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Effect of Environmental Factors on Rock-inhabiting Fungal Communities from Brazilian Soapstone Samples

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Abstract: The present study aims to evaluate the environmental effect on fungal community composition associated with biodeterioration occurring in stones (soapstone) at two distinct locations in Minas Gerais State, Brazil: Congonhas city and Sanctuary of Caraça. Four collections of fungal communities over one year were obtained from both research sites from the soapstone block surfaces exposed for over two decades. The molecular diversity profile of the fungal community at the two localities was obtained by DGGE (Denaturing Gradient Gel Electrophoresis), and the genomes of the most representative population were sequenced. DGGE showed the formation of two clusters with filamentous fungal communities. Sequencing of the most representative bands revealed the presence of fungi associated with the biodeterioration of soapstone. In addition, many of the identified species were associated with photobionts that could generate lichens, indicating that environmental characteristics affect the occurrence of filamentous fungi, which leads to biodeterioration of stones. Authors' study focused on an environmental variation of an extreme habitat for fungi associated with soapstone in the state of Minas Gerais, Brazil and identified the presence of interesting rock-inhabiting fungal communities including species related to lichens, which can accelerate the deterioration of stones by the production of organic acids.

Key words: Fungal community, biodeterioration, soapstone, cultural heritage, Minas Gerais state.

1. Introduction

Minas Gerais State is among the most representative examples of religious and civil architecture from the Brazilian colonial period; it is so significant that some structures such as the Sanctuary of Senhor Bom Jesus de Matosinhos, Congonhas, are considered a Cultural Heritage of Humanity, by the UNESCO (United Nations Educational, Scientific and Cultural Organization) [1] (Fig. 1A).

Studies on the biodeterioration of buildings exposed to the outdoor environment in Latin America [2] and the historic buildings in Brazil, especially in cities from the State of Minas Gerais, such as Ouro Preto and Tiradentes, have revealed that cyanobacteria and fungi usually constitute the major biomass in these stones [3].

Stones are subject to multiple stress factors such as oligotrophic conditions, variability temperatures, UV-radiation and osmotic stress, and are very hard to colonise [4].

The occurrence of microbial growth on stones is initially an aesthetic problem since the visible microbial growth may alter the appearance of buildings and

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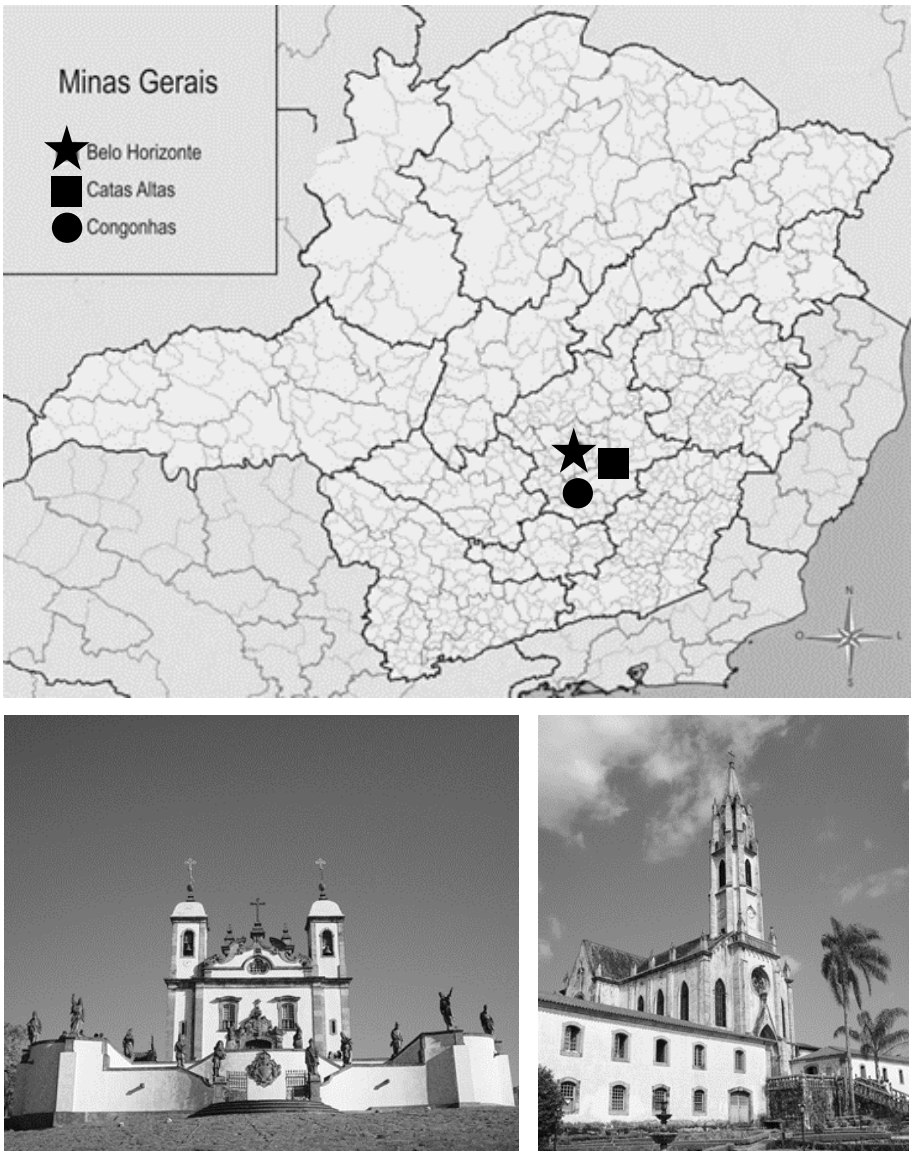

Fig. 1 (A) Map of the State of Minas Gerais, highlighting the cities of study in relation to the city Belo Horizonte, Brazil; (B) Sanctuary of Senhor Bom Jesus de Matosinhos, in Congonhas; (C) Sanctuary of Caraça.

sculptures [5]. However, in many cases, their metabolic activity is the main cause of the deterioration of stones [6] and even overlaps physical weathering [7]. Italian sculptures in marble exposed to the outdoor environment can also have a significant aesthetic problem due to the presence of deteriorating agents, especially microcolonial black fungi [8].

Biological deterioration consists of an undesirable change in the properties of a material, which is caused by the metabolic activity of an organism involved in the biotransfer of chemical elements in biogeochemical cycles [9]. Thus, the biodeterioration process in stones is directly related to the availability of water and nutrients for the microorganisms [6]. Thus, specific material parameters such as porosity and permeability, and the environmental conditions of the architectural ensemble to which they are exposed, are the factors that will determine the rate of biodeterioration attack by microorganisms [5].

Among the fungal groups, the RIF (Rock-Inhabiting Fungi) have been studied because of their capacity to survive on stone surfaces, which are unique habitats

where rapid changes in radiation, temperature, water and nutrients represent a challenge to microbial survival in different environments throughout the world [10]. Originally, RIF have been found in stones from the Atacama Desert, Chile [11], Antartic Deserts [12], islands of the Antarctic Peninsula [13], Australian Alps [14] and historic monuments in the Mediterranean area [15].

According to Isola, D., et al. [4], RIF residing on monuments must face sun exposure, prolonged periods of dryness and nutrient deficiency as well as the additional stress of pollution, since monuments are often located in urban environments. They include specific species in the asexual states, small colonies and compact forms showing the presence of melanin, species occurring in hot and cold deserts, and in other temperate ecosystems of the planet [16]. Moreover, RIF are actually among the most destructive groups of investigated microorganisms that cause deterioration of monuments exposed to outdoor conditions [17].

Filamentous fungi may cause both mechanical and chemical deterioration of stone [18]. Mechanical deterioration of stone is achieved via hyphal penetration, which causes breakup and fragmentation [19]. On the other hand, chemical deterioration by filamentous fungi involves damage of mineral materials by various mechanisms such as acid production, changes in tension due to salts, metal complexing activity of biofilms action [20], and chemoorganotrophic metabolism with simple nutritional requirements [21]. They cause aesthetical damages because of melanin production and mechanical destruction leading to exfoliation of stone artwork [22].

Others studies demonstrated the isolation of organic acids producing fungal species such as *Verticillium lecanii*, *Penicillium expansum*, *P. nigricans*, *Aspergillus japonicus*, *A. ochraceus*, *A. puniceus*, *Mucor hiemalis*, and *Fusarium oxysporum* from quartzite and soapstone samples from the cities Congonhas [6], Ouro Preto and Caraça, also in Brazil [23].

In order to study the composition of the fungal community associated with the deterioration process, the authors chose the Sanctuary of Senhor Bom Jesus de Matosinhos and the Architectonical Assemblage of Caraça. The Sanctuary of Senhor Bom Jesus de Matosinhos in Congonhas city, Minas Gerais state, where some of the most famous professionals of the age worked, is located in an industrialised region with many mining companies in its proximity and for this reason, it still presents incipient alterations. The Architectonical Assemblage of Caraça is protected from big cities and industrial plants, and was chosen for this study for its clean air quality as a comparable site in a low pollution area. Furthermore, it is located in a region little affected by negative environmental influences.

Therefore, this study aimed to evaluate the dynamics of the RIF community that causes biodeterioration on exposed outdoor stones and to establish the degree of influence of environmental pollution, temperature variation, wind direction, topography and the type of metabolic stress levels on the dynamic fungal community.

This work discusses the environmental impact on the fungal community that damages stone, making it an extremely relevant tool to conserve and preserve the cultural heritage in the stone architecture of Minas Gerais State. According to the environmental situation described, determination of the factors that initiate the biodeterioration of stones is essential for the preservation of Brazilian cultural heritage.

2. Material and Methods

2.1 Studied Areas

The Sanctuary of Senhor Bom Jesus de Matosinhos is situated on a hill above the town of Congonhas in the State of Minas Gerais and was registered in the UNESCO world cultural heritage list (Fig. 1B). The city of Congonhas is situated in the southwest of the Quadrilátero Ferrífero in the upper São Francisco Basin, at a latitude of 20º29'59" S and a longitude of 43º51'28" W, at a lower altitude than the rest of the

region, between 850 and 1,000 meters. Despite this, its topography is irregular, with hills often showing drops of over 20% and lying mostly in the NW-SE direction, reflecting the schist and granular diorite substrate [24].

In the mountains of Caraça, within a vast natural reserve, the Sanctuary is situated far away from everyday activities; it is a priority region for conservation and was recognised by IPHAN (Instituto do Patrimônio Histórico e Artístico Nacional) in 1955 (Fig. 1C).

The Architectonical Assemblage of Caraça is situated in the east of the Quadrilátero Ferrífero in the middle of the Caraça mountain chain at the edge of the river with the same name, which belongs to the basin of the Doce River, at an altitude of 1,280 meters.

On the floor of this low area, sections with basic stones have been weathered and colonised by species of the semi deciduous seasonal forest. The highest peaks of the Caraça mountain range lie above 600 meters of the Caraça River plain and surround it in a horseshoe shape, with its opening to the northwest [24].

2.2 Object of Study

The stone samples from the Sanctuary of Caraça were exposed outdoors on November 26th, 1993, and were located in two tables on the cover of the art gallery. In this locale, the samples were obtained from three blocks of soapstone. The samples from the Sanctuary of Senhor Bom Jesus de Matosinhos in Congonhas were exposed outdoors on November 20th, 1993 and were also located on two exposure tables on the cover attached to the Basilica Bom Jesus de Matosinhos (Fig. 2).

2.3 Soapstone Characterization

The main mineral component of soapstone is talc, along with variable proportions of magnesium chlorite, carbonates and dolomite. It shows granolepidoblastic texture and grey colour ranging from tonality according to the distribution of constituent minerals. When weathered, it is brownish due to the oxides and hydroxides of iron, and exhibits macroscopic pores and cavities resulting from the leaching of carbonate [25]. The geological specifications are shown in Table 1.

2.4 Sample Collection

The samples were collected during all four seasons of the years 2013 and 2014, aiming to relate the occurrence of fungi with the degree of deterioration in the samples. Collection was performed with a sterile spatula, with the aim of obtaining the pulverised material through the stone surface. The material was then placed in sterile Petri dishes and subsequently closed and transported to the Mycology Laboratory of the Federal University of Minas Gerais for processing.

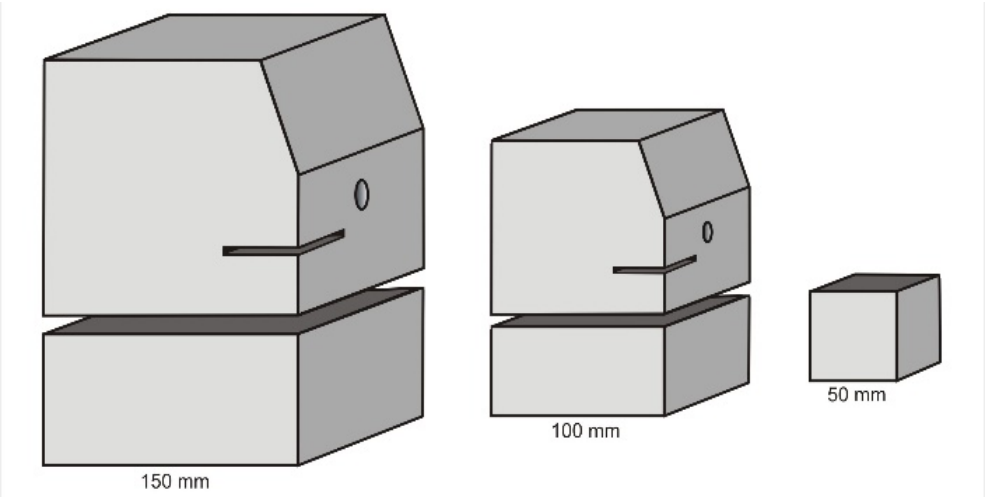

Fig. 2 Schematic drawing of three variable dimensions of the samples in soapstone [1].

Table 1 Specifications of the soapstone blocks on outdoor exposure located in Caraça and the city of Congonhas [1].

Characteristics locality	Caraça	Congonhas
Dimensions blocks (mm)	L: 150 × 150 M: 100 × 100 S: 50 × 50	L: 150 × 150 M: 100 × 100 S: 50 × 50
Area (mm^2)	L: 104,700 M: 46,600 S: 7,500	L: 104,700 M: 46,600 S: 7,500
Lithological composition (%)	Talc: 57.6 Chlorite: 19.2 Dolomite: 20.4	Talc: 55.8 Chlorite: 18 Dolomite: 18.6
Density (g/cm^3)	2.9	2.95
Opacity (%)	2.8	7.6
Ultrasonic pulse (km/s)	3.69	3.67

Legend: L: Large; M: Medium; S: Small.

2.5 Total DNA Extraction

DNA extraction was performed using the Power Soil DNA kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA), following the manufacturer's instructions.

2.6 PCR (Polymerase Chain Reaction) Amplification

The PCR for examining the fungal community was performed using one primer pair for the first reaction and another primer pair for the second reaction. For a final volume of 25 μL, the first reaction used 5 μL of PCR buffer (5 X), 3 μL of $MgCl_2$ (25 mM), 1 μL of dNTPs (10 pmoles), 0.5 μL of NS1 (10 pmoles) and EF3 (10 pmoles) (Invitrogen™, Carlsbad, CA, USA), 5 U/μL of Go*Taq* DNA Polymerase (Promega, Fitchburg, Wiscosin, USA), and 30 ng of template DNA [26, 27]. The first reaction product (0.5 μL) was used as the template for the second PCR, and contained the same reagents in the same proportion, except for the use of primer pair 2, FF390 (10 pmoles) and FR1GC (10 pmoles) (Invitrogen™) [28].

The amplification parameters of the first reaction consisted of 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 47 °C for 1 min, and extension at 72 °C for 2 min. In the first cycle, denaturation was performed at 94 °C for 4 min and in the last cycle, final extension lasted for 10 min at 72 °C. The second reaction consisted of 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 50 °C for 1 min and extension at 72 °C for 1 min. In the first cycle, denaturation was performed at 94 °C for 4 min and in the last cycle, final extension lasted for 10 min at 72 °C.

2.7 DGGE (Denaturing Gradient Gel Electrophoresis)

The amplified products were separated by DGGE [29], using the Dcode Universal Mutation Detection System (BioRad, Richmond, USA), in 8% polyacrylamide gels, prepared with 150 μL of ammonium persulphate (10%) and 18 μL of TEMED (Tetramethylethylenediamine) in 1 X TAE buffer (10 mM tris-acetate, 0.5 mM EDTA, pH 8), with a denaturation gradient of 35-55% urea and formamide. Aliquots of 25 μL PCR products were applied to each slot and the matrix was subjected to electrophoresis at 60 V for 20 h at 60 °C. The gel was stained with gel red (Biotium, Hayward, CA, USA), and visualised in ultraviolet light using L-PIX (Loccus Biotecnologia, Cotia, SP, Brazil). The results were presented as a dendrogram constructed using UPGMA (Unweighted Pair Group Arithmetic Mean) and were analysed by Pearson Correlation Coefficients in BioNumerics software (Applied Maths, Belgium), through the similarities between the obtained DNA profile bands. The similarities were evaluated based on the presence or absence of bands in DGGE.

2.8 DNA Sequencing

The bands that were more representative and exclusive in the profiles were excised and eluted in 50 µL of ultra-pure water overnight at 4 °C, and were homogenised and centrifuged for 1 min at 3,293 g. A volume of 5 µL of the obtained supernatant was used as a template for PCR with the same primer pairs used in the second reaction but without the GC clamp. The PCR product was purified using the ExoSAP-IT PCR Clean-up kit (GE, Healthcare, Sunnyvale, CA, USA) according to the manufacturer's instructions. The purified bands were sequenced in a Sequenciator DNA Analyser (Applied Biosystems, Carlsbad, CA, USA). The obtained sequences were analysed using SeqManP with Lasergene software (DNAstar Inc., Madison, WI, USA), and a consensus sequence was obtained using Bioedit v.7.0.5.3 software (Carlsbad, ON, Canada). The representative consensus sequences of the fungal taxa were deposited into the GenBank database by BLASTN (Nucleotide Basic Local Alignment Search Tool) similarity search, algorithm available from the NCBI (National Centre for Biotechnology Information) [30].

2.9 Nucleotide Sequence Accession Numbers

The 18S and ITS (Internal Transcribed Spacer) sequences were deposited at the NCBI database under the accession numbers KU951524, KU951525, KU951526, KU951527, KU951528, KU951529, KU951530, KU951531, KU951532, KU951533, KU951534, KU951535, KU951536, KU951537, KU951538, KU951538, KU951540, KU951541, KU951542, KU951543, KU951544, KU951545, KU951546, KU951547, KU951548, KU951549, KU951550, KU951551, KU951552, KU951553, KU951554, KU951555, KU951556, KU951557, and KU951558.

2.10 Measurement of Environmental and Chemical Parameters

Physical parameters such as air temperature, relative humidity, and wind speed and direction were obtained and recorded at the exact locations of collection, by checking on the website of INMET (National Institute of Meteorology). Direct analysis of chemical parameters such as quantification of pollutant gases emitted as TSP (Total Suspended Particles), IP (Inhalable Particles), SO_2 (Sulphur Dioxide), CO (Carbon Monoxide), O_3 (Ozone), TH (Total Hydrocarbons), NO_2 (Nitrogen Dioxide), NO (Nitrogen Monoxide), NO_x (Nitrogen Oxide), CH_4 (Methane), NCH_4H (Non-methane Hydrocarbons), VOC (Volatile Organic Compounds) and BTEX (Benzene, Toluene, Ethylbenzene, Xylene) was performed and analysed by comparing to the parameters established by the Brazilian Resolution CONAMA (National Environment Council) and 3/1990 and supplemented by Resolution 8/1990. Statistical analyses were performed using GraphPad Prism, version 5.00, for Windows (GraphPad Software, San Diego, CA, USA). ANOVA (Analysis of Variance) was used to test the significance of differences in other assays. Statistically significant results were considered when $p < 0.05$.

2.11 PCA (Principal Component Analysis)

In order to obtain a relationship between environmental parameters and the occurrence of some obtained fungal species related to biodeterioration, PCA was used. Thus, PCA calculations were performed using the PAST (Paleontological Statistics) computer program, version 2.16 [31].

3. Results

3.1 Composition and Specifications of the Soapstone Samples

Analysis of the stone samples composition (Table 1) showed that the soapstone is a metamorphic stone comprised of talc, chlorite, and dolomite. On an average approximately 55% of the sample was composed of talc, being the component that gives low mineral hardness and high density to soapstone

(2.9 g/cm^3). Due to this dense feature, the surface of the stone is susceptible to fungal colonization and physico-chemical reactions [32].

3.2 DGGE and Community Profile Analyses

Previous studies of fungal identification from extreme environments [4, 14, 33, 34] have shown that RIF groups require specific conditions of humidity, temperature, pH and time for the formation of reproductive structures, which are used for their morphological identification. However, many of these fungi do not produce spores, making their micro-morphological identification. In order to address this issue, molecular tools based on obtaining the profile of the soapstone colonising fungal community by DGGE and searching GenBank were used. Thirty-five populations of filamentous fungi distributed in a range of four collections over the years 2013 and 2014 were obtained (Table 2). The DGGE gel (Fig. 3) shows a pattern of diversity and composition of fungal species in addition to the formation of two groups of microorganisms, the first one corresponding to collections 1 and 2, and the second group corresponding to collections 3 and 4. Many species were dominant in some populations (Figs. 3-b1, b6, b7) in relation to others. Furthermore, some populations were demonstrated to be unique from one location, such as *Sarcinomyces* sp. (Fig. 3-b7) that occurred only in Congonhas and *Trapelia glebulosa* (Fig. 3-b1) that was unique to Caraça.

3.3 Interaction between Environmental Parameters and Biodeterioration

The environmental conditions of the four collections (Table 3) show a wide variation in the parameters that influence the RIF community. Wind direction is a relevant factor for the variable spread of fungal spores during the four seasons of the year in Brazil. Moreover, the difference in relative humidity proved relevant to both locations, and is one of the main physical parameters that directly affect the development of fungal colonization [35].

Observations regarding the evolution of emissions and particulate matter in the atmosphere are shown in Table 4. Since the 90s, a remarkable increase in some air pollution parameters until the year 2013 has been observed. Sulphur dioxide was the parameter that showed a striking difference between the two cities, since the beginning of data collection, with the highest values always recorded in Congonhas. The difference in nitrogen dioxide emission rates in 2012 surpassed all other years of the sampled period, with higher values in the city of Congonhas, when compared to those in Caraça.

Fig. 4 shows the values of the main environmental parameters from the collection periods in Congonhas including TSP, IP, SO_2 and NO_2. The month of August showed the highest TPS, NO_2 and SO_2 emission values, corresponding to collection 3. TPS levels always outperformed all the other parameters analysed at all the recorded times, and this fact may have contributed to the establishment of some fungal species that had not been detected before in stone samples from Congonhas, as observed in the DGGE profile (Figs. 3-b6 and b7). Regarding collection 1, statistical differences were observed for all other collections considering the values obtained for the NO parameter.

The SO_2 parameter showed a positive correlation with moisture on the fungal population of *Sarcinomyces* sp. and *Pseudocyphellaria coriifolia*. NO_2, CO and TSP parameters acted synergistically on *Physcia aipolia*, whereas only temperature influenced the occurrence of the species *Capnobotryella* sp., *Diploschistes scruposus* and *Rhizocarpon geographicum* and species from the class Dothideomycetes and the order Capnodiales (Fig. 5).

4. Discussion

This study shows that the vast majority of RIF, settlers of soapstone from the two localities, Congonhas and the Sanctuary of Caraça, belong to a

Table 2 Fungi identified based on the partial SSU rDNA fragments sequence analysis of DGGE bands.

Best Blast match [GenBank accession number]	Collection 1						Collection 2						Collection 3						Collection 4						GenBank Accession number	Simil (%)
	CAR			CON			CAR			CON			CAR			CON			CAR			CON				
	S	M	L	S	M	L	S	M	L	S	M	L	S	M	L	S	M	L	S	M	L	S	M	L		
Bjerkandera adusta [KC176310.1]															x										KU951545	97
Capnobotryella sp. [AJ972856.1]							x	x																	KU951541	99
Order Capnodiales [KC315868.1]							x						x												KU951540	99
Cetradonia linearis [AF085470.1]											x														KU951552	99
Diploschistes scruposus [KJ766708.1]																				x	x				KU951530	96
Class Dothideomycetes [KC315860.1]			x																						KU951556	99
Extremus antarcticus [GU250314.1]				x										x											KU951546	99
Meria laricis [HQ634818.1]			x																						KU951527	97
Microcyclosporella parkii [GU214551.1]	x		x						x	x															KU951548	97
Mycosphaerella endophytica [GU2114538.1]				x																					KU951529	98
Mycosphaerella marasasii [GU214548.1]								x																	KU951544	99
Passaflora bellynckii [GU214556.1]													x											x	KU951554	98
Passaflora perplexa [GU214559.1]													x	x	x	x	x	x	x	x	x	x	x	x	KU951524	99
Passaflora sequoiae [GU214667.1]																x	x								KU951538	100
Peccania fontqueriana [AF336902.1]										x															KU951547	90
Phyllobaeis erythrella [AF491848.1]				x		x																			KU951533	98
Physcia aipolia [AF088244.1]																x	x	x							KU951542	99
Pseudevernia cladonia [KJ766762.1]				x																					KU951531	99

Table 2 continued

Best Blast match [GenBank accession number]	Collection 1						Collection 2						Collection 3						Collection 4						GenBank Accession number	Simil (%)
	CAR			CON			CAR			CON			CAR			CON			CAR			CON				
	S	M	L	S	M	L	S	M	L	S	M	L	S	M	L	S	M	L	S	M	L	S	M	L		
Pseudocyphellaria coriifolia [EU3600885.1]					x																				KU951535	99
Rhizocarpon geographicum [AF088246.1]						x																			KU951537	100
Sarcinomyces sp. [JN695021.1]																x	x					x	x		KU951558	100
Sclerotinia homoeocarpa [HQ449698.1]	x																								KU951525	97
Stereocaulon alpinum [JN941179.1]										x															KU951549	97
Stereocaulon sasakii [JN941185.1]																		x							KU951538	99
Stereocaulon tomentosum [JN941181.1]										x															KU951550	98
Stereocaulon vesuvianum [AF085466.1]												x													KU951555	98
Stigmina platani [GU214598.1]																								x	KU951551	97
Trapelia glebulosa [KJ766806.1]							x	x	x																KU951543	97
Trapelia involuta [AF119499.2]																					x				KU951534	97
Trapeliopsis glaucopholis [KJ766808.1]													x	x					x	x					KU951532	97
Uncultured Eukaryote [GQ462948.1]		x																							KU951526	97
Uncultured Fungus [GQ462948.1]			x																						KU951528	97
Xanthomendoza borealis [AJ535293.1]																								x	KU951553	97
Xanthoparmelia conspersa [AF117992.1]					x																				KU951536	99
Xanthoparmelia semiviridis [KJ766816.1]			x																						KU951557	100

Legend: CAR = Caraça; CON = Congonhas; S = Small; M = Medium; L = Large; Simil = Similarity.

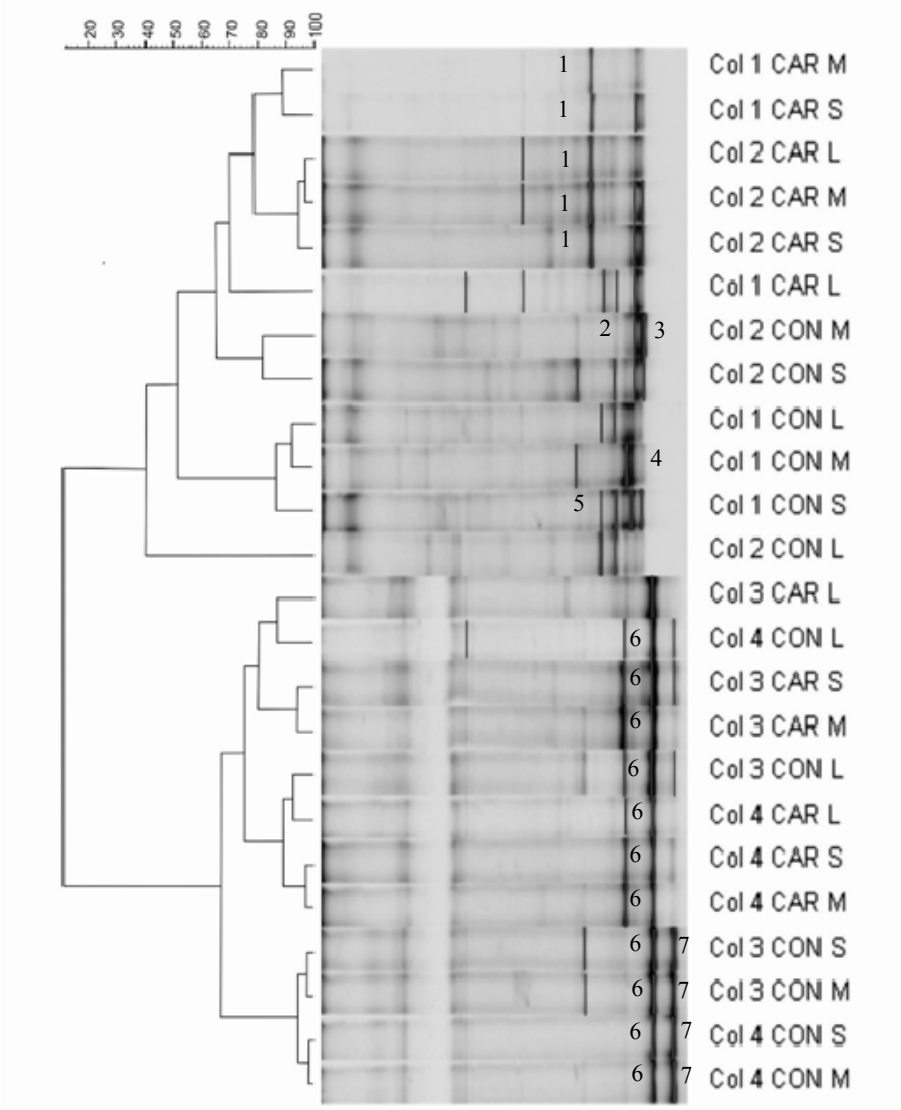

Fig. 3 Grouping analysis of fungal community profile obtained by DGGE technique with 35-55% urea and formamide (UF) and containing amplified sections of SSU rDNA fragments. Dendograms were determined by UPGMA and Pearson Correlation. The highlighted bands were excised for sequencing (b1 = *Trapelia glebulosa*; b2, b3, b4, b5 = Capnodiales; b6 = *Passaflora perplexa*; b7 = *Sarcinomyces* sp.) b = band; CON = Congonhas; CAR = Caraça; S, M, L = Small, Medium and Large.

Table 3 Physical parameters of the research sites, Congonhas and Caraça for collections 01, 02, 03 and 04.

Physical parameters	Collection/Location							
	Collection 01		Collection 02		Collection 03		Collection 04	
	CON	CAR	CON	CAR	CON	CAR	CON	CAR
Direction of the winds	N	N	SE	NW	E	E	SE	NE
Rainfall probability (%)	52	49	14	48	2	8	0	27
Relative humidity (%)	35	83	88	49	72	47	83	42
Temperature (°C)	26	23	22	29	12	23	20	28
Thermal sensation (°C)	31	23	22	29	12	23	20	28
Wind speed (Km/h)	10	11	10	10	10	24	13	13

Legend: CON = Congonhas; CAR = Caraça; N = north; SE = southeast; NW = northwest; E = east; NE = northeast.

Table 4 Atmospheric emissions data of polluting gases and particles in the years 1993, 2000, 2012 and 2013 in the regions of Congonhas and Caraça. CON = Congonhas; CAR = Caraça. Data obtained by mining companies in the Minas Gerais State and website of INMET, Brazil.

Atmospheric emissions	Year: 1993		Year: 2000		Year: 2012		Year: 2013	
	CON	CAR	CON	CAR	CON	CAR	CON	CAR
BTEX (ppb)	Nd	Nd	Nd	Nd	Nd	Nd	7.5	Nd
CH_4 (ppm)	1.68	69	1.44	0.08	Nd	Nd	2.15	Nd
CO (ppm)	0.29	73	1	0.5	221.79	1.67	425	209
VOC (ppm)	Nd	Nd	Nd	Nd	0.1	Nd	Nd	Nd
NCH_4H (ppm)	0.39	42	0.07	8,4	Nd	Nd	0.74	0.005
NO (ug/m^3)	13	13	Nd	Nd	Nd	Nd	100	25.6
NO_2 (ug/m^3)	2	3	Nd	Nd	58.3	2.18	27	25.5
NO_X (ug/m^3)	15	23	Nd	Nd	Nd	Nd	69.5	7.4
O_3 (ug/m^3)	36	29	52	3.1	58.3	0.71	85	18.75
IP (ug/m^3)	Nd	Nd	Nd	Nd	22.93	10.09	39	40.5
TSP (ug/m^3)	72	Nd	29	Nd	52.76	8.78	110.5	6.4
SO_2 (ug/m^3)	4	5	25	0.02	3.5	0.01	15	2.3
TH (ppm)	2.07	Nd	1.51	Nd	Nd	Nd	1.9	0.005

Legend: CON = Congonhas; CAR = Caraça; Nd = not detected; BTEX = benzene, toluene, ethylbenzene, xylene; CH_4 = methane; CO = carbon monoxide; VOC = volatile organic compounds; NCH_4H = non-methane hydrocarbon; NO = nitrogen monoxide; NO_2 = nitrogen dioxide; NO_X = nitrogen oxide; O_3 = ozone; IP = inhalable particles; TSP = total suspended particles; SO_2 = sulfur dioxide; TH = total hydrocarbon.

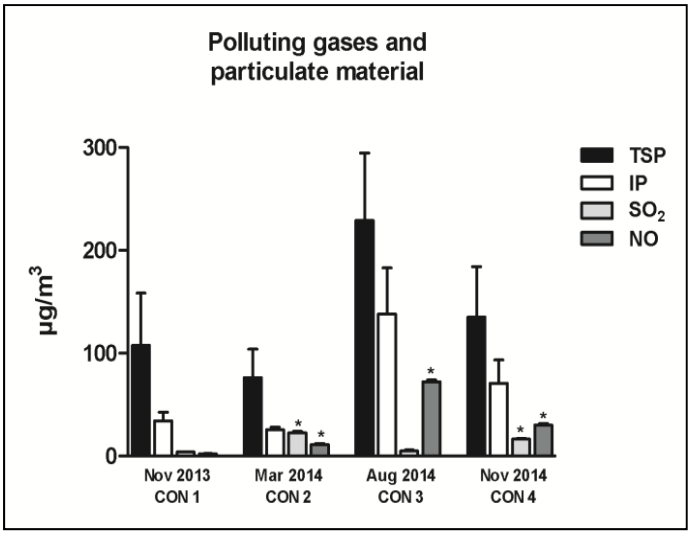

Fig. 4 Average month of gas emissions and particulate matter (µg/m^3) in the town of Congonhas for the months of collections. *p < 0.05. CON = Congonhas; TSP = Total Suspended Particles; IP = Inhalable Particles; SO_2 = Sulfur Dioxide; NO_2 = Nitrogen Dioxide.

group establishing symbiotic relationships of the lichen type. In addition, other fungi obtained, such as those belonging to the class Dothideomycetes, and acting as symbiotic species, are also associated with rocks in extreme environments. However, few studies have shown the ecological role of these fungal species in such environmental conditions [11].

Despite the limitations of DGGE, the analysis of fungal community profiles (Fig. 3) revealed a cluster among populations of the same locality, which coincides with the fact that these organisms are susceptible to the same environmental conditions and therefore favour the colonisation of the same fungal species. Regarding the collection periods, the profiles

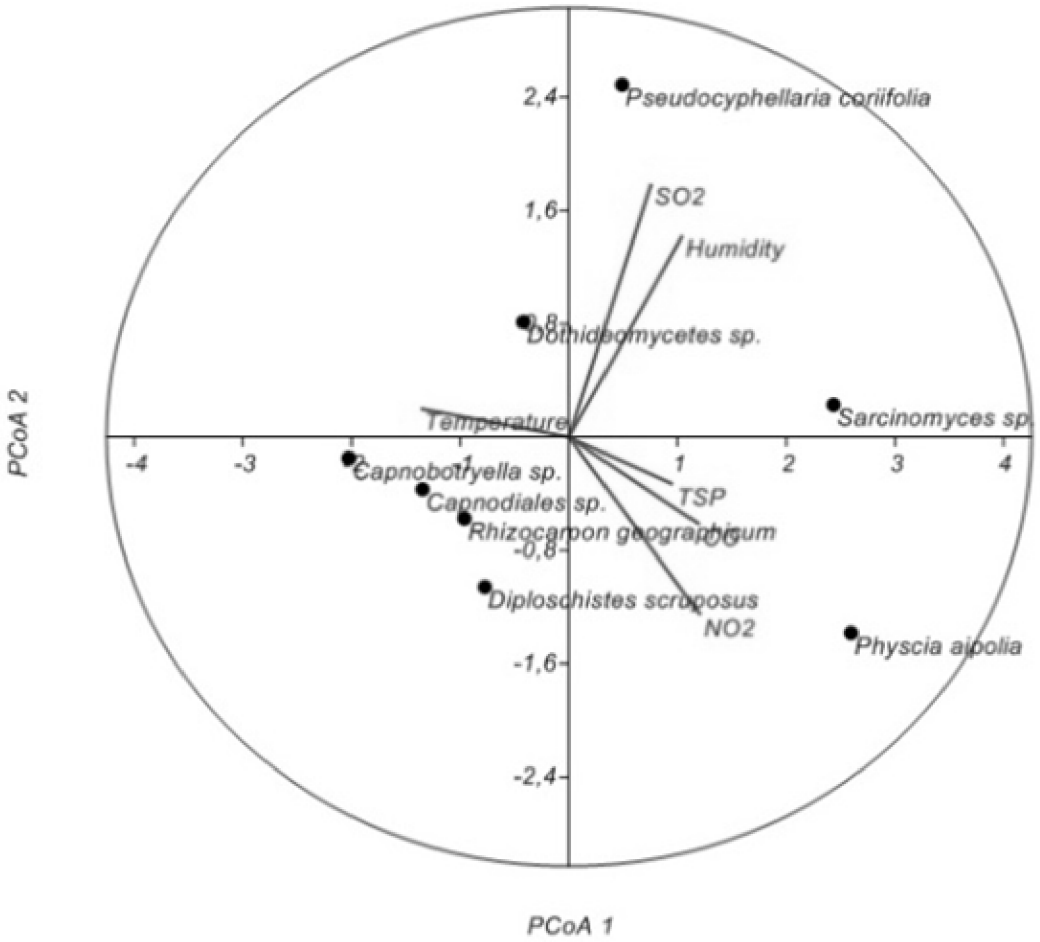

Fig. 5 Principal component analysis plot calculated among the physicochemical local parameters in Congonhas and Caraça [temperature, humidity, NO_2, SO_2, TSP and CO] of the fungal communities associated soapstone in Minas Gerais State.

of the obtained populations revealed two groups, the first one shows similarity between collections 1 and 2, and the second group shows proximity between communities of collections 3 and 4.

The collections 1 and 2 from Caraça highlight the occurrence of the species *Trapelia glebulosa* (Fig. 3-b1), which proved to be dominant in both clusters. *Trapelia glebulosa* is often associated with the lichen mycobiont species, which occurs in the Amazon region and in the Atlantic Forest of Northeast Brazil [36] and in a type of sandstone [37]. Approximately 20% of the fungal species found in stones are associated with photobionts, and this is one of the most relevant issues in the context of heritage preservation, because of the consequences that lichen association can cause on stone [36, 38]. Moreover, the occurrence of *T. glebulosa* in two seasons suggests their affinity with the specific environmental conditions of Caraça in periods of high humidity and temperature.

Fungal communities from collections 1 and 2 from Congonhas showed a wide variety of populations, specifically, *Cetradonia linearis*, *Pseudocyphellaria coriifolia* and *Phyllobates erythrella* species and the one specie of the order Capnodiales (Figs. 3-b2, b3, b4, b5), which showed dominance. Earlier reports suggest that these fungal species are also present in lichen thallus, which explains the occurrence of lichenised fungi on the surface of the studied stones [14, 34, 39, 40].

Furthermore, the specie of the order Capnodiales has shown the metabolic ability to colonise stones

associated with photobiont organisms resulting in lichen [41] or non-symbiotic association [16].

The species *Passalora perplexa* (Fig. 3-b6) was only identified in samples 3 and 4 from both research sites. *Sarcinomyces* sp. (Fig. 3-b7), which seem to be unique to the community of Congonhas, also occurred only in the collections 3 and 4 and had a genetic profile very similar to *P. perplexa*. Literature shows that the representatives of *Sarcinomyces* sp. may be commonly associated with plant pathogenic species [42]. However, despite the similarity of the genetic profile with *P. perplexa*, there are no reports of a synergistic action of both species in the deterioration of stones.

Sarcinomyces are black meristematic yeasts capable of producing pigments such as melanin [43] and may cause irreversible damage to stone sculptures [44]. Since many black fungi are pleomorphic and have anamorphic life cycles with widely divergent types of propagation, morphology offers only a presumptive identification at the genus level [16]. This fungus may be free living or may be associated with lichens and exhibit adaptive capacity to colonise terrestrial environments such as stones and soil [45].

Some of the identified fungal populations have a direct relationship with the biodeterioration of stone, especially in soapstone. Moreover, many of these species have an association with photobionts, establishing an ecological lichenic relationship that is very damaging to the stone surface owing to the acid metabolites produced by these organisms. The occurrence of the species *Bjerkandera adusta* in stone from Caraça results in a rare event, since literature often describes its association with wood deterioration [46]. Species of *Mycosphaerella*, regarded as an endophytic body associated with eucalyptus leaves [47], were also detected on the stone samples, and can be justified by the presence of numerous eucalyptus species in the Caraça region. The *Stereocaulon* sp. occurred as four different species: *S. alpinum, S. sasakii*, *S. tomentosum* and *S. vesuviana*, all of which present the feature of a lichen association relationship [48], this symbiosis being the precursor of negative and deleterious effects on the biodeterioration process. Moreover, there are species that can produce melanin, such as *Capnobotryella* sp. and *Sarcinomyces* sp., which may compromise the surface of the stones from the aesthetic and structural perspective [43]. Thus, the vast majority of species can also present a lichen association and be susceptible to air pollution, such as *Physcia aipolia* [49].

The rate and type of biological colonisation of stone is influenced by a wide array of environmental factors in addition to substrate characteristics. Thus, biocolonisation on stone can generally be attributed to two main groups of contributing factors: those relating to the substrate properties such as the bioreceptivity of stone and the environmental factors [50].

The interaction between the environmental influences and the composition of the RIF species obtained is extremely important to understand the dynamics of the fungal community associated with the biodeterioration process. Caneva, G., Nugari, M. P. and Salvadori, O. [51] considered the quantity and availability of moisture as the main determining factors for the speed at which a surface is colonised. Furthermore, a stone surface in outdoor conditions can be an inhospitable environment for organisms, with rapid changes in surface temperature and variation in moisture availability [50]. Moreover, it is necessary to consider the atmospheric factors and the geography of the regions studied, to obtain information about the state of preservation of the monuments and the sites belonging to the cultural heritage, and these have rarely been analysed in literature [52].

In urban environments, atmospheric pollution by motor traffic is generally considered a major factor of building material decay [53]. In addition, these environments have shown a significant increase in the rates of most dangerous pollutants such as nitrogen dioxide and fine particles [54]. Particulate pollutants such as sulphur dioxide and hydrocarbons present in

the urban environments of Congonhas (Table 4), act synergistically with the fungi present accelerating the decay process [55]. Thus, the burning of fossil fuels in the urban centers for transportation and energy production is a key source of destructive geological heritage [56].

The data shown in Table 4 are staggering, since they demonstrate the discrepancy between the values of the two places, especially for the more relevant parameters, such as SO_2, NO_2, IP and TSP and this reflects in the clustering of fungal populations as revealed by DGGE (Fig. 3).

High levels of SO_2 observed in the region of Congonhas (Fig. 4) facilitate the formation and precipitation of acid rain, due to the oxidation of this compound. The resultant sulphuric acid causes harmful and irreversible damage upon contact with the outdoor-exposed stones. Consequently, the resulting acidity has been shown to cause severe decay [55]. Furthermore, sulphuric acid from acid rain also reacts with limestone promoting the formation of gypsum and leading to the formation of a crust on its surface. Thus, once exposed to the particles suspended in the atmosphere, these scabs promote the adhesion of particulate materials leading to the formation of black crusts [57]. Because of these chain reactions, black crusts are enriched as particulate matter from air pollution by providing the resources and ideal conditions for new microbial colonization [58]. The possibility of black crust formation is also related to NO_x, CO_2, suspended hydrocarbons and organic compounds and inhalable particulates [59], as detected at high levels in Congonhas as compared with those in Caraça (Table 4).

Relative humidity greater than 80% coupled with the probability of precipitation at both research sites (Table 3), results in a scenario that could lead to irreversible damage due to an annual average of 15 $\mu g/m^3$ SO_2 observed in Congonhas (Table 4), an event which can lead to acid rain precipitation in this site.

The wind speed and direction are prevailing parameters on the spread of fungal spores, acting as one of the determining factors in microbial colonization [60]. However, the geographical location and the surrounding local relief of exposure of the studied stone samples positively influence the colonization of new fungal species [61]. Moreover, the dispersion of pollutant gases and particulate matter was reflected in the changes in composition of different species of fungal communities studied over 12 months.

PCA showed that high levels of moisture and sulphur dioxide in the atmosphere influence the occurrence of *Sarcinomyces* sp. and *Pseudocyphellaria coriifolia* on the stones of Congonhas (Fig. 5). In addition to microbial activity, both factors, when combined can lead to the formation and precipitation of acid rain which constitutes a negative scenario at the study site.

Although *Physcia aipolia* is associated with photobionts and is sensitive to environmental pollution, this species was also detected in Congonhas and factors such as total suspended particles, nitrogen dioxide and carbon monoxide, acted synergistically on this population showing the resistance of this fungus to polluting factors of anthropogenic origin.

A group consisting of three species of fungi (*Capnobotryella* sp., *Diploschistes scruposus*, *Rhizocarpon geographicum*, the class Dothideomycetes and organism of the order Capnodiales) had shown sensitivity only to the variation of temperature, since this location shows no vulnerability to industrial actions.

5. Conclusions

The geological characteristics of soapstone associated with RIF's communities suggest that soapstone may provide a favourable microenvironment to fungal colonisation, survival and dispersal in extreme conditions. Environmental data revealed that in the last two collections there was an increase in the levels of TSP, IP, SO_2 and NO_2 in

Congonhas. This event occurs at the same time as the establishment of *Sarcinomyces* sp., which is a fungus capable of producing black pigment that acts in deteriorating the stone surface, compromises its integrity and aesthetics. In addition, literature describes its association with photobionts and promoting a lichen association, therefore supporting an active production of strong lichen acids. Considering these data in particular, this event could be indicative of the direct influence of environmental parameters providing colonisation and the establishment of fungal species with the potential for deterioration.

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