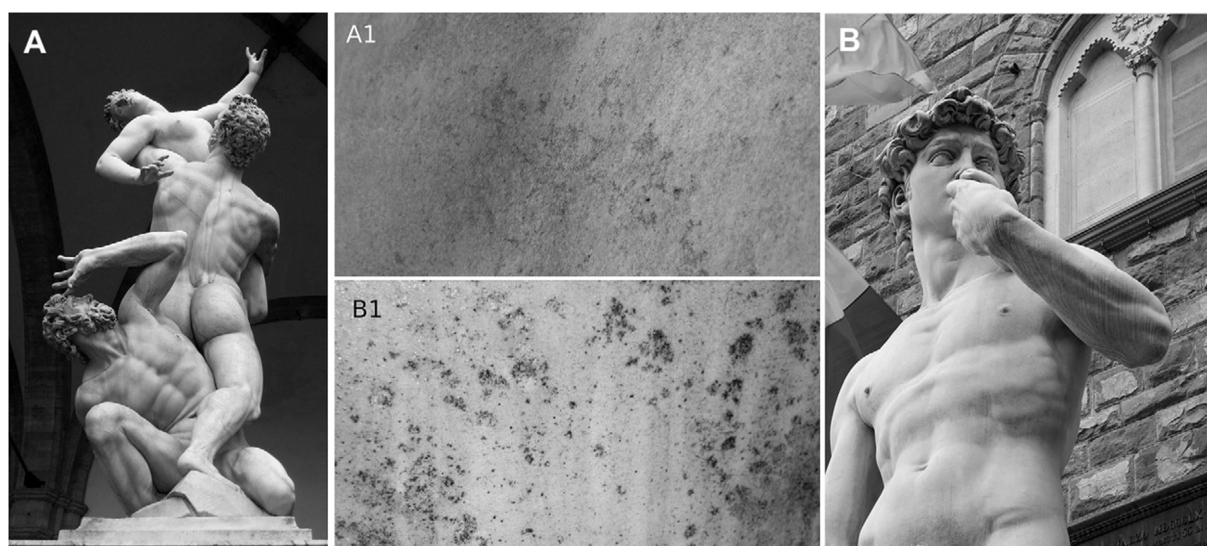

Fig. 1. Marble statues and sampled areas showing dark gray spots. A, "Ratto delle Sabine," Loggia dei Lanzi, Piazza della Signoria, Firenze, Italy; A1, magnification of the 2007 sampled area on the left lower abdomen. B, "Copia del David," outside the main façade of Palazzo Vecchio, Piazza della Signoria, Firenze, Italy; B1, magnification of the area sampled in 2008 on the right buttock.

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and 72 °C for 2 min. The reaction was completed with a final extension at 72 °C for 5 min, then cooled and held at 8 °C.

For ITS PCR, conditions consisted of an initial denaturing step of 2 min at 94 °C followed by 35 cycles at 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 2 min. The reaction was completed with a final extension at 72 °C for 10 min and then cooled and held at 8 °C.

PCR was carried out with 1 unit of *EuroTaq* polymerase (Euroclone) on a Primus Thermal Cycle machine (MWG). A small aliquot of all PCR products was analyzed by electrophoresis on 0.8% agarose gel in TBE buffer. All PCR products were purified with a high product purification kit (ROCHE).

The 18S rDNA and ITS fragments were sequenced using an ABIPrism 310 analyzer (Applied Biosystems). To sequence the 18S rDNA, primer NS1 was used and, in some cases, primers NS3, NS5, and NS8 (Noda et al., 1995; Schabereiter-Gurtner et al., 2001) were used as well. ITS fragments were sequenced by using primers ITS4 and EF3RCNL (Lord et al., 2002). All sequences were assembled and edited with Chromas software and phylogenetic analyses inferred with Mega 4 (Tamura et al., 2007). Highly similar sequences were interrogated with and downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/). Newly generated 18S and ITS sequences were deposited at the NCBI database under the accession numbers FJ556905, FJ556910, JF749182–JF749185, JF702915, JF702916, JF758859, and JF758860.

2.3.2. Phylogenetic analysis by ITS

ITS sequences of isolated fungi and other ITS sequences retrieved from the database on criteria described in the Results Section (3.2) were aligned with the ClustalW software included in the MEGA 4 package (Tamura et al., 2007). The resulting alignments were checked manually and corrected if necessary. The software MEGA 4 was used to construct an unrooted phylogenetic tree, using the neighbor-joining method (Saitou and Nei, 1987). Sequence divergences among strains were quantified with the Kimura-2-parameter distance model (Kimura, 1980). For treatment of gaps, the "Complete Deletion" option was chosen. Bootstrap analysis (1000 replicates) was used to test the topology of the neighbor-joining method data.

2.3.3. AFLP analysis

Amplified fragment length polymorphisms (AFLP) were visualized as described by Vos et al. (1995), with a slight modification. Briefly, purified DNA was digested simultaneously with EcoRI and MseI; afterward, EcoRI and MseI adaptors were ligated. PCR products were amplified with EcoRI-A (6-carboxyfluorescein-5′-GACTGCGTACCAATTCA) and MseI-G (5′-GATGAGTCCTGAGTAAG) primers, with selective A and G nucleotides at the 3′ end, respectively. Amplified fragments were separated by capillary electrophoresis on an ABI 310 bioanalyzer (Applied Biosystems), and fragment size was determined with a 50–400-bp internal standard (Rox 400HD). Only fragments of 60–380 bp were considered in profile analyses performed by GeneMapper 4.0 software (Applied Biosystems). Cluster analysis of the profiles was performed using the unweighted pair group method with arithmetic mean (UPGMA) of the Numerical Taxonomy and Multivariate Analysis System NTsys-pc v.2 software (Exeter Software). The percentage of similarity between profiles was calculated using the Dice correlation coefficient.

2.4. Acid test

Isolated strains from marble were tested for their ability to form metabolic acid products. Fungal isolates were plated on malt extract agar containing 0.5% of finely powdered $CaCO_3$. The ability to dissolve $CaCO_3$ results in a clear halo around the colonies (Diakumaku et al., 1995). Plates were incubated for one month at 27 °C.

2.5. MCF culturing on marble samples

A slightly decayed Carrara marble specimen (Gioia type with 2% porosity; 5 × 5 × 1.8 cm) was broken with a hammer into little pieces, sterilized, placed in a 250-ml flask containing 30 ml Sabouraud liquid medium (OXOID), and inoculated with cells from one of the MCF isolates (M4). The culture was incubated at 27 °C statically and checked visually until black cell clusters appeared to adhere to the marble. Afterward, marble pieces were aseptically removed, rinsed with physiological solution, and dried for microscopic analysis. Marble samples were observed with a stereomicroscope, an optical phase contrast microscope, and an electron microscope. Observations were documented photographically.

3. Results

3.1. Microscopic analysis of marble

Marble powder sampled in 2006 and 2007 from RdS was directly observed with an optical microscope. Eleven small marble fragments with dark areas were observed both with the stereomicroscope and the contrast phase OM at increasing magnifications in order to understand the origin of the dark spots: one fragment from the 2006 sampling, five fragments from before, and five after the 2007 cleaning procedure. All the observed dark spots corresponded to black microcolonies (Fig. 2A and B) with a yeast-like morphology.

All the microscopically observed black spots were transferred with a sterile needle onto MEA medium where they generated dark colonies within 1–2 wk (see Section 3.2).

Marble particles showing dark gray spots from RdS and CdD were also analyzed by ESEM. All the observed dark spots corresponded to microcolonies composed of clusters of yeast-like spherical cells about 5–7 μm in diameter (Fig. 2C and D). Microscopic examination clearly demonstrated that the black discoloration on both statues was associated with black fungal structures and that dirt and fly ash were not present in any of the observed samples.

3.2. Fungi isolated from the marble sculptures

After inoculation and incubation of marble powder from sampled areas on MEA, black fungal colonies and strongly pigmented orange yeast-like fungi grew as a closely confluent patina after 1–2 wk. Two black strains were isolated from RdS: M4-2006 and M4-2007, the latter being a strain found after cleaning, and an orange yeast-like fungus designated RS. From CdD, two black strains, D1 and D3, were isolated from two different sampled areas, and an orange yeast-like fungus designated D. All black colonies showed cauliflower-like morphology typical of rock-inhabiting meristematic fungi. Colonies initially were moist; later they became dry and produced a thin, gray-whitish aerial mycelium on the colony surface (Fig. 2E). M4 colonies grown on MEA were also observed by ESEM. They appeared constituted of predominantly spherical cells of about 5 μm in diameter, tightly packed, organized in chains and clusters with chains of elongated cells (Fig. 2F) and a shape very similar to that of colonies observed on marble (Fig. 2C and D). Such tissue-like organization has already been described for rock-dwelling microcolonial black fungi (Gorbushina et al., 2003). Strains M4, D1, and D3 were referred to black MCF on the basis of their morphology. Microscopically, their cells showed yeast-like features; they appeared as single spherical cells and larger spherical cells aggregated in bulb-like clusters, or in

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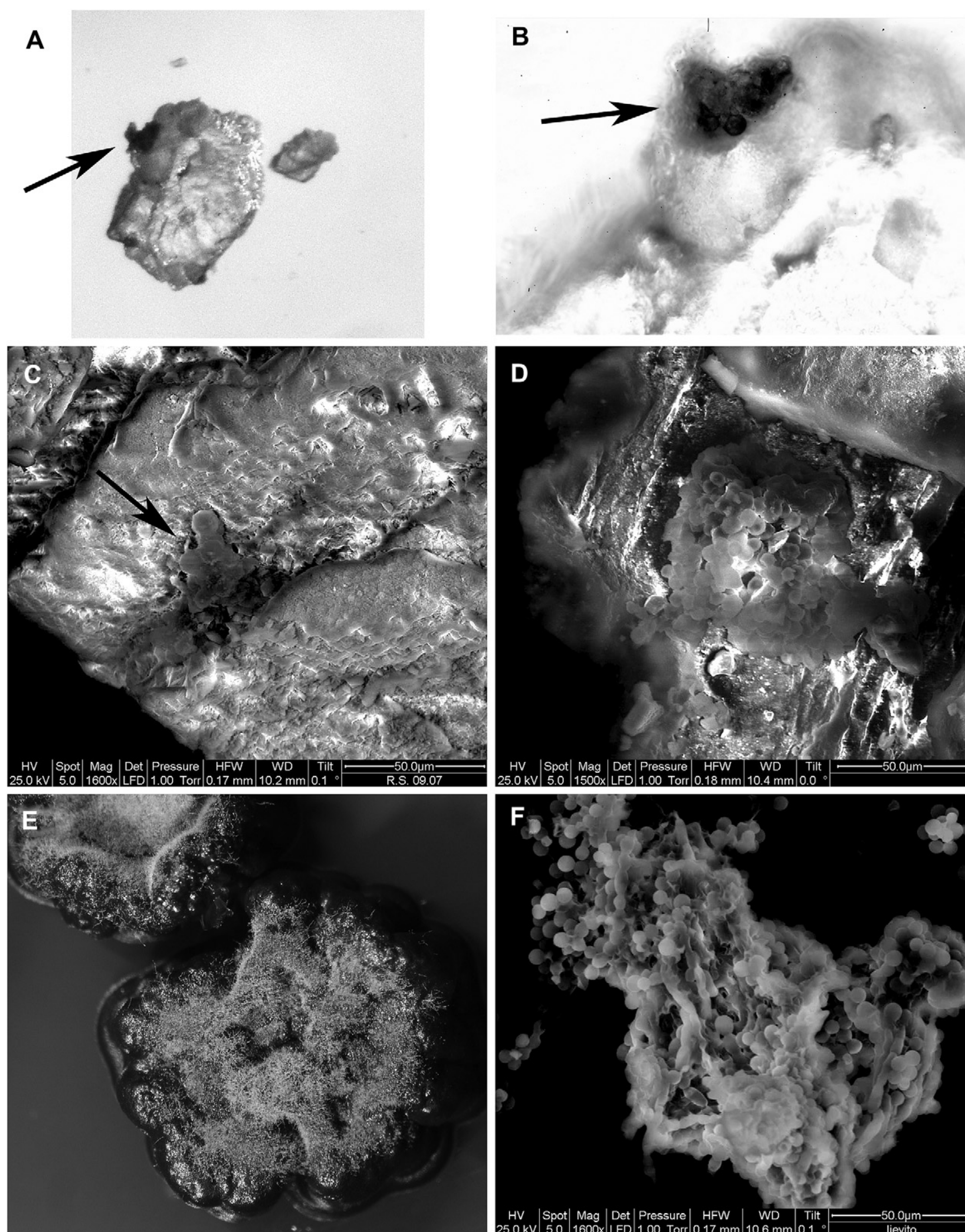

Fig. 2. Microscopic observations of marble particulate and colonies isolated from it. A, optical microscope observation of the particulate sampled from "Ratto delle Sabine"; the dark spot (arrow) is a fungal microcolony (50×) with, B, spherical yeast-like cells (200×). C, D, ESEM observation of the particulate from areas sampled in 2007 of "Ratto delle Sabine" and in 2008 of "Copia del David," respectively. Fungal microcolonies (arrow in C) are still visible after the cleaning intervention. E, M4 colony grown on MEA observed with the stereomicroscope and, F, with the ESEM.

hyphae of swollen cells (not shown). The yeast-like strains RS and D were observed by light microscopy; cells were ellipsoidal to subglobose with polar budding (not shown). Ballistoconidia were produced on cornmeal agar.

Identification of black fungi usually must be confirmed with molecular data. Therefore, the black fungal isolates were characterized using the sequence of the small subunit and internal transcribed spacer regions of rDNA and compared to each other and

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related species by AFLP. The 18S rDNA was entirely sequenced for strains M4-2006 and M4-2007, while the 5′ region of about 600 nt was sequenced for the 18S of D1 and D3. The entire 18S of M4-2006 and M4-2007, the 5′ region of the 18S, and the ITS region of all the four black strains M4-2006, M4-2007, D1, and D3 were 100% identical. However, database interrogations with the 18S and the ITS sequences yielded different results. The 18S sequences showed 100% identity to sequences from *Coniosporium perforans*, *Glyphium elatum*, and *Knufia cryptophialidica*, while ITS sequences showed 100% identity to *Sarcinomyces petricola* and 99% identity to *Phaeococcomyces chersonesos.* Since *Glyphium* shows a very different morphology and habitat from those of M4 (Lorenzo and Messuti, 2005) it was excluded from further comparative analysis. *K. cryptophialidica* – originally isolated from *Populus tremuloides* – was described by Hutchison et al. (1995) as a so-called dematiaceous hyphomycete with high similarity to *Phaeococcomyces catenatus* and *Ph. nigricans*. However, *K. cryptophialidica* exhibits phialides and phialoconidia when grown on MEA. These structures clearly differentiate *K. cryptophialidica* from *Phaeococcomyces* as well as from the strains isolated in this study. *Knufia*, moreover, was placed closely to the order of *Capnodiales/Pleosporales* whereas *Coniosporium*, and *Sarcinomyces* (and thus *Phaeococcomyces*), are phylogenetically distinct and belong to the *Chaetothyriales* and *Hysteriales*, respectively.

Coniosporium, *Phaeococcomyces*, and *Sarcinomyces* are well-known rock-inhabiting MCF genera (Sterflinger and Prillinger, 2001; Bogomolova and Minter, 2003; Sert et al., 2007) with high morphological similarity to M4, D1, and D3.

Phylogenetic relationships were reconstructed to determine the genetic distances between black yeast-like fungi M4, D1, and D3 and other related microcolonial fungi. A phylogenetic tree was constructed using ITS sequences (ITS I/5.8S/ITS II) of M4 and reference strains retrieved from the NCBI database. The ITS sequences were chosen using the following criteria: the most similar sequences to the M4 as resulted from the BLAST output (90% identity minimum); other sequences from referenced strains belonging to the genera *Coniosporium*, *Phaeococcomyces*, and *Sarcinomyces* not overlapping the former ones. The phylogenetic tree (Fig. 3) showed that strains M4, D1, and D3 belonged to the cluster of *S. petricola*, originally classified as an anamorph member of the *Ascomycota*, order *Chaetothyriales*, family *Herpotrichiellaceae* (Wollenzien et al., 1997). In particular, strain M4 was essentially identical to *S. petricola* holotype strain CBS 726.95 (sequence accession number AJ244275.1), originally isolated from an angel statue of Carrara marble in Messina, Italy (Wollenzien et al., 1997). The three genera, *Sarcinomyces* as well as *Coniosporium* and *Phaeococcomyces*, appear as polyphyletic because they are distributed among several distinct clades in the tree (Fig. 3) and according to other ITS-based phylogenetic analyses (Sert et al., 2007; Ruibal et al., 2008).

To better characterize the genetic relationship between the MFC strains and the most similar reference strains, the genomic DNAs of M4, D1, and D3, and of *P. chersonesos* CABI 389175, *C. perforans* (MA 5723) CBS 885.95, and *S. petricola* (MA 5722) CBS 726.95 were examined by AFLP analysis. The results showed that the MFC strains belonged to the same species, with a high level of similarity (85%), and were closely related to *P. chersonesos* and *S. petricola*. Moreover, these two reference strains were highly similar to each other (Fig. 4A). On the other hand, *C. perforans* was observed as an outgroup.

Molecular analysis was also carried out on the orange yeast-like fungi. The 5′ region of 18S (about 600 nt) and the ITS region of the two strains RS and D were 100% identical. Moreover, the molecular analysis based on ITS and 18S was consistent; 18S sequences were 100% and ITS 99% identical to those of *Erythrobasidium hasegawianum* and *Sporobolomyces yunnanensis*. Analysis with AFLP showed that the orange yeast-like fungal isolates RS and D were genetically closer to *S. yunnanensis* HB1275 than to *E. hasegawianum* HB61 (Fig. 4B).

3.3. Biodegradative potential of the MCF and yeast-like fungal strains

All the isolated MCF and the orange yeast-like fungal isolates were plated on MEA supplemented with 0.5% $CaCO_3$ to determine if they were able to dissolve calcium carbonate (Diakumaku et al., 1995). No strain generated a halo due to $CaCO_3$ dissolution after one month of incubation.

To study the interactions between M4 colonies and the marble substratum, Gioia marble pieces were inoculated as described in the materials and methods section. Colonies grew on the marble. To check colony adhesion to the substratum, we tried to remove them with a needle. After one month of incubation, colonies appeared well adhered to the substratum and resistant to the needle pressure (Fig. 5A). Observation by ESEM showed that colonies grew preferentially inside fine cracks and crevices and were anchored to the substratum by chains of elongated cells resembling hyphae (Fig. 5B–D).

4. Discussion

Discoloration of two marble statues with high cultural value – “Ratto delle Sabine” and “Copia del David” – was associated with microcolonial fungi shown to be responsible for typical dark black spots. Microscopic analysis of samples taken in 2006, 2007, and 2008 proved the biogenic origin of this discoloration, since black spots always corresponded to fungal microcolonies and could clearly be distinguished from other black crusts due to gypsum, fly ash, and dirt deposits. Black fungi have been described before as biodeteriogens of natural and artistic marbles and limestones exposed to outdoor environments, causing discoloration and material loss by their biopitting activity (Diakumaku et al., 1995; Sterflinger and Krumbein, 1997). These fungi are commonly isolated from monumental stones in the Mediterranean Basin and are considered the most harmful microorganisms associated with stone and monument biodeterioration (Urzí et al., 2000).

Since many black fungi are pleomorphic, having anamorph life cycles with widely divergent types of propagation, morphology offers only a presumptive identification at the genus level. To reliably identify and determine their phylogenetic position, molecular methods are needed (Urzí et al., 2000; Sterflinger and Prillinger, 2001; Sterflinger, 2006; Ruibal et al., 2009). For this reason, the MFC isolates were characterized by morphological and molecular rDNA based methods. The 18S rDNA sequence is generally regarded as a marker for resolution at the genus level. In this case, however, the results were ambiguous and did not yield a conclusive identification. Analyses of ITS sequences, on the other hand, permitted a clear identification of the black fungi as *P. chersonesos*/*S. petricola* (Fig. 3). The result was confirmed by AFLP fingerprints. Both species were described as rock-inhabiting fungi isolated from natural rocks and from marble of monumental buildings (Wollenzien et al., 1997; Bogomolova and Minter, 2003). *S. petricola* was originally isolated from monumental marble in Mediterranean environments (Wollenzien et al., 1997) and subsequently north of the Alps (Sterflinger and Prillinger, 2001). It exhibits yeast-like growth, hyphal formation, and meristematic growth, and was originally classified as an anamorph member of the *Chaetothyriales* (Wollenzien et al., 1997). According to Wollenzien et al. (1997), *S. petricola* has not been found on substrata other than stone, and this might be its natural ecological niche, although its exact role in biodeterioration has yet to be

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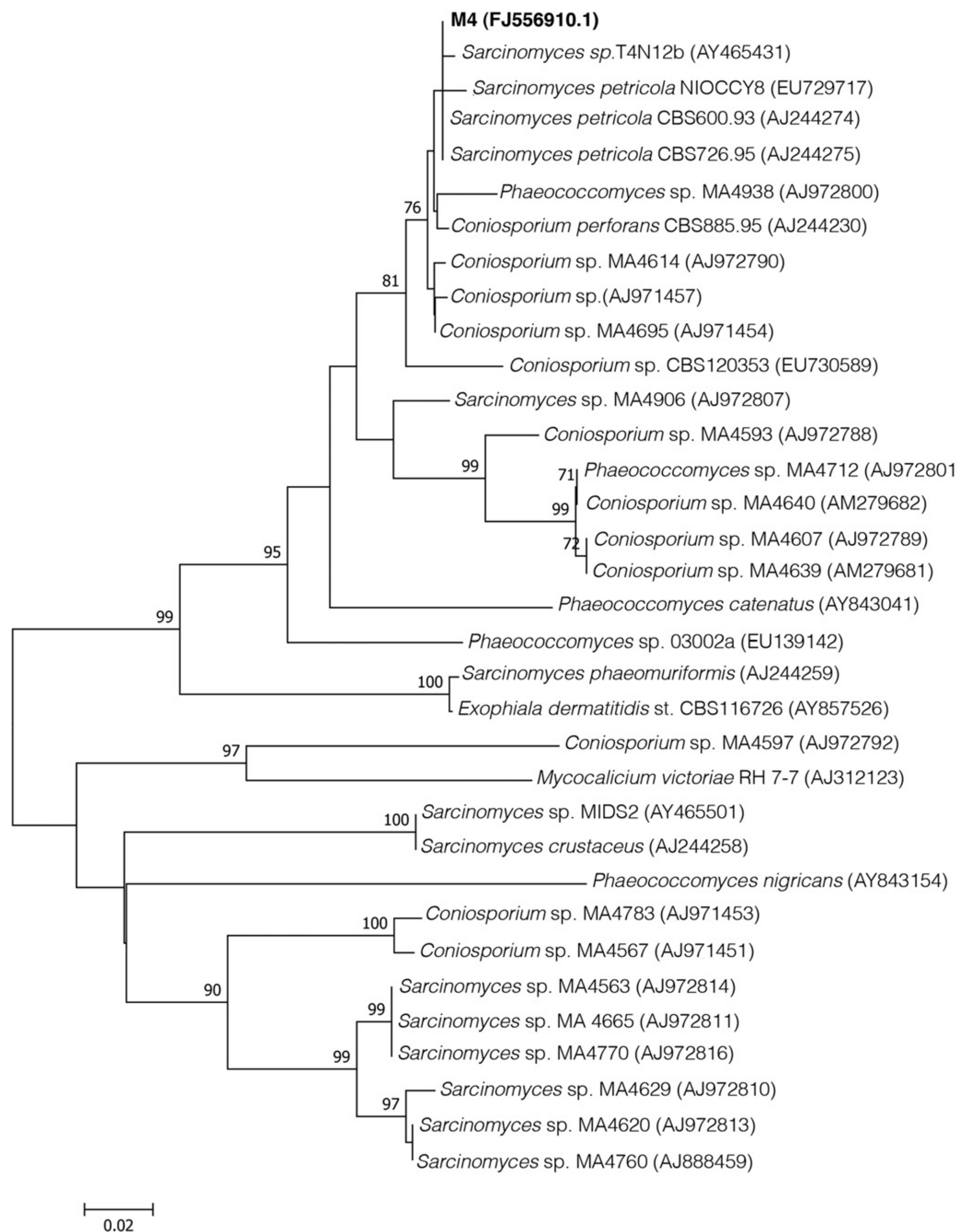

Fig. 3. Phylogenetic tree constructed from the ITS sequences (ITS I/5.8S/ITS II) of strain M4 (in bold) and 33 reference strains retrieved from the NCBI database. Numbers indicate bootstrap confidence percentages. The scale bar indicates substitutions per nucleotide. The accession numbers of the ITS sequences are reported after the strain name.

established. Some species of the genus *Phaeococcomyces* are the synanamorphs of *Sarcinomyces*. *Phaeococcomyces* spp. are characterized by torulose growth with yeast-like budding, whereas *Sarcinomyces* spp. form sarcinic packages with isodiametric division of subdividing cells (Ruibal et al., 2008).

Amplified fragment length polymorphism analysis is a very sensitive tool that compares closely related species and detects genomic differences at the subspecies level (Vos et al., 1995). In this study, AFLP analysis showed that all strains of the isolated black meristematic fungi differed very little and could be regarded as identical at the species level.

Interestingly, the same strain was isolated before and immediately after the cleaning treatment with ammonium carbonate. This observation further demonstrates the high resistance of these fungi toward various physical and chemical methods and emphasizes the challenge for the restorers to clean such types of decay. Ammonium carbonate treatment is commonly used in restoration to remove black gypsum crusts from rock surfaces. In the case of the two sculptures analyzed here, fungi seem to be responsible for the blackening of the white marble surfaces rather than gypsum. Therefore, restoration treatments should be sought to address the agents of biogenic deterioration.

In addition to marble discoloration, black fungi may well be responsible for serious material losses. Although it was impossible to collect deep marble samples from these valuable statues, it can be imagined that black fungi could penetrate into the substratum and cause loss of cohesion in marble crystals. The potential of black fungi to damage marble by mechanical attack, with cracking and

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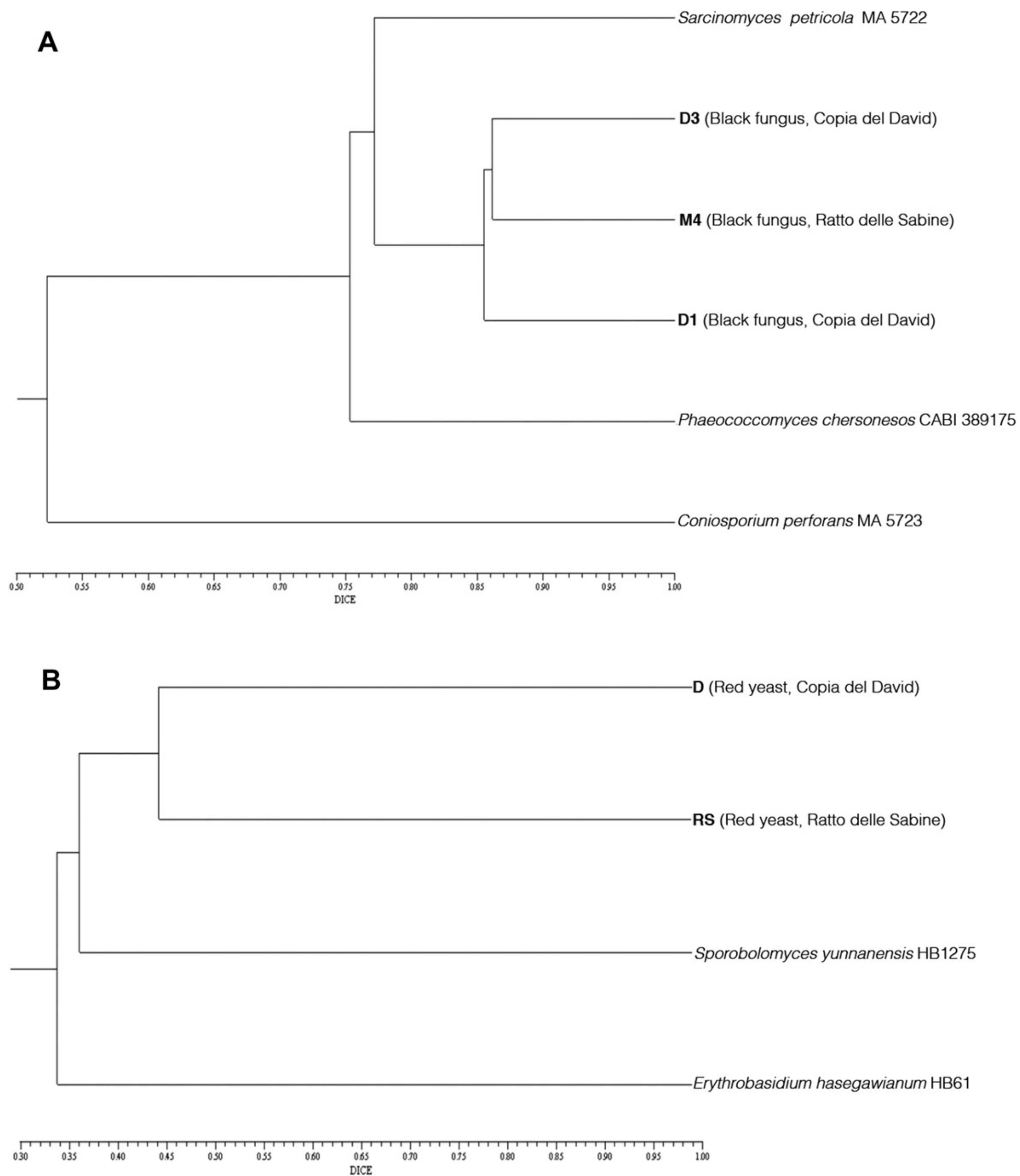

Fig. 4. AFLP analysis dendrogram of isolated (in bold) and reference fungal strains. A, black MCF strains; B, orange yeast-like fungal strains. Percentage values of DICE coefficient of similarity are shown under the dendrogram.

fissure formation, has been described (Diakumaku et al., 1995; Wollenzien et al., 1995; Sterflinger and Krumbein, 1997).

Penetration of marble by black fungi is not yet understood and, in accordance with laboratory results on carbonate agar, calcium carbonate dissolution must be excluded (Diakumaku et al., 1995; Wollenzien et al., 1995). According to the multiple-stages model of fungal mechanical attack proposed by Sterflinger and Krumbein (1997), the stages of pre-penetration (when the fungal cells reach the substratum) and penetration (when thin penetration hyphae are extended into the inner part of the rock through the discontinuities) could be deduced from the microscopic observation of M4 cells grown on marble samples (Fig. 5). On the other hand, the presence of melanin in the cell wall has been associated with an increase in hyphal rigidity “hardness” and fungal turgor pressure, which would confer pathogenicity by facilitating plant penetration by plant pathogen fungi (Money and Howard, 1996; Money et al., 1998). An analogous penetration potential conferred by wall melanization could be hypothesized for marble by MCF.

It is not possible, from our data, to establish whether a post-penetration stage (with fungi developing internal colonies that lift the overlying rock crystals and with the rock surface becoming deteriorated and roughened) occurred. Since the pieces of inoculated marble presented rough surfaces and discontinuities as inoculation started, it is difficult to assess if fissures where colonies were located were either initiated or deepened by fungal growth. Interestingly, the orange yeast-like fungal isolate, identified as

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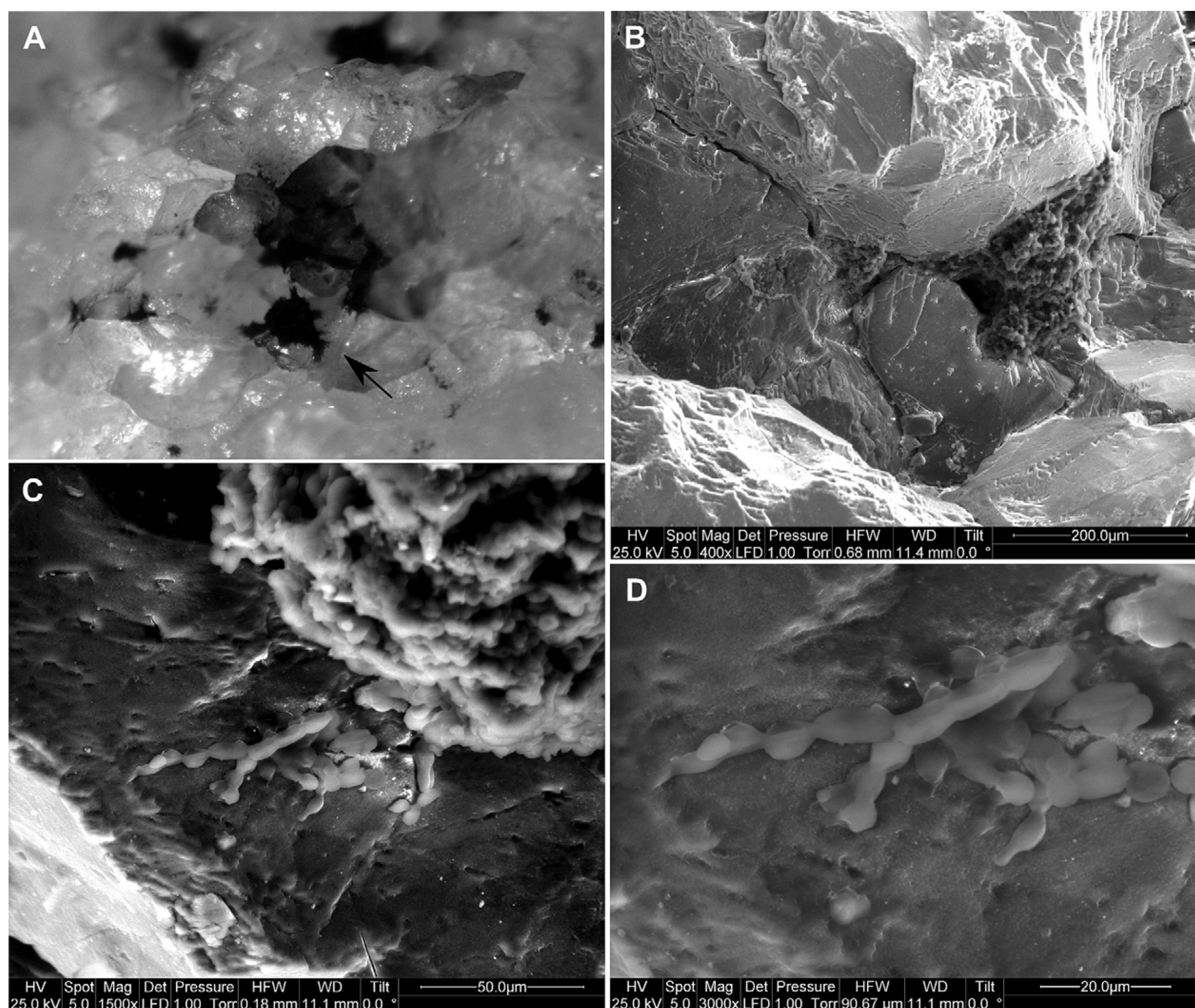

Fig. 5. Microscopic observations of M4 colonies on Gioia marble particles after one month of incubation. Colony growth follows the discontinuities and the fissures of marble (penetration stage, according to Sterflinger and Krumbein, 1997). A, M4 colonies grown in the cracks of marble (arrow) observed with the stereomicroscope (57×), and, B, with the ESEM. C, ESEM micrograph of an M4 colony anchored on marble; the anchoring structures are magnified in D.

S. yunnanensis, was isolated together with the black fungi from both statues. This fungus was observed to occur frequently when other fungi are isolated from rock surfaces, and it seems to be closely associated with black microcolonial fungi (unpublished data, Sterflinger); its identity was clarified for the first time in this study. Because no red or orange patina was visible on deteriorated areas of the statues, it can be concluded that its growth on marble is limited. Furthermore, this orange fungus did not exhibit lytic potential on calcium carbonate. A possible functional interaction with black fungi on stone merits more study and will be further investigated.

5. Conclusions

The fungus *S. petricola* is responsible for the formation of grayish dark spots on several areas of the two valuable statues “Ratto delle Sabine” and “Copia del David.” Understanding the biogenic cause will aid new formulations for a proper scheduling of restoration and conservation interventions. Based on ITS and AFLP analysis, it was shown that strains isolated from both statues at different times are identical and able to survive chemical treatments with ammonium carbonate. The results of AFLP analysis further suggest an intimate association between *Phaeococcomyces chersonesus* and *S. petricola,* which appear to be synanamorphs of the same organism.

Acknowledgments

The monitoring and restoration of the “Ratto delle Sabine” and the “Copia del David” were commissioned by the Polo Museale Fiorentino and the Assessorato alla Cultura del Comune di Firenze, respectively. Both interventions were carried out by the restoration company Meridiana Restauri. We would like to thank the restorer Alberto Casciani for the on-site assistance in collecting the marble samples from both statues; Dr. Cristiana Giordano and Dr. Cristina Salvatici of Centro di Microscopie Elettroniche CEME-CNR, Sesto Fiorentino, for ESEM analysis; and Mrs. Cristina Indorato for technical assistance.

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