sustainability

MDPI

Article

Preliminary Studies on Fungal Contamination of Two Rupestrian Churches from Matera (Southern Italy)

Stefania Mirela Mang [1], Laura Scrano [2,*] and Ippolito Camele [1,*]

[1] School of Agricultural, Forestry, Food and Environmental Sciences (SAFE), University of Basilicata, Viale dell'Ateneo Lucano 10, 85100 Potenza, Italy; stefania.mang@unibas.it

[2] Department of European and Mediterranean Cultures (DICEM), University of Basilicata, Via Lanera, 75100 Matera, Italy

* Correspondence: laura.scrano@unibas.it (L.S.); ippolito.camele@unibas.it (I.C.); Tel.: +39-0912-05231 (L.S.); +39-0971-205544 (I.C.)

Received: 4 August 2020; Accepted: 25 August 2020; Published: 27 August 2020

Abstract: The Sassi, a UNESCO World Heritage Site and its rupestrian churches, are richly decorated and visited by thousands of visitors every year. It is important to preserve this heritage which shows signs of deterioration due to abiotic and/or biotic factors. Aiming to carry out in the future an environmental-friendly restoration, a screening of the fungi present on walls and frescoes of two rupestrian churches "*Santa Lucia alle Malve*" and "*La Madonna dei derelitti*" located, respectively, in the "Sasso Caveoso" and in the "Sasso Barisano" was performed. Isolation and characterization of fungal species from investigated sites was carried out. Total genomic DNA (gDNA) was extracted from pure fungal cultures and subsequently utilized in PCRs using primers that amplify a portion of the ribosomal DNA (ITS5/ITS4) or the β-tubulin gene (Bt2a/Bt2b). The amplicons were directly sequenced. Obtained nucleotide sequences were compared to those present in the GenBank (NCBI) showing a very high similarity (99–100%) with the following species: *Parengyodontium album*, *Alternaria alternata*, *Cladosporium cladosporioides*, *Lecanicillium psalliotae*, *Meyerozyma guilliermondii* and *Botryotrichum atrogriseum*. All sequences from this study were deposited in the EMBL database. Detailed knowledge about fungi isolated from stone is indispensable not only to counter/reduce the structural and aesthetic damage but also to protect the health of both guardians and visitors who may develop different pathologies due to the spores diffused in the environment.

Keywords: ancient frescoes; fungi; molecular identification; rupestrian churches; Sassi of Matera

1. Introduction

A great number of Historical Cultural Heritage assets selected and protected by UNESCO is composed of monuments made of stone or other materials and among these edifices, the rupestrian churches are the oldest ones. One of the UNESCO World Heritage Sites (WHS) containing rupestrian churches is the Sassi and the Park of the Rupestrian Churches of Matera, inscribed in 1993 situated in the southern part of Italy, within the town of Matera (Basilicata Region, Italy). This WHS is one of the most superb, integral examples of troglodyte settlement found in the Mediterranean region harmoniously inserted into the natural landscape and ecosystem. All rupestrian churches located there can be considered without any doubt as historical treasures being richly decorated and visited by thousands of visitors every year, testifying the human presence from very old times and the strong desire to transmit to the next generations traditions and culture. In this view, the safeguarding of the cultural heritage located in Matera, The European Capital of Culture in 2019, is of primary importance not only for the cultural aspects but also for the economic ones.

Amongst the historical and cultural assets of Sassi and the Park of the Rupestrian Churches of Matera, two churches named "Santa Lucia alle Malve" and "La Madonna dei derelitti" also known as "della Scordata" are also included. The first church, located in the "Sasso Caveoso" site, was the first female monastery of the Benedictine Order from the VIII century. It is one of the most important churches of Matera providing access to only one part to the public. This church is beautifully decorated and holds a few antique frescoes. "La Madonna dei derelitti" is situated in the "Sasso Barisano" site. It is a very simple and poorly structured architectural monument with its internal walls decorated with frescoes. However, at present only one fresco, named Saint Nicholas, is still visible. Both churches were excavated into stone blocks mainly composed of calcite, in approximately the same period (Late Middle Ages).

Unfortunately, these precious historical and cultural possessions of humanity, which can provide scientific information and emotional connection, had been exposed over the years to a heavy degradation process. This was caused by abiotic and/or biotic factors (biodeterioration) that depreciated stone surfaces and frescoes [1]. The negative actions of these factors can often be observed as aesthetic changes (discoloration) and structural damages (defects and cracks). In addition, harmful effects of the microbial growth such as biofilm formation, biomineralization, degradation of organic binders are also very common [2–8].

The presence of microorganisms including fungi on the walls and frescoes, particularly inside closed areas, influenced the air quality and could also be linked to several human health problems such as asthma and other respiratory illnesses especially in people with immune system deficiencies, [9–12].

The planning and application of prevention and restoration strategies to protect the cultural/historical assets is impossible without a complete understanding of the entire microbial diversity present. Therefore, the identification of microorganisms is compulsory being the first step within this complex process [13–18]. The cultivation and subsequent identification of the microbes colonizing the cultural/historical monuments which will offer some knowledge for the biodeterioration nevertheless, need to be further supported by other functional tests assessing the microbiological effects on decay.

Although, studies on microbial community such as fungi and bacteria responsible for deterioration of stone temples or churches were already undertaken [19–21] investigations on the edifices from The Sassi and the Park of the Rupestrian Churches of Matera are still very few or absent. A recent study reported by Caneva et al. [22] on the Crypt of the Original Sin (Matera) dealt with changes in biodeterioration patterns (BPs) on mural paintings. Microscopy and biomolecular methods aid to finally obtain effective restoration strategies. However, to our knowledge, studies on biodeterioration of the two rupestrian churches "*Santa Lucia alle Malve*" and "*La Madonna dei derelitti*" have not been reported so far.

The objective of this study was to perform an initial screening of the fungal species present on the interior walls and frescoes of the two above mentioned rupestrian churches with the final aim to accomplish, in the near future, their environmental-friendly restoration and also to evaluate the possible effects of fungi on human health.

2. Materials and Methods

2.1. Materials and Sampling

Two rupestrian churches,"*Santa Lucia alle Malve*" and "*La Madonna dei derelitti*", showing advanced signs of deterioration such as discoloration, cracks, fissures and exfoliation (Figure 1a–d) were investigated to eventually identify fungi present (Figure 1).

A non-destructive sampling from interior walls was carried out using a small cotton sterile swab soaked in sterile distilled water. The sampling was gently applied once on the surface of four different points (S1–S4) for each rupestrian church.

The cotton swabs were immediately placed into sterile vials containing 1 mL of double distilled water. Then, they were brought to the laboratory and stored in the fridge at 4°C until used.

Figure 1. General view of the environmental context (**a**,**b**) and interior (**c**,**d**) of the two rupestrian churches "*Santa Lucia alle Malve*" and "*La Madonna dei derelitti*". Photos from the interior (**c**,**d**) of the two rupestrian churches also show the S1-1, S1-2, S1-3, S1-4, S2-1, S2-2, S2-3 and S2-4 sites from where non-invasive sampling was performed. To investigate overall fungal species present, material from four sampling points of each site was combined into two samples corresponding to (A) "*Santa Lucia alle Malve*" church and (B) "*La Madonna dei dereliti*" church.

2.2. Fungi Isolation and Morphological Analyses

In order to isolate the cultivable fungal species, from each sample, 100 µL of suspension was directly plated on Petri dishes with Potato Dextrose Agar (PDA) amended with kanamycin (1 mg/L) and streptomycin (1 mg/L).The plates were placed in an incubator at 24 ± 1 °C in dark and incubated for 7 days. The pure fungal cultures obtained were used for further morphological and molecular analyses. Preliminary identification of pure fungal isolates was carried out using a microscope (Axioscope, Zeiss—Germany) according to the macroscopic and microscopic features of the isolates.

2.3. Molecular Analyses

2.3.1. Genomic DNA Isolation

Mycelium was scraped from the surface of the pure fungal colony and finely ground using liquid nitrogen.The genomic DNA (gDNA) was extracted from approximately 100 mg of each sample using the NucleoSpin Plant II™ (Macherey-Nagel, Germany) kit following manufacturer's instructions.

The quantity and quality of the recovered gDNAs was checked by readings at Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) at 260 nm and 260/230 nm wavelength. The gDNA was stored at −20 °C until further analyses.

2.3.2. PCR Amplification, Sequencing and Sequences Analyses

The gDNA of each pure fungal isolate was subjected to Polymerase Chain Reaction (PCR) using oligos ITS5 and ITS4 [23] or primers Bt2a and Bt2b [24] which amplified a fragment of the Internal Transcribed Spacer (ITS) and the beta tubulin gene (TUB-2), respectively. For PCR reaction, 4–6 µL (100 ng) of each gDNA extracted was employed as template in 25 µL mixture using 1 unit of Phire Hot Start II DNA Polymerase Enzyme (Thermo Scientific Inc., Wilmington, USA). The cycling protocol used for ITS amplification was the following: an initial denaturation at 98 °C for 5 min (1 cycle); followed by denaturation at 98 °C for 5 s; annealing at 58 °C for 5 s; extension at 72 °C for 20 s (40 cycles) and a final extension at 72 °C for 1 min (1 cycle). In case of β-tubulin gene the cycling protocol was the same as for ITS only with a slight modification of the annealing temperature which was set at at 62 °C. All PCR reactions were carried out using a Bio-Rad T100™ Thermal Cycler (Hercules, California, USA). PCR products were detected by electrophoresis in 1.2% (w/v) agarose gels, pre-stained with SYBR Safe DNA Gel Stain (Invitrogen Inc., Carlsbad, CA, USA) and photographed.

The amplicons were sequenced by BMR Genomics (Padua, Italy), using Illumina technology and the same primers as for the PCR. All nucleotide sequences obtained in this study were compared to those already existing in the GenBank (NCBI) public database for similar genes/regions using the Basic Local Alignment Search Tool (BLASTn) program [25]. In order to correctly choose the identified taxa available in public database, nucleotide sequences of fungi from uncultured/unidentified samples and those with questionable identification were excluded.

2.4. Phylogenetic Investigations

The ITS region nucleotide sequences from this study along with other ITS sequences of the same species downloaded from the GenBank (NCBI) were used in phylogenetic investigations. Only three/four nucleotide sequences/species were carefully chosen with a very high identity (99–100%).

Nucleotide sequences obtained in this study along with those retrieved from the GenBank database (Table 1) were aligned by ClustalW multiple sequence alignment program (http://www.ebi.ac.uk/clustalw) [26–28] followed by manual correction for same length adjustment. The phylogenetic analyses were performed in the MEGA-X phylogeny package [29].

Table 1. List of the species, isolates/strains and their GenBank accession number downloaded from the NCBI database and used in the phylogenetic analysis.

Taxon	Isolate/Strain	Acc. No. (ITS/TUB2 *)	Isolation Source as Described in GenBank	Country	Year [a]
Parengyondontium album	R33	MK513850	limestone walls of old Cathedral Coimbra UNESCO World Heritage Site	Portugal	2019
-"-	1111ARD4D1	LN808868	wall of tourist grottoes "Cueva de Ardales"	Spain	2015
-"-	MC_A31	HQ115665	wall after water damage	Austria	2011
-"-	CBS 368. 72	MH860502	not mentioned	Romania	2020
Alternaria alternata	A63	MH042810	wall paintings in cave temples in grottoes	China	2018
-"-	A70	MH042815	wall paintings in cave temples in grottoes	China	2018
-"-	MF18_15	MK367425	deteriorated walls from the 11th c. St. Sophia Cathedral	Ukraine	2019

Table 1. *Cont.*

Taxon	Isolate/Strain	Acc. No. (ITS/TUB2 *)	Isolation Source as Described in GenBank	Country	Year [a]
-"-	R33	MK513820	limestone walls of old Cathedral Coimbra UNESCO World Heritage Site	Portugal	2019
Cladosporium cladosporioides	A42	MH042761	wall paintings in cave temples in grottoes	China	2018
-"-	A60	MH042807	wall paintings in cave temples in grottoes	China	2018
-"-	A64	MH042811	wall paintings in cave temples in grottoes	China	2018
Lecanicillium psalliotae	KYK00165	AB360367	soil	Japan	2014
-"-	KYK00175	AB360364	not mentioned	Japan	2014
-"-	TZT-18-37	MH922821	not mentioned	China	2018
Meyerozyma guilliermondii	BOEFB3000m	MH671320	cultural heritage conservation facility	Serbia	2018
-"-	JY 45	KM014587	continental shelf sediments	India	2014
-"-	MAS-63	MG846135	soil	India	2018
Botryotrichum atrogriseum *	CBS 130.28	KX976931	dung of rabbit	Netherlands	2017
-"-	CBS 604.69	KX976932	corn field soil	Canada	2017
-"-	CCF 5752	LR584034	air in the restroom	USA	2019
Subramaniula thielavioides **	CBS 122.78	KP900708	not mentioned	Netherlands	2015

Note: * The β-tubulin (TUB2) partial gene was amplified only for this fungal species. ** This fungal species was used as an outgroup only for the phylogenetic analysis involving *B. atrogriseum* isolates. [a] The year refers to the publication year found in the NCBI GenBank database for the respective nucleotide sequence.

In case of the phylogenetic analysis of ITS region, the final dataset had 50 nucleotide sequences with 403 positions. All sites were treated equally for the analysis and gaps were treated as missing data. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated (complete deletion option).The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model [30]. In order to test the phylogenetic tree robustness statistical verification by bootstrap analysis (1000 replicates) was performed [31].

The β-tubulin sequences from this study along with three similar sequences downloaded from the NCBI database, were processed in a separate phylogenetic analysis similarly to the ITS [29–31] with the only difference that the final nucleotide sequences alignment had seven nucleotide sequences and there were a total of 401 positions in the final dataset. The nucleotide sequence (Acc. no. KP900708) of *Subramaniula thielavioides* (Arx, Mukerji and N. Singh), strain CBS 122.78, a closer species from the *Chaetomiaceae*, was chosen as an outgroup.

3. Results

3.1. Fungi Isolation and Morphological Characterization

A total number of 36 pure fungal culture isolates were obtained on PDA media from the two rupestrian churches in Matera as shown in Table 2. The observation of morphological features by light microscopy allowed the identification of six fungal genera (Table 2).

Table 2. Fungi recovered from the two rupestrian churches with their phylogenetic resemblance and GenBank accession numbers.

Isolate	Closest Species	Length (bp) [a]	Sequence Similarity (% Identity)	GenBank Acc. No.
A5_1	*Parengyondontium album*	575	99.83	LR778136
A5_2	-"-	575	99.83	LR778137
A5_3	-"-	575	99.83	LR778138
A5_4	-"-	575	99.83	LR778139
S2_9_1	-"-	589	100	LR782547
S2_9_2	-"-	589	100	LR782548
S2_9_3	-"-	589	100	LR782549
L5_1	-"-	578	100	LR778170
L5_2	-"-	578	100	LR778171
L5_3	-"-	578	100	LR778172
L5_4	-"-	578	100	LR778173
D2_1	*Alternaria alternata*	543	100	LR778167
D2_2	-"-	543	100	LR778168
D2_3	-"-	543	100	LR778169
F2_1	-"-	547	100	LR778181
F2_2	-"-	547	100	LR778182
F2_3	-"-	547	100	LR778183
F2_4	-"-	547	100	LR778184
G2_1	-"-	548	100	LR778186
G2_2	-"-	548	100	LR778187
G2_3	-"-	548	100	LR778188
G2_4	-"-	548	100	LR778189
H2_1	-"-	560	100	LR778214
H2_2	-"-	560	100	LR778215
I5_1	*Cladosporium cladosporioides*	531	100	LR778218
I5_2	-"-	531	100	LR778219
I5_3	-"-	531	100	LR778220
I5_4	-"-	531	100	LR778221
K5_1	*Lecanicillium psalliotae*	586	100	LR778251
K5_2	-"-	586	100	LR778252
O4_1	*Meyerozyma guilliermondii*	565	100	LR794849
O4_2	-"-	565	100	LR794850
O4_3	-"-	565	100	LR794851
2Ipg_1	*Botryotrichum atrogriseum*	452	98.99	LR794852
2Ipg_2	-"-	452	98.99	LR794853
2Ipg_3	-"-	452	98.99	LR794854

[a] Number showing the length of the PCR products (base pairs) obtained in this study, sequenced and further analyzed.

3.2. Molecular Identification of the Fungal Species

The PCR performed with the ITS primers produced a single amplicon of 531 to 589 bp in size. Instead, PCR employing Bt primers, yielded a unique amplicon of 452 bp (Table 2).

The ITS sequences, from this study, after comparison with those present in the GenBank (NCBI), showed a 99–100% sequence identity with the ITS sequences belonging to the following species: *Parengyodontium album* (Limber) C.C. Tsang, J.F.W. Chan, W.M. Pong, J.H.K. Chen, A.H.Y. Ngan, Cheung, C.K.C. Lai, D.N.C. Tsang, S.K.P. Lau, P.C.Y. Woo; *Alternaria alternata* (Fr.) Keissl.; *Cladosporium cladosporioides* (Fresen.) G.A. de Vries; *Lecanicillium psalliotae* (Treschew) Zare and W. Gams.; *Meyerozyma guilliermondii* (Wick.) Kurtzman and M. Suzuki.

The β-tubulin nucleotide sequences from this study had a 98.99% sequence identity with the same gene belonging to *Botryotrichum atrogriseum* J.F.H. Beyma species (accession numbers are presented in Table 1).

DNA sequence information for all fungal isolates generated from the present study was deposited in the European Nucleotide Archives (ENA; https://www.ebi.ac.uk/ena) of the EMBL database (Table 2).

3.3. Phylogenetic Identification of the Fungal Species

Phylogenetic trees based on ITS nucleotide sequence data constructed with both distance and maximum parsimony (MP) using the Mega-X phylogeny package gave identical results. Thus, only the phylogenetic tree based on Maximum Likelihood (ML) analysis will be presented.

In particular, the unrooted ML tree showing the phylogenetic position of the ITS rRNA gene sequences, retrieved from the internal walls and frescoes of the two rupestrian churches in Matera, in relation to their closest type species is shown in Figure 2.

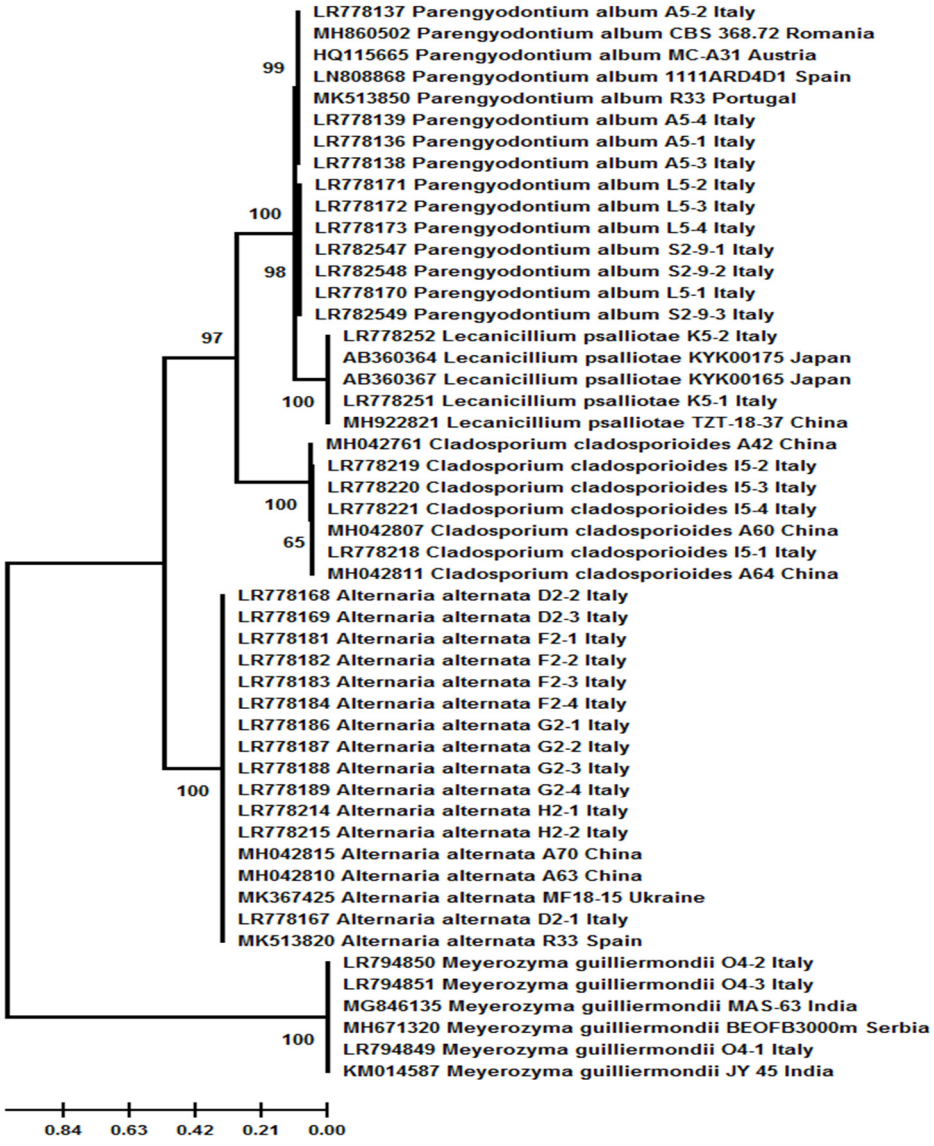

Figure 2. Phylogenetic tree based on Maximum Likelihood method and Kimura 2-parameter model, analyzed for partial Internal Transcribed Spacer (ITS) sequence (total of 403 positions in the final dataset) of samples from two rupestrian churches in Matera identified in this study and closely related sequences downloaded from the NCBI database. The tree with the highest log likelihood (−2108.93) is shown. Statistical evaluation of the tree topology was performed by bootstrap analysis (bootstrap values are reported as a percentage of 1000 replications). Bootstrap values > 50% were indicated at the nodes. The scale bar indicates the number of expected changes/site.

All identified isolates of a given genus closely affiliated (bootstrap value of 100%) to the species already present in the GenBank database (Table 1) are simultaneously well separated from all other analyzed genera (Figure 2).

Phylogenetic analysis based on β-tubulin gene, demonstrated that the three isolates of *B. atrogriseum* from this study are closely related to the same species already present in the GenBank databasegrouping all in the same clade (Figure 3). In addition, the three *B. atrogriseum* isolates, originated from the rupestrian churches of Matera, clustered in the same subclade with an excellent bootstrap value of 99%. They were also clustering with one of the *B. atrogriseum* CCF-5752 downloaded from the GenBank with relatively high (90%) bootstrap support.

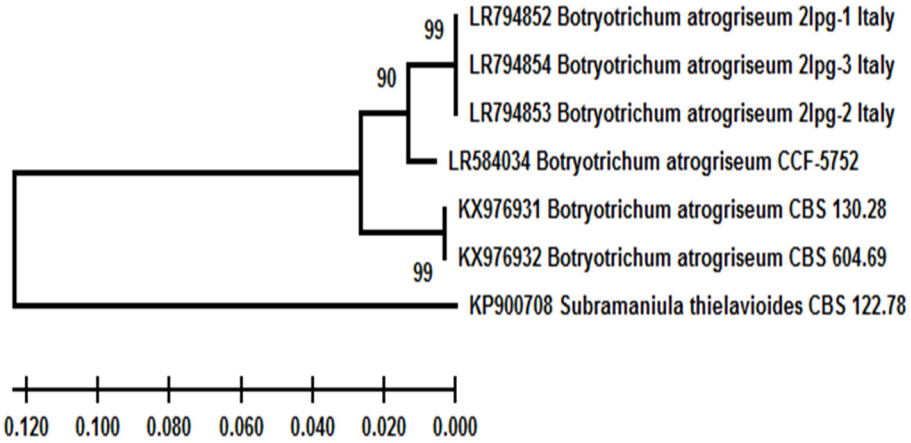

Figure 3. Phylogenetic tree based on Maximum Likelihood method and Kimura 2-parameter model, analyzed for partial β-tubulin sequence (total of 401 positions in the final dataset) of samples from two rupestrian churches in Matera identified in this study and closely related sequences downloaded from the NCBI database. The tree with the highest log likelihood (−926.30) is shown. Statistical evaluation of the tree topology was performed by bootstrap analysis (bootstrap values are reported as percentage of 1000 replications). Bootstrap values >70% were indicated at the nodes. The scale bar indicates the number of expected changes/site.

4. Discussion

It is well known that the abiotic (wind, water, ice, thermal oscillations etc.) and biotic factors (e.g., fungi, bacteria, algae, lichens) can cause structural and esthetic damage to the historical monuments matrix exhibited as degradation, deterioration and decay phenomena [32–34].

More precisely, fungi are frequently associated to biodeterioration of stone monuments [35–37]. They are responsible for various modifications of the substrate observed as discolorations, exfoliations, loss of material, bioweathering, surface deposits, etc. due to acid corrosion, enzymatic degradation and mechanical attack [33,35,36]. Several genera, like *Alternaria*, *Cladosporium*, *Verticillium*, *Penicillium*, *Engyondontium* are commonly linked to biodeterioration of cultural and historic heritage [6,38–41]. Therefore, identifying the main features of these fungi, assess their nutritional needs and collect information related to their growth and development will help to select future specific protocols to be applied in order to contrast any present degradation process or prevent a forthcoming one.

Furthermore, indoor mycoflora can have negative effects on human health causing respiratory illnesses, keratosis, and pulmonary diseases especially in immune-suppressed patients [42,43]. The presence of fungal spores in elevated concentrations inside of edifices increases the risk to human health. One of the most often fungal genera linked with allergic asthma and allergic rhinitis are *Alternaria* and *Cladosporium* [44,45] which were both found in our study.

A. alternata is a very common fungal species widely present on different substrates and environments [9,34,35]. This species was extensively found in cultural and historical heritage sites associated with substrate alterations like discolorations [6,14,46,47]. Furthermore, *A. alternata* spores are recognized as being very common powerful aeroallergens [48,49]. Usually intense exposure to *A. alternata* allergens happens in the open-air. However, this fungus is very often found in indoor environments where the level of exposure to aeroallergen increases. In addition, human exposure to

fungal spores of *A. alternata* has strongly been associated with increased asthma severity [44,50,51]. Thus, the presence of *A. alternata* may be considered as a potential threat for the health of guardians and visitors in cultural heritage sites.

Previous studies by Gutarowska et al. [18], who reported the presence of *P. album* from cultural heritage assets, are in agreement with the outcomes from this study. Fungi from genus *Engyondontium* (*Parengyondontium*) are linked to biodegradation phenomena [18,52] observed on different matrices and also connected to human health like fungaemia in immunocompromised patients [53] endocarditis [54], brain abscess [55] or keratitis [56].

C. cladosporioides, another fungal species isolated from the two investigated sites in Matera, is a common microbial agent on stone monuments and often associated with the accelerated degradation process of ancient wall painting discoloration [4–6,18]. Exposure of humans to *Cladosporium* spores is also reported to be linked to allergic reactions [57].

Results from this study, reporting the presence of the *L. psalliotae* species are in accordance with the research of Sareela et al. [4] who already described *L. aranerarum* (another species of genus *Lecanicillium*) from marble surface, frescoes and biofilm of Roman catacombs of St. Domitilla and St. Callistus in Rome.

M. guilliermondii was also detected from both walls and frescoes in this study. To our knowledge, there are no reported studies on *M. guilliermondii* from cultural heritage monuments. Maciel et al. [58] revealed the *M. guilliermondii* involvement in human candidose disease. The same fungus was reported by Corte and co-workers to be present on food and other environmental niches [59]. Its diversity was investigated and also some clues for its speciation were provided. Furthermore, very recently De Marco et al. [60] investigated the genomes of another *Meyerozyma* species and presented new and significant data regarding the *M. guilliermondii* species-complex.

The recent work by Wang et al. [61] revealed few different species of *Botryotrichum B. piluliferum* (originated from walls), *B. murorum* (isolated from ceiling tiles) and *B. peruvianum* (isolated from walls) while *B. atrogriserum* examined in their study originated even from corn field soil, dung of rabbit or mountains but not from walls.

The results from this study report the presence of *B. atrogriseum* on interior walls and frescoes from the two rupestrian churches in Matera.

Moreover, some fungal species identified in this study, *A. alternata*, *C. cladosporioides* and *P. album*, were previously associated with walls [4,18,41,52]. To our knowledge, there are no reported studies on *M. guilliermondii*, *B. atrogriseum* and *L. psalliotae* from cultural heritage monuments. However, a close relative of *L. psalliotae* and *B. atrogriseum* namely *L. aranerarum* and *B. piluliferum*, and *B. peruvianum* were showed to originate from wall samples [61].

The *Chaetomium* genus, that *B. atrogriseum* also belongs to, is known to produce potentially harmful metabolites which may have an effect on the health of the visitors and curators, therefore its investigation is of great importance [62,63].

In order to avoid problems for human health, due to spores spread [64], it would be appropriate to improve the air quality inside heritage sites through the use of air-filters or by controlling fungal presence. Besides, a seasonal variation of fungal populations may occur over time and consequently the risks for human health can vary [65,66].

The control of fungi in cultural heritage assets is an important issue which was investigated using various ways to limit their development and growth such as the use of natural substances employed as green conservation strategies and the utilization of engineered nanomaterials [67–69].

Results from this preliminary study contributed to furnish new and useful knowledge about the presence, morphology and molecular characterization of fungi found on the walls and frescoes of two rupestrian churches of Matera. Considering the information obtained here about these fungi, along with future programmed culture-independent studies and investigations concerning their implication in the degradation of the two cultural heritage sites and their effects on the health of guardians and visitors, it will be possible to start a concrete planned and preventive conservation

strategy of the mural paintings from the two stone churches. Therefore, important actions like mural paintings "cleaning", capable of getting rid of the revealed biological patina without having to kill the fungi and then "biocidal" treatments intended to eliminate them, should be undertaken.

Author Contributions: Conceptualization, I.C., L.S. and S.M.M.; methodology, L.S. and S.M.M.; software, S.M.M.; validation, S.M.M., I.C. and L.S.; investigation, L.S., S.M.M., I.C.; data curation, S.M.M., I.C. and L.S.; writing—original draft preparation, S.M.M.; writing—review and editing, S.M.M., I.C. and L.S.; supervision, I.C. and L.S.; project administration, L.S.; funding acquisition, L.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministero dell'Università e della Ricerca (Italy), grant number SNC 00520, project: Smart Cities—Product and Process Innovation for Maintenance, Preservation and Sustainable Programmed Restoration of Cultural Heritage.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Scheerer, S.; Ortega-Morales, B.O.; Gaylarde, C.C. Chapter 5 Microbial Deterioration of Stone Monuments—An Updated Overview. *Adv. Appl. Microbiol.* **2009**, *66*, 97–139. [CrossRef]
2. Warscheid, T.; Braams, J. Biodeterioration of stone: A review. *Int. Biodeterior. Biodegrad.* **2000**, *46*, 343–368. [CrossRef]
3. Karbowska-Berent, J. Microbiodeterioration of mural paintings: A review. In *Art, Biology and Conservation: Biodeterioration of Works of Art*; Koestler, R.J., Koestler, V.H., Charola, A.E., Nieto-Fernanadez, F.E., Eds.; Metropolitan Museum of Art: New York, NY, USA, 2003; pp. 266–302.
4. Saarela, M.; Alakomi, H.-L.; Suihko, M.-L.; Maunuksela, L.; Raaska, L.; Mattila-Sandholm, T. Heterotrophic microorganisms in air and biofilm smaples from Roman catacombs, with special emphasis on actinobacteria and fungi. *Int. Biodeterior. Biodegrad.* **2004**, *54*, 27–37. [CrossRef]
5. Saiz-Jimenez, C. Microbiological and environmental issues in show caves. *World J. Microbiol. Biotechnol.* **2012**, *28*, 2453–2464. [CrossRef] [PubMed]
6. Sterflinger, K.; Piñar, G. Microbial deterioration of cultural heritage and works of art—Tilting at windmills? *Appl. Microbiol. Biotechnol.* **2013**, *97*, 9637–9646. [CrossRef] [PubMed]
7. Rivera, L.E.C.; Ramos, A.P.; Sánchez, J.I.C.; Serrano, M.E.D. Origin and Control Strategies of Biofilms in the Cultural Heritage. In *Antimicrobials, Antibiotric Resistance, Antibiofilm Strategies and Activity Methods*; Kirmusaoğlu, S., Ed.; Intech Open Science: London, UK, 2018. Available online: https://www.intehopen.com/books/antimicrobials-antibiotic-resistance-antibiofilm-strategies-and-activity-methods/origin-and-control-strategies-of-biofilms-in-the-cultural-heritage (accessed on 16 May 2020). [CrossRef]
8. Ponizovskaya, V.B.; Rebrikova, N.L.; Kachalkin, A.V.; Antropova, A.B.; Bilanenko, E.N.; Mokeeva, V.L. Micromycetes as colonizers of mineral building materials in historic monuments and museums. *Fungal Biol.* **2019**, *123*, 290–306. [CrossRef] [PubMed]
9. Singh, J.; Singh, J. *Building Mycology: Management of Decay and Health in Buildings*; Chapman & Hall: London, UK, 1994; pp. 1–269.
10. Bornehag, C.G.; Blomquist, G.; Gyntelberg, F.; Järvholm, B.; Malmberg, P.; Nordvall, L.; Nielsen, A.; Pershagen, G.; Sundell, J. Dampness in Buildings and Health. Nordic Interdisciplinary Review of the Scientific Evidence on Associations between Exposure to "Dampness" in Buildings and Health Effects (NORDDAMP). *Indoor Air* **2001**, *11*, 72–86. [CrossRef]
11. Baxi, S.N.; Portnoy, J.M.; Larenas-Linnemann, D.; Phipatanakul, W.; Barnes, C.; Grimes, C.; Horner, W.E.; Kennedy, K.; Levetin, E.; Miller, J.D.; et al. Exposure and Health Effects of Fungi on Humans. *J. Allergy Clin. Immunol. Pract.* **2016**, *4*, 396–404. [CrossRef]
12. Pettigrew, H.D.; Selmi, C.; Teuber, S.S.; Gershwin, M.E. Mold and Human Health: Separating the Wheat from the Chaff. *Clin. Rev. Allergy Immunol.* **2009**, *38*, 148–155. [CrossRef]
13. Karakasidou, K.; Nikolouli, K.; Amoutzias, G.D.; Pournou, A.; Manassis, C.; Tsiamis, G.; Mossialos, D. Microbial diversity in biodeteriorated Greek historical documents dating back to the 19th and 20th century: A case study. *MicrobiologyOpen* **2018**, *7*, e00596. [CrossRef]

14. Pinheiro, A.C.; Mesquita, N.; Trovão, J.; Soares, F.; Tiago, I.; Coelho, C.; De Carvalho, H.P.; Gil, F.; Catarino, L.; Piñar, G.; et al. Limestone biodeterioration: A review on the Portuguese cultural heritage scenario. *J. Cult. Herit.* **2019**, *36*, 275–285. [CrossRef]
15. Gonzales, J.M. Overview on existing molecular techniques of potential interest in cultural heritage. In *Molecular Biology and Cultural Heritage*; Gonzales-Jimenez, C., Ed.; Swets & Zeitlinger B.V. Publishers: Lisse, The Netherlands, 2003.
16. Adamiak, J.; Otlewska, A.; Tafer, H.; Lopandic', K.; Gutarowska, B.; Sterflinger, K.; Piñar, G. First evaluation of the microbiome of built cultural heritage by using the Ion Torrent next generation sequencing platform. *Int. Biodeterior. Biodegrad.* **2018**, *131*, 11–18. [CrossRef]
17. Grottoli, A.; Beccaccioli, M.; Zoppis, E.; Fratini, R.S.; Schifano, E.; Santarelli, M.L.; Uccelletti, D.; Reverberi, M. Nanopore Sequencing and Bioinformatics for Rapidly Identifying Cultural Heritage Spoilage Microorganisms. *Front. Mater.* **2020**, *7*, 14. [CrossRef]
18. Gutarowska, B.; Celikkol-Aydin, S.; Bonifay, V.; Otlewska, A.; Aydin, E.; Oldham, A.L.; Brauer, J.I.; Duncan, K.E.; Adamiak, J.; Sunner, J.A.; et al. Metabolomic and high-throughput sequencing analysis—Modern approach for the assessment of biodeterioration of materials from historic buildings. *Front. Microbiol.* **2015**, *6*, 979. [CrossRef] [PubMed]
19. Trovão, J.; Gil, F.; Catarino, L.; Soares, F.; Tiago, I.; Portugal, A. Analysis of fungal deterioration phenomena in the first Portuguese King tomb using a multi-analytical approach. *Int. Biodeterior. Biodegrad.* **2020**, *149*, 104933. [CrossRef]
20. Zhang, X.; Ge, Q.; Zhu, Z.; Deng, Y.; Gu, J.-D. Microbiological community of the Royal Palace in Angkor Thom and Beng Mealea of Cambodia by Illumina sequencing based on 16S rRNA gene. *Int. Biodeterior. Biodegrad.* **2018**, *134*, 127–135. [CrossRef]
21. Duan, Y.; Wu, F.; Wang, W.; Gu, J.-D.; Li, Y.; Feng, H.; Chen, T.; Liu, G.; An, L.-Z. Differences of Microbial Community on the wall paintings preserved in situ and ex situ of the Tiantishan Grottoes, China. *Int. Biodeterior. Biodegrad.* **2018**, *132*, 102–113. [CrossRef]
22. Caneva, G.; Bartoli, F.; Fontani, M.; Mazzeschi, D.; Visca, P. Changes in biodeterioration patterns of mural paintings: Multi-temporal mapping for a preventive conservation strategy in the Crypt of the Original Sin (Matera, Italy). *J. Cult. Herit.* **2019**, *40*, 59–68. [CrossRef]
23. White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In *PCR Protoc.*; Innis, M.A., White, T.J., Sninsky, J.J., Gelfand, D.H., Eds.; Academic Press Inc.: New York, NY, USA, 1990; pp. 315–322.
24. Glass, N.L.; Donaldson, G.C. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* **1995**, *61*, 1323–1330. [CrossRef]
25. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, J.D. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403–410. [CrossRef]
26. Madeira, F.; Park, Y.; Lee, J.; Buso, N.; Gur, T.; Madhusoodanan, N.; Basutkar, P.; Tivey, A.R.N.; Potter, S.C.; Finn, R.D.; et al. The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Res.* **2019**, *47*, W636–W641. [CrossRef] [PubMed]
27. CLUSTAL. Available online: http://www.ebi.ac.uk/clustalw (accessed on 23 April 2020).
28. Larkin, M.; Blackshields, G.; Brown, N.P.; Chenna, R.; Mcgettigan, P.; McWilliam, H.; Valentin, F.; Wallace, I.; Wilm, A.; López, R.; et al. Clustal W and Clustal X version 2.0. *Bioinformatics* **2007**, *23*, 2947–2948. [CrossRef] [PubMed]
29. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef]
30. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **1980**, *16*, 111–120. [CrossRef] [PubMed]
31. Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **1985**, *39*, 783–791. [CrossRef]
32. Gaylarde, C.C.; Morton, L.H.G. Deteriogenic biofilms on buildings and their control: A review. *Biofouling* **1999**, *14*, 59–74. [CrossRef]
33. Dakal, T.C.; Cameotra, S.S. Microbially induced deterioration of architectural heritages: Routes and mechanisms involved. *Environ. Sci. Eur.* **2012**, *24*, 36. [CrossRef]

34. Jain, A.; Bhadauria, S.; Kumar, V.; Chauhan, R.S. Biodeterioration of sandstone under the influence of different humidity levels in laboratory conditions. *Build. Environ.* **2009**, *44*, 1276–1284. [CrossRef]
35. Braams, J. Ecological Studies on the Fungal Microflora Inhabiting Historical Sandstone Monuments. Ph.D. Thesis, University of Oldenburg, Oldenbury, Germany, 1992.
36. Grbić, M.L.; Vukojevic, J. Role of fungi in biodeterioration process of stone in historic buildings. *Zb. Matice Srp. Prir. Nauk.* **2009**, 245–251. [CrossRef]
37. Salvadori, O.; Municchia, A.C. The Role of Fungi and Lichens in the Biodeterioration of Stone Monuments. *Open Conf. Proc. J.* **2016**, *7*, 39–54. [CrossRef]
38. Mazzoli, R.; Giuffrida, M.G.; Pessione, E. Back to the past: "Find the guilty bug—microorganisms involved in the biodeterioration of archeological and historical artifacts". *Appl. Microbiol. Biotechnol.* **2018**, *102*, 6393–6407. [CrossRef] [PubMed]
39. Di Carlo, E.; Chisesi, R.; Barresi, G.; Barbaro, S.; Lombardo, G.; Rotolo, V.; Sebastianelli, M.; Travagliato, G.; Palla, F. Fungi and Bacteria in Indoor Cultural Heritage Environments: Microbial-related Risks for Artworks and Human Health. *Environ. Ecol. Res.* **2016**, *4*, 257–264. [CrossRef]
40. Abdel-Ghany, T.M.; Omar, A.; Elwkeel, F.M.; Al Abboud, M.A.; AlAwlaqi, M. Fungal deterioration of limestone false-door monument. *Heliyon* **2019**, *5*, e02673. [CrossRef] [PubMed]
41. Jurado, V.; Sanchez-Moral, S.; Saiz-Jimenez, C. Entomogenous fungi and the conservation of the cultural heritage: A review. *Int. Biodeterior. Biodegrad.* **2008**, *62*, 325–330. [CrossRef]
42. Pulimood, T.B.; Corden, J.M.; Bryden, C.; Sharples, L.; Nasser, S.M. Epidemic asthma and the role of the fungal mold *Alternaria alternata*. *J. Allergy Clin. Immunol.* **2007**, *120*, 610–617. [CrossRef]
43. Peat, J.K.; Dickerson, J.; Li, J. Effects of damp and mould in the home on respiratory health: A review of the literature. *Allergy* **1998**, *53*, 120–128. [CrossRef] [PubMed]
44. Crameri, R.; Garbani, M.; Rhyner, C.; Huitema, C. Fungi: The neglected allergenic sources. *Allergy* **2013**, *69*, 176–185. [CrossRef]
45. Simon-Nobbe, B.; Denk, U.; Pöll, V.; Rid, R.; Breitenbach, M. The Spectrum of Fungal Allergy. *Int. Arch. Allergy Immunol.* **2007**, *145*, 58–86. [CrossRef]
46. Ma, Y.; Zhang, H.; Du, Y.; Tian, T.; Xiang, T.; Liu, X.; Wu, F.; An, L.; Wang, W.; Gu, J.-D.; et al. The community distribution of bacteria and fungi on ancient wall paintings of the Mogao Grottoes. *Sci. Rep.* **2015**, *5*, 7752. [CrossRef]
47. Mohammadi, P.; Maghboli-Balasjin, N. Isolation and molecular identification of deteriorating fungi from Cyrus the Great tomb stones. *Iran. J. Microbiol.* **2014**, *6*, 361–370.
48. Kustrzeba-Wojcicka, I.; Siwak, E.; Terlecki, G.; Wolanczyk-Medrala, A.; Medrala, W. *Alternaria alternata* and Its Allergens: A Comprehensive Review. *Clin. Rev. Allergy Immunol.* **2014**, *47*, 354–365. [CrossRef] [PubMed]
49. Habibi, A.; Safaiefarahani, B. Indoor damp surfaces harbor molds with clinical significance. *Curr. Med. Mycol.* **2018**, *4*, 1–9. [CrossRef] [PubMed]
50. De Ana, S.G.; Torres-Rodríguez, J.M.; Ramírez, E.A.; García, S.M.; Belmonte-Soler, J. Seasonal distribution of *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium* species isolated in homes of fungal allergic patients. *J. Investig. Allergol. Clin. Immunol.* **2006**, *16*, 357–363. [PubMed]
51. Gabriel, M.; Postigo, I.; Tomaz, C.; Martinez, J. *Alternaria alternata* allergens: Markers of exposure, phylogeny and risk of fungi-induced respiratory allergy. *Environ. Int.* **2016**, *89*, 71–80. [CrossRef]
52. Berner, M.; Wanner, G.; Lubitz, W. A comparative study of the fungal flora present in medieval wall paintings in the chapel of the castle Herberstein and in the parish church of St Georgen in Styria, Austria. *Int. Biodeterior. Biodegrad.* **1997**, *40*, 53–61. [CrossRef]
53. Macêdo, D.P.C.; Neves, R.P.; De Souza-Motta, C.M.; Magalhães, O.M.C. Engyodontium album fungaemia: The first reported case. *Braz. J. Microbiol.* **2007**, *38*, 110–112. [CrossRef]
54. Augustinsky, J.; Kammeyer, P.; Husain, A.; DeHoog, G.S.; Libertin, C.R. *Engyodontium album* endocarditis. *J. Clin. Microbiol.* **1990**, *28*, 1479–1481. [CrossRef]
55. Seeliger, H.P. Infections of man by opportunistic molds—their identification and nomenclature of their diseases. *Mykosen* **1983**, *26*, 587–598. [CrossRef]
56. McDonnell, P.J.; Werblin, T.P.; Sigler, L.; Green, W.R. Mycotic keratitis due to *Beauveria alba*. *Cornea* **1984**, *3*, 213–216. [CrossRef]
57. Hasnain, S.M.; Al-Frayh, A.S.; Al-Suwaine, A.; Gad-El-Rab, M.O.; Fatima, K.; Al-Sedairy, S. *Cladosporium* and respiratory allergy: Diagnostic implications in Saudi Arabia. *Mycopathologia* **2004**, *157*, 171–179. [CrossRef]

58. Maciel, N.O.; Johann, S.; Brandão, L.R.; Kucharíková, S.; Morais, C.G.; Oliveira, A.P.; Freitas, G.J.; Borelli, B.M.; Pellizzari, F.M.; Santos, D.A.; et al. Occurrence, antifungal susceptibility, and virulence factors of opportunistic yeasts isolated from Brazilian beaches. *Mem. Inst. Oswaldo Cruz* **2019**, *114*, e180566. [CrossRef] [PubMed]
59. Corte, L.; di Cagno, R.; Groenewald, M.; Roscini, L.; Colabella, C.; Gobbetti, M.; Cardinali, G. Phenotypic and molecular diversity of *Myerozyma guilliermondii* strains isolated from food and other environmental niches, hints for an incipient speciation. *Food Microbiol.* **2015**, *48*, 206–215. [CrossRef] [PubMed]
60. De Marco, L.; Epis, S.; Capone, A.; Martín, E.; Bozic, J.; Crotti, E.; Ricci, I.; Sassera, D. The Genomes of Four *Meyerozyma caribbica* Isolates and Novel Insights into the *Meyerozyma guilliermondii* Species Complex. *G3 Genes Genomes Genet.* **2018**, *8*, 755–759. [CrossRef] [PubMed]
61. Wang, X.; Houbraken, J.; Groenewald, J.; Meijer, M.; Andersen, B.; Nielsen, K.; Crous, P.; Samson, R.A. Diversity and taxonomy of *Chaetomium* and chaetomium-like fungi from indoor environments. *Stud. Mycol.* **2016**, *84*, 145–224. [CrossRef] [PubMed]
62. Miller, J.D.; McMullin, D.R. Fungal secondary metabolites as harmful indoor air contaminants: 10 years on. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 9953–9966. [CrossRef] [PubMed]
63. Došen, I.; Nielsen, K.F.; Clausen, G.; Andersen, B. Potentially harmful secondary metabolites produced by indoor *Chaetomium* species on artificially and naturally contaminated building materials. *Indoor Air* **2016**, *27*, 34–46. [CrossRef]
64. Lin, W.-R.; Chen, Y.-H.; Lee, M.-F.; Hsu, L.-Y.; Tien, C.-J.; Shih, F.-M.; Hsiao, S.-C.; Wang, P.-H. Does Spore Count Matter in Fungal Allergy? The Role of Allergenic Fungal Species. *Allergy Asthma Immunol. Res.* **2016**, *8*, 404–411. [CrossRef]
65. Medrela-Kuder, E. Seasonal variations in the occurrence of culturable airborne fungi in outdoor and in indoor air in Craćow. *Int. Biodeterior. Biodegrad.* **2003**, *52*, 203–205. [CrossRef]
66. Sharma, K. Seasonal variation and ecological study on fungi in relation to biodeterioration. *Recent Res. Sci. Technol.* **2012**, *4*, 6–8.
67. Caneva, G.; Tescari, M. Stone biodeterioration: Treatments and preventive conservation. In Proceedings of the International Symposium on Stone Conservation, Conservation Technologies fort Stone Cultural Heritages: Status and Future Prospects, Seoul, Korea, 1 September 2017; pp. 95–114.
68. Caneva, G.; Fidanza, M.R.; Tonon, C.; Favero-Longo, S.E. Biodeterioration Patterns and Their Interpretation for Potential Applications to Stone Conservation: A Hypothesis from Allelopathic Inhibitory Effects of Lichens on the *Caestia* Pyramid (Rome). *Sustainability* **2020**, *12*, 1132. [CrossRef]
69. Palla, F.; Bruno, M.; Mercurio, F.; Tantillo, A.; Rotolo, V. Essential Oils as Natural Biocides in Conservation of Cultural Heritage. *Molecules* **2020**, *25*, 730. [CrossRef] [PubMed]