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ENVIRONMENTAL MICROBIOLOGY - SHORT COMMUNICATION

Isolation and molecular identification of a strain belonging to the new species *Zalaria obscura* from a deteriorated wooden artwork

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Abstract
We report the case of an outdoor deteriorated wooden sculpture of Madonna, completely blackened in the face, and thus suspected of fungal attack. A multi-disciplinary approach, including microbiological analysis, molecular biology, and Fourier transform infrared (FT-IR) spectroscopy, was applied to understand the real nature of the observed alteration. FT-IR showed that the blackening was due to the application of a natural terpene resin subjected to alteration over time. The microbiological assay allowed to isolate a particular black fungus that has been recovered in the vegetative phase, growing as the only species adapted to the examined substrate. Basic Local Alignment Search Tool (BLAST) analysis of the ITS (internal transcribed spacer) region sequence identified the fungus (LS31012019) as *Zalaria obscura*, a black yeast belonging to the new genus *Zalaria*, family *Dothideales*. Overall, this study evidenced the importance of a multi-disciplinary approach to understand the real causes of observed deterioration of artworks. More interestingly, the recovery of a strain identified as *Z. obscura* from this type of substrate is never reported in the literature and this finding could offer the possibility to investigate the role of this microorganism in the deterioration process of cultural heritage.

Keywords Artwork · Deterioration process · Multidisciplinary approach · Identification · *Zalaria obscura*

Introduction

Cultural heritage has an inestimable value in historical, artistic, and cultural terms for each generation [1]. However, no material can be considered insusceptible to microbial attack since organic and inorganic materials of artworks often represent suitable environment for microbial colonization, which consequently esthetic and, in some cases, structural damages [2]. In the literature, it has been well reported the damage of monuments, mural paintings, and frescoes due to different microorganisms [3–5], while few studies related to canvas [6, 7] or wood artworks have been described [8]. Fungi represent the principal actors of wood degradation in terrestrial sites because these are able to degrade cellulose, hemicellulose, and lignin [9]. These microorganisms can be divided in three categories on the basis of the produced wood decay. The first two groups of fungi are taxonomically classified as *Basidiomycota* and are represented by white- and brown-rot fungi. White-rot fungi (*Pleurotus ostreatus* and *Phanerochaete chrysosporium*) degrade simultaneously all the components of the plant cell wall and, in some cases, more extensive degradation of lignin and hemicellulose occurs, leaving the wood whit a "white appearance" due to remaining cellulose. In contrast, brown-rot fungi (*Serpula lacrymans*, *Fibroporia vaillantii*, *Coniophora puteana*) are able to circumvent the lignin barrier, removing the hemicellulose and cellulose with only minor modification to the lignin component. For the rapid loss of cellulose and/or hemicellulose, the wood undergoes a significant loss of strength, often before decay features are evident by naked eyes [10]. The third category is represented by fungi causing soft-rot, taxonomically classified as *Ascomycota* and *Deuteromycota*. These fungi are

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able to colonize wood under conditions that are not favorable for brown- or white-rot (hot, cold, or wet) and are represented by fungi such as *Chaetomium*, *Ceratocystis*, and *Kretzschmaria deusta*. The soft rot is generally characterized by a soft decayed wood surface in contact with excessive damp, distinctive of dry environments, and macroscopically similar to brown rot [11].

Different environmental factors influence fungal growth and their activity of wood decomposition. Oxygen (lower oxygen level, lower decay process), pH, temperature, and nitrogen can be taken in account for a faster or slower wood degradation due to a microbial population [12]. Nevertheless, the most important requirement is moisture level [10]; indeed, for white- and brown-rot fungi, the optimum moisture levels are approximately 40–80%, while soft-rot fungi can tolerate a wider range of moisture conditions, as present in excessively wet or dry woods. Once recognized, the presence of fungi in a damaged artistic wooden object, the correct identification is difficult by the standard microbiological procedures (they can reach only the genus-level identification), but more suitable techniques can be considered and applied, such as the molecular ones [1, 13].

Considering all that, in this investigation, we report the case of a deteriorated outdoor wooden sculpture suspected of fungal attack. A multi-disciplinary approach, including Fourier transform infrared spectroscopy (FT-IR), microbiological analysis, and bio-molecular techniques, was applied to understand the real nature of the observed alteration.

Materials and methods

Samples collection

The examined artwork (a wooden sculpture of Madonna) is placed in an outdoor niche of a local road (Loreto, Ancona, Italy). The artwork, completely blackened, appeared remarkable damaged (Fig. 1a) and, for this, was subjected to different microbiological and chemical analyses. The samples utilized for the different analyses were represented by fragments spontaneously detached from the sculpture and collected, with the permission from the local owner and authorities, by a local restorer before starting the restoration activity (Fig. 1b).

FT-IR-ATR analysis

FT-IR spectra were acquired in attenuated total reflectance mode (ATR) using a Perkin-Elmer Spectrum Two FT-IR™ equipped with a ZnSe crystal. The sample was kept in contact with the crystal at controlled pressure and the spectrum was acquired with 4 scan. Air background was firstly acquired.

Microbiological sampling and characterization

Each collected wooden fragment was transferred onto Petri-dishes containing different selective solid media for fungi, Sabouraud Dextrose Agar (SDA), Malt Extract Agar (MEA), and Potato Dextrose Agar (PDA) (Liofilchem). Cultures were incubated at 25 °C for 5–7 days and regularly observed to verify the presence of growth. Then, the isolated fungi were identified to genus level using biometric parameter (in particular colony diameter) and microscopic features [14–16].

DNA extraction, PCR amplification, DNA sequencing, and BLAST alignment

Genomic DNA of the fungal isolate was extracted from its mycelia grown on PDA. Mycelia were harvested and transferred to a 1.5 mL Eppendorf tube containing 500 μL of PBS centrifuged for few minutes at 8.000 rpm. The supernatant was removed ant the pellet was crushed in liquid nitrogen using a sterilized micro-pestle. The powder was suspended in 100–300 μL of a buffer containing 200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, and 0.5% w/v SDS, vortexed for 10 s. The suspension was clarified by centrifugation for 10 min at the max speed (14.000 rpm), and the aqueous phase was placed to a new tube. Nucleic acids were precipitated by adding to it an equal volume of isopropanol and kept for 30 min at − 20 °C before the centrifugation for 15 min at 14,000 rpm. The DNA pellet was air-dried and solubilized in 10–50 μL of ultrapure water. The DNA sample was loaded on an 0.8% agarose gel and its concentration was deduced

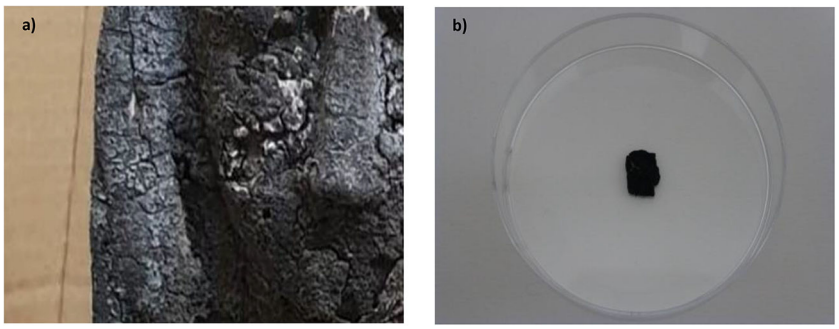

Fig. 1 Particular of the blackened wooden artwork suspected of fungal attack (**a**) and one of the related fragments (micro-samples) used for the different analysis (**b**)

against the Lamda DNA-HindIII digest (New England, BioLabs, USA).

The rDNA regions comprising ITS1, 5.8S rRNA gene, and ITS2 were amplified using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCT CCGCTTATTGATATGC-3′) [17]. The reaction mixture, 25 μL each sample, was set up using the TaKaRa Taq™ (TaKaRa Shuzo Co., Ltd., Tokyo, Japan) and the buffers supplied, and the final concentration of primers was 800 nM. The PCR reactions were performed in a Verity Thermal cycler (Applied Biosystems). Cycling parameters were 95 °C for 2 min, followed by 35 cycles of 95 °C for 15 s, 52 °C for 15 s, and 72 °C for 1.5 min, with a final extension at 72 °C for 10 min. Reactions were performed in duplicate and in 1:5 dilution, and negative controls (without DNA) were included. The PCR products were checked by electrophoresis in 1.5% (w/v) agarose gels containing 1X Midori Green Advance (Nippon Genetics, Japan), that was photographed using the GelDoc 2000 (Bio-Rad, Richmond, CA). Positive amplification products were purified by using a GeneAll ® Expin™ PCR SV, and DNA concentration was quantified using NanoDrop 2000C (Thermo Fisher Scientific, Wilmington, DE, USA), following the manufacturer's instructions. Before purification, the PCR products were sequenced in duplicate by Eurofins Genomics (Ebensburg, Germany) with the same primer sets used for PCR. After edition, final sequence was submitted to the GenBank under the accession number MN480547. The nucleotide sequence (ITS1, 5.8S rRNA gene, ITS2) was compared in GenBank using BLASTN search [18], and the algorithm was run through the NCBI website.

Results and discussion

To really understand the biodeterioration process it is important to know the relationships between microorganisms and environment, in order to relate the different microbial populations to the physical-chemical conditions of the artwork and surroundings. Indeed, the growth dynamics of microbial communities are influenced by the materials of the artwork substrate itself and by environmental factors such as water, chemical composition of the air, humidity, temperature, light, and pollution level.

In this investigation, we present the case of a Madonna wooden sculpture that for its outdoor placement was exposed to different environmental conditions, thus resulting remarkably deteriorated and apparently suspected of fungal attack (Fig. 1a). Our first concern was to understand if the observed blackened face of the Madonna was induced by biological or chemical agents. The applied FTIR-ATR technique allowed to verify as the face of the Madonna was covered by a natural terpene resin, as evident by the typical signal pattern: a carbonyl (C=O) signal at 1716 cm^{-1}, a single C–O bond signal at 1239 cm^{-1}, and the hydrocarbon backbone signals constitute by the peaks at 2917 and 2849 cm^{-1}, attributed to symmetric and asymmetric C–H stretching of methylic and methylenic groups and that at 1454 and 1397 cm^{-1} due to C–H bending vibration modes [19] (Fig. 2). The applied resin was also subject to deterioration over time, as evident by the macroscopic observation. This result is not surprising considering that the iconographic representation of the Madonna in some areas, including Loreto, is typical with blackface. In some cases, the origin of the dark color is simply due to alteration by smoke (candles or fire) or by the alteration of the lead-based pigments of the painting itself; moreover, the micro thickness silver leaf finish of face may have oxidized over time, leaving the surface black. In other cases, the origin remains uncertain because many images have been repainted several times, or even, radically altered during restorations [20]. In the examined sculpture, it is not simple to define the time when the resin was applied or the period necessary for the decay, but in any case, this covering may have offered a suitable substrate for microbial growth. As regards, the cultural methods, different selective media (SDA, PDA, and MEA), were used. Several fragments from the darkened face of the Madonna wooden sculpture, as well as someones recovered from the back of the artwork were analyzed. In the latter case, after incubation of samples, no growth was observed. On the contrary, the cultures of the darkened fragments evidenced the growth of black smooth and slimy colonies on MEA after 7 days (Fig. 3a), developing an aerial mycelium after the prolonged incubation period (28 days) (Fig. 3c). Fungal isolation cultures, performed with the three-point method, revealed the presence of yeast-like darker colonies on PDA but not on SDA (Fig. 3b). To obtain more information on the morphology of this microorganism, microscopic observations carried out (after 14 and 28 days) evidenced aseptate, dark brown chlamydospores, hyaline hyphae and yeast-like, hyaline, globose to ellipsoidal conidia (Fig. 4). On the basis of the analyzed macroscopic and microscopic parameters, as well as the morphological parameters including the presence of spores and conidia, we have initially hypothesized that the fungal isolate could be ascribed to the *Aureobasidium*-like species. However, all these observations were not sufficient to reach a satisfactory identification of the microorganism, and therefore, to really understand the species of the black-fungus recovered from the wooden sculpture (named as LS31012019), molecular methods were applied. Genomic DNA was then extracted from mycelium and ITS region amplified and sequenced and deposited in the GenBank (MN480547). The ITS regions are nested in the nuclear rDNA repeat and, owning a high variation between taxonomically different fungal species and even within the species, have proved to be a suitable tool to investigate the fungal diversity in different

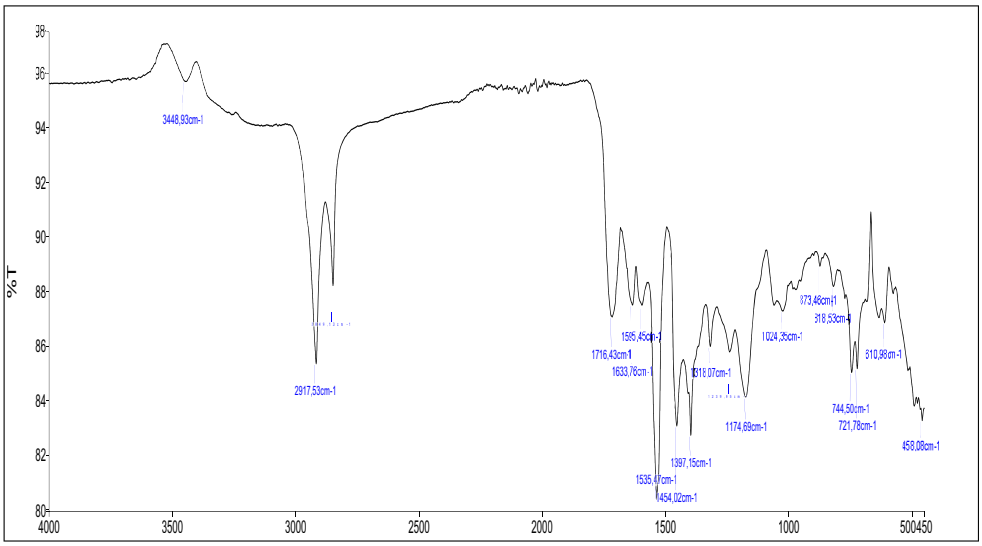

Fig. 2 FT-IR spectra of the analyzed sample obtained following the technique reported by Derrick [19]

materials [21]. In our case, the edited sequence was compared via BLAST to GenBank nucleotide collections, and the obtained highest-scoring pair (100% of identity) allowed the identification of the isolated microorganism as *Zalaria obscura*. In addition, our sequence showed a 100% identity to the sequences reported by Humphries et al. [22] that have introduced a new family (*Zalariaceae*) and a new genus (*Zalaria*) with two species, *Z. alba* and *Z. obscura*, to accommodate this relatively novel species.

The need of new approaches in the study of microbial community in cultural heritage was widely described by Gonzales and Saiz-Jiménez [23] that stressed as the microbial diversity of artworks required appropriate detection methods and appropriate controlling strategies to preserve them from microbial colonization. As regards our experience, we can state that cultural method revealed the presence of black fungus, in a vegetative state, as the only species adapted to the analyzed substrate and the applied molecular method allowed the

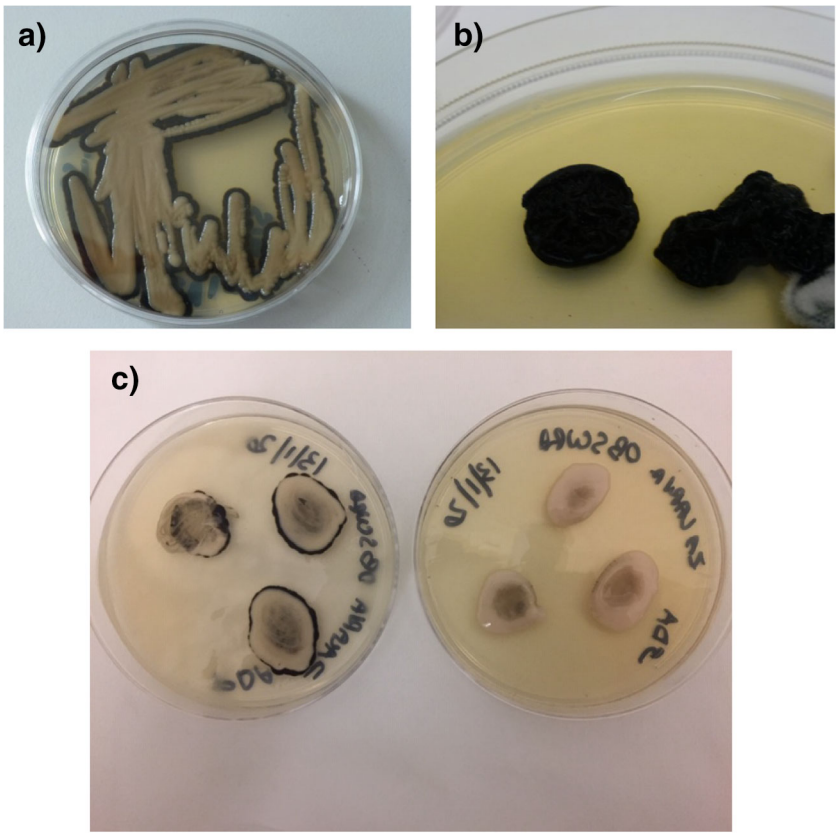

Fig. 3 Darkly pigmented yeast-like colonies observed on MEA after 7 days of incubation (**a**) and following development of melanized aerial mycelium after 4 weeks of incubation (**b**). Representative image of the three-point inoculation of the darkly pigmented yeast-like on PDA (right) and SDA (left) as appear after 14 days of incubation (**c**)

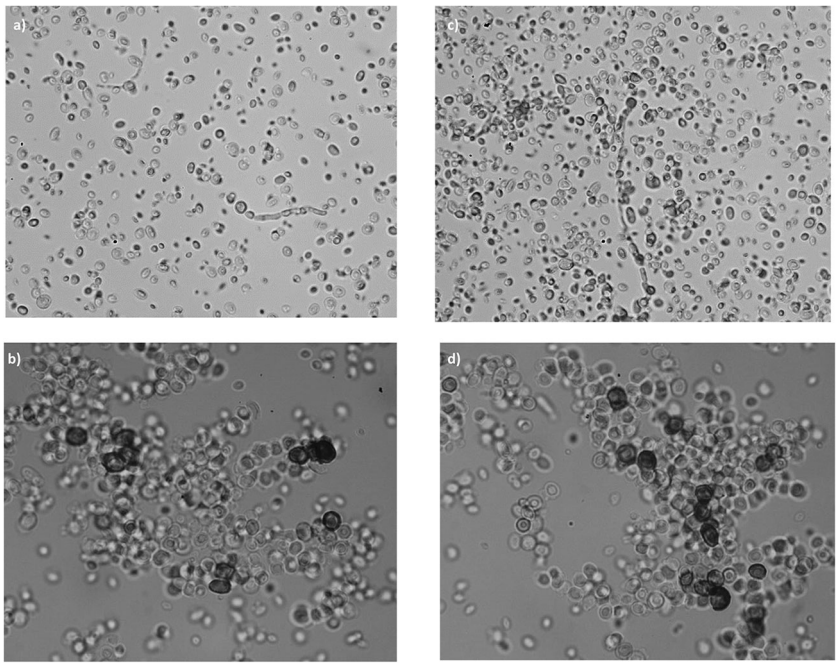

Fig. 4 Microscopic images of LS31012019 isolate as appears after 14 (**a**, **b**) and 21 days (**c**, **d**) of incubation on MEA. Samples were previously stained with blue lactophenol solution. (**a** and **c** magnification × 20/0.45; **b** and **d** magnification × 40/0.65; binocular optical microscope, Axiolab RE 31509, Zeiss)

correct identification of the microorganism at species level (*Zalaria obsura*). Indeed, the important role of melanin in protecting fungi in diverse hostile environments is well debated [24, 25], and in our case, the recovery of the black fungus *Z. obscura* (LS31012019) can be explained for the presence of this particular pigment. A combined approach was recently used to identify the filamentous fungi in a Brazilian artwork with a compromised status of conservation and was able to correlate the presence of *Aspergillus niger* with the observed deterioration of painting [26]. Similarly, Liu et al. [27] isolated two *Fusarium* species from a waterlogged artwork, identifying them as *F. solani* and *F. oxysporum* using molecule method.

In conclusion, the present investigation demonstrated that the combination of culturing methods and molecular techniques can facilitate the study of natural microbial communities in biodeteriorated artworks. In our case, *Z. obsura* LS31012019 resulted to be the dominant microorganism in the examined artistic sample that can be thought as a "peculiar ecological niche" allowing the growth and the survival of this black yeast. It can be noted that the recovery of *Z. obscura* from a wooden artwork is never reported in the literature and, in the future, could be interesting to deep study the possible role of this microorganism in the biodeterioration process of artworks, by investigating its enzymatic profile on different wooden components, as well as its ability to grow under diverse environmental conditions or substrates.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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