Fungal Diversity

Extremotolerant rock inhabiting black fungi from Italian monumental sites

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Corresponding Author:	Laura Selbmann, Ph.D. Università degli Studi della Tuscia Viterbo, VT ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Università degli Studi della Tuscia
Corresponding Author's Secondary Institution:	
First Author:	Daniela Isola, PhD
First Author Secondary Information:	
Order of Authors:	Daniela Isola, PhD
	Laura Zucconi, Professor
	Silvano Onofri, Professor
	Giulia Caneva, Professor
	Sybren G de Hoog, Professor
	Laura Selbmann, Ph.D.
Order of Authors Secondary Information:	
Funding Information:	
Abstract:	A wide sampling for isolating highly destructive and extremotolerant black fungi was performed from Italian monuments in selected historical sites which include the "Cortile della Pigna", Vatican Museum and the St Peter colonnade (Vatican City State), the monumental cemetery of Bonaria (Cagliari), and other monuments in the city of Cagliari. Thirty out of seventy-four strains isolated were identified basing on ITS Blastn comparison. Based on multilocus phylogeny and morphological data, one new genus and species Lithophila guttulata, five new species Knufia marmoricola, K. vaticanii, K. karalitana, K. mediterranea and Exophiala bonariae, order Chaetothyriales and one new genus and species, Saxophila tyrrhenica, and two new species Vermiconia calcicola and Devriesia sardiniae, order Capnodiales, were proposed. Ecological considerations are put forward.
Response to Reviewers:	Reviewer #1: This is a quite substantial paper that extends the knowledge about the diversity of rock inhabiting black fungi significantly. It is generally well prepared includes a couple of new taxonomic descriptions and is well suited for the journal after revision. - What I still miss is a description how species can be generally delimited, either by sequence data or by morphological discontinuities. This would help to recognize species or genera, particularly in these phenotypically difficult and character-poor groups. It often seems a matter of taste…. I suggest the authors include some more information on concepts of taxa. Answer: This is actually a difficult matter. These fungi are invariably asexual and very scarcely morphological differentiated. It is tricky to define a species in this group. For this reason it was defined by Egidi et al. (2014) a “standard” for establishing species

delimitation based on some well represented groups. We referred to that standard for species delimitation in this work too, as specified in pag. 8 lines 15-16..

-Table 1 is quite long. Perhaps the editor might consider to shift this into supplementary mat.

Answer: Tab 1 may be shifted to supplementary material if the editor decides

- What are „bioderiogens" ? page 4

Answer: “Bioderiogens” was corrected in “biodeterioges”

-A genus cannot be "defined" in a tree: "The genus Knufia was clearly defined and supported in the tree". page 8

Answer: The sentence was changed as follows: “The genus Knufia was well supported in the tree”

-The part on stress-induced mutation is speculative and rates have not been tested here… "stress-increasing mutational rate in RIF might therefore be regarded as a genetic strategy to improve survival rates…" ???? seriously, this should be deleted. [if it is true what the authors claim then I would be careful with description sterile taxa, as future more extensive studies might then find countless intermediate forms] page 16

Answer: The sentence was deleted

-"clustered in a sufficiently supported separate position to justify the assignment to a new species," this sounds very strange and. when is a position sufficiently separate ? page 17

Answer: The sentence was changed as follows:”Two of the RIF isolated, CCFEE 6202 and 6210, clustered in a separate position and were assignment to a new species, D. sardiniae.”

-I always thought Hortaea is halotolerant rather than halophilic (and dependent on salt). page 18

Answer: The species H. werneckii is actually halophilic since it grows best at 10% NaCl concentration rather that at 0 and with an optimal growth at 7% as reported by Sterflinger 1998 (Temperature and NaCl- tolerance of rock-inhabiting meristematic fungi Antonie van Leeuwenhoek 74: 271–281).

-It would here also appropriate to consider Gostincar et al. 2010 (FEMS Microbiol Ecol 71) page 18

Answer: The reference has been added

-I don't know what the authors want to say with „ dispersed as dry dust and transported by winds" Many conidia are found in dusts (which is not a propagule by itself) and all spores are transported by wind ? this part again sounds speculative. and the last sentence of the paper sounds trivial. try to find a better finale. page 18

Answer: Rock fungi do not sporulate as a rule; yet, they disperse quite efficiently since, in many cases, the same species is distributed worldwide as for Elasticomyces elasticus. These fungi strictly adhere to the substratum even thanks to the production of extracellular polymeric substances. It is well known that they are quite skilled in destroying rocks, carving mechanically stone monuments of the Mediterranean area and powdering the substratum in natural conditions. In the Antarctic, for instance, they take part of the cryptoendolithic microbial communities and the biological weathering of the rock is reported as main mechanism of dispersal for these organisms (Friedmann, 1982; Nienow and Friedmann, 1993)

-Correct to Teratosphaeriaceae. page 36 in figure

	Answer: The figure has been corrected Reviewer #2: This paper described two new genera and 7 new species of rock-inhibiting fungi from Italian monumental sites. The paper was well organized and written. -There were two gene loci applied in the Figure 2 and three gene loci applied in the figure 3 for the phylogenic analysis. However, currently, 4-5 gene loci generally applied for distinguishing the species of RIF. At least the authors should discuss this issue. Answer: For the phylogeny in Fig 2, we referred to Chomnunti et al. 2012 for resolving families in the Chaetothyriales, who based the analysis on ITS LSU. Yet, the groups we have got in the Chaetothyriales are well resolved and perfectly comparable with what obtained by Guiedan et al. 2014 to allocate orders and families in the class Chaetothyriomycetidae. For the Phylogeny in Fig 3 we have referred to Quaedvlieg et al., 2014 who used to resolve the positions in the tree including the same three families (Neodevrisiaceae, Extremaceae and Theraosphaeriaceae) two genes only (LSU and RPB2); we used three (ITS, LSU and RPB2) To explain this concept, the following sentences have been added in the results, section Multi-locus phylogeny, “The topology of our tree is in accordance with the most recent phylogenetic studies on the Chaetothyriales (Chomnunti et al. 2012, Guiedan et al. 2014).” and “The topology of the tree is in accordance with the most recent phylogenetic studies allocating species in the Teratosphaeriaceae and related families (Quaedvlieg et al. 2014).” -Another point is the that damagement of RIF on the monuments should be investigated and evaluated, or at least discussed. Answer: The following sentence in the conclusions has been added: “ They actively penetrate and break up the substratum by a mechanical and chemical actions by the production of siderophores (Marvasi et al. 2012); powdered rock, on which RIF strictly adhere by the production of extracellular polymeric substances, are then dispersed as dry dust and transported by winds.” -The last paragraph on the disperse of RIF seems superficial and should be deleted. Answer: The sentence has been modified as above and explained in deeper details the connection between dispersal and mechanism of biodeterioration of monuments.

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Extremotolerant rock inhabiting black fungi from Italian monumental sites

D. Isola - L. Zucconi - S. Onofri - G. Caneva - G.S. de Hoog - L. Selbmann

L. Selbmann (✉) - D. Isola - L. Zucconi - S. Onofri
DEB, Università degli Studi della Tuscia, Largo dell'Università, Viterbo, Italy
e-mail: selbmann@unitus.it

G. Caneva
Department of Sciences, Università Roma Tre, Viale Marconi 446, 00146 Roma, Italy

G.S. de Hoog
CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

Abstract A wide sampling for isolating highly destructive and extremotolerant black fungi was performed from Italian monuments in selected historical sites which include the "Cortile della Pigna", Vatican Museum and the St Peter colonnade (Vatican City State), the monumental cemetery of Bonaria (Cagliari), and other monuments in the city of Cagliari. Thirty out of seventy-four strains isolated were identified basing on ITS Blastn comparison. Based on multilocus phylogeny and morphological data, one new genus and species *Lithophila guttulata*, five new species *Knufia marmoricola*, *K. vaticanii*, *K. karalitana*, *K. mediterranea* and *Exophiala bonariae*, order Chaetothyriales and one new genus and species, *Saxophila tyrrhenica*, and two new species *Vermiconia calcicola* and *Devriesia sardiniae*, order Capnodiales, were proposed. Ecological considerations are put forward.

Taxonomic novelties: *Devriesia sardiniae* Isola & de Hoog **sp. nov.**; *Exophiala bonariae* Isola & Zucconi **sp. nov.**; *Knufia karalitana* Isola & Onofri **sp. nov.**; *Knufia marmoricola* Onofri & Zucconi **sp. nov.**; *Knufia mediterranea* Selbmann & Zucconi **sp. nov.**; *Knufia vaticanii* Zucconi & Onofri **sp. nov.**; *Lithophila* Selbmann & Isola **gen. nov.**; *Lithophila guttulata* Selbmann & Isola **sp. nov.**; *Saxophila* Selbmann & de Hoog **gen. nov.**; *Saxophila tyrrhenica* Selbmann & de Hoog **sp. nov.**; *Vermiconia calcicola* de Hoog & Onofri **sp. nov.**

Key words
Extremotolerance – Monuments – Microcolonial fungi – Multilocus phylogeny

Introduction

'Black yeasts and relatives', 'meristematic fungi', or 'microcolonial fungi' are frequently used terms to indicate a group of fungi having highly melanized cell walls, producing daughter cells by yeast-like, or monopolar or multilateral budding, and being able to shift from a mycelial to a meristematic growth by longitudinal and transversal septum formation. These fungi are phylogenetically heterogeneous, including entities that belong to different orders of Ascomycota (de Hoog and McGinnis 1987). Meristematic growth is infrequent in the fungal kingdom and can be viewed as a specific response to external stress. Conversion towards isodiametric expansion can be induced in hyphal or yeast-like thalli of herpotrichiellaceous fungi by environmental factors such as acidification of the culture medium (Mendoza et al. 1993). Similar observations have been made in Dothideomycetes (de Hoog et al. 1999). In some cases, this can become a stable character (Matsumoto et al. 1986) as for black yeasts living under permanent stress (Selbmann et al. 2005, 2008). Also permanently meristematic mutants are known in species that normally grow as filamentous fungi (Xi et al. 2009). Black yeasts are typically extremotolerant or extremophilic, being able to tolerate stress factors such as high and low temperatures, drought, osmotic stress, high solar and UV radiation or prolonged period of water deficiency (Sterflinger 2006, Selbmann et al. 2008, 2011, 2013, 2014a, Zakharova et al. 2013); some of them may even survive simulated and real space conditions (Onofri et al. 2008, 2012). Their ecology reflects these abilities and they occur in environments mostly precluded to others as saltpans (Plemenitaš and Gunde-Cimerman 2005), acidic and hydrocarbon-contaminated sites (Baker et al. 2004, Seyedmousavi et al. 2011, Selbmann et al. 2013, Isola et al. 2013) and exposed rocks. Rocks are characterized by multiple stress factors as oligotrophic conditions, high or low and changing temperatures, UV-radiation, osmotic stress and are, therefore, very hard to colonize. Nevertheless, rock is one of the most recurrent substrates for specialized melanized fungi, and these lithobionts, also known as 'rock inhabiting fungi' (RIF), are the most extremotolerant among black yeasts (Selbmann et al. 2014b). Originally they have been found in the rocks of the hot Arizona desert (USA) and of the extremely cold Antarctic deserts (Friedmann 1982, Staley et al. 1982), but were later commonly isolated in the Mediterranean area from exposed natural rocks (Ruibal et al. 2005) and monuments (Sert et al. 2007a, b, c, Marvasi et al. 2012, Onofri et al. 2014) and other temperate bioclimates (Wollenzein et al. 1995, Salvadori 2000, Ascaso et al. 2008, Caneva et al. 2013). When live on monuments, RIF must cope with sun exposition, prolonged periods of drought and nutrient deficiency, as well as with the additional stress of pollution, since monuments are often located in urban environments. RIF are actually one of the most destructive and scarcely investigated groups of microorganisms causing deterioration of monuments exposed to outdoor conditions (Isola et al. 2013b, Onofri et al. 2014). They cause esthetical damage as a consequence of melanin production and mechanical destruction leading to exfoliation of stone artworks and biopitting (Sterflinger and Krumbein 1997, Lombardozzi et al. 2012). They represent a serious challenge for restorers because of the strong destructive potential and the high resistance to many types of chemical treatment (Isola et al. 2013b).

Because of their role in deterioration of monuments, a great deal of interest is being paid to species growing exclusively on rock and causing visible biodeterioration patterns. Only in relatively few cases the fungal communities on rock surfaces were completely inventoried and still fewer species have been described and characterized phylogenetically. Earlier studies concerned marble monuments from historical sites in Greece (Sterflinger and Krumbein 1997, Wollenzien et al. 1995, 1997), more recently from Ukraine and Turkey (Bogomolova and Minter 2003, Sert et al. 2007a, b, c) and, to a less extent, Italy (De Leo et al. 1999, 2003, Zucconi et al. 2012).

Due to its long and varied history, Italy hosts the largest number of UNESCO World Heritage Sites; high art and monuments are distributed everywhere along the country. Despite this huge cultural heritage and the risk that black fungi may represent, a deep and accurate sampling in historical areas has never been conducted. In the frame of a collaboration for the preservation of art-works in some Italian historical sites, the Vatican, the monumental cemetery of Bonaria (Cagliari) and some monuments in the city of Cagliari, have been sampled for isolating biodeteriogens. The aim of the present study was to document the biodiversity of extremotolerant RIF on stone monuments of these sites; here rock colonizers must cope with additional strong stresses of high levels of pollution in the Vatican and high salt concentrations due to the proximity of the sea in the other sites. New genera and species were defined by multilocus phylogeny.

Materials and methods

Studied areas

Vatican City State

The Vatican City State is a walled enclave located within the city of Rome, being comprised in the transitional Mediterranean region; it is characterized by medium Meso-Mediterranean thermo type with hot and dry summers, mild winter temperatures and rainfalls that are concentrated in autumn and winter (Blasi, 2001).

It is the smallest country in the world and, being the center of catholic religion, is characterized by invaluable monuments and historical buildings. Over centuries, the Vatican has had official artists conceiving masterpieces in the name of the Catholic Church and these nowadays are exposed in the Vatican Museum together with roman stone-works of art and others coming from ancient Egypt, Middle East and Etruria.

Two famous areas were sampled in the Vatican City State; the first sampling concerned 8 ancient Roman stone artworks: a sarcophagus, four capitals, the Albani family's coat of arms in exedra designed by Pirro Ligorio, and two memorial funeral stones exposed along the perimeter of the "Cortile della Pigna"; this is one of the three courtyards of the Vatican Museum, named after the four meters high bronze pine cone placed on a double staircase, dated I-II century a. C., found in the middle age at the Thermae of Agrippa. The second sampling was performed at St Peter square from the Bernini's colonnade. Known worldwide, the square was built by Bernini for Pope Alexander VII in 1656-67. The colonnade consists of 284 majestic Doric columns and 88 pillars of white, porous travertine carved from "Piani di San Clemente", Tivoli, Rome.

Cagliari - Cemetery of Bonaria and historical buildings sampled

The city of Cagliari is situated in the south of Sardinia and occupies the flat spaces between the hills and the sea to the south and south-east, in an articulate landscape including beaches, lagoons and mountains surrounding the east part of the city. Cagliari is characterized by a thermo-Mediterranean bioclimate, with a low pluviometry, hot, dry and long summers and very mild winters (Grapow and Blasi 1998). The humidity may be high due to the proximity of the sea, ranging between 65% and 95% in winter and between 55% and 85% in summer. Dominant winds blow from south (Sirocco, Libeccio) and north-west (Mistral) transporting red dust from Sahara and marine aerosols, that constitute abundant natural pollutant across the urban area.

<u>The monumental cemetery of Bonaria</u> rises on the homonymous hill less than 400 meters from the sea; it is located in the proximity of a necropolis remains in an area populated since the Punic, Roman and early Christian times. The cemetery, inaugurated on January 1829, is included in the Association of Significant Cemeteries in Europe (ASCE) and preserves a huge number of funeral monuments created by many artists. Beyond the artistic value of stone-works, the cemetery of Bonaria also represents the open-air memory of many political, cultural, literary, scientific, military, religious and commercial people that made the history of Cagliari at the turn of 1800-1900. As other monumental cemeteries, it is reach of vegetation; among the botanical species present, cypresses are dominant with the species *Cupressus sempervirens, C. cashmeriana*, and *C. macrocarpa*. The vegetation also includes species of palms such as *Phoenix canariensis, P. dactilifera, Dracaena indivisa*, evergreen-oaks, Japanese maples (*Acer palmatum*), carobs (*Ceratonia siliqua)*, pines (*Pinus pinea*, *P. halepensis*), roses, hibiscus (*Hybiscus mutabilis*), pittosporum (*Pittosporum tobira*), oleanders (*Nerium oleander*), mastic trees (*Pistacia lentiscus*), Judas' trees (*Cercis siliquastrum*), and *Clerodendrum trichotomum*.

<u>Boyl Palace</u> is one of the most representative noble buildings of the district of "Castello" in Cagliari. It was erected in 1840 by Carlo Boyl Pilo, Marquis of Putifigari. The palace has a marble

balustrade, decorated with four statues representing the seasons and, in a central position the coat of arms.

The Cagliari Cathedral, or the Cathedral of Santa Maria, was built in the 13th century in Pisan-Romanesque style, obtaining cathedral status in 1258. In the 17th and 18th centuries it was renovated along Baroque lines and the current façade was constructed in the 1930s in Neo-Romanesque style. The other monuments sampled in Cagliari were Bastion Saint Remy, the lions outside of the Cathedral of Santa Maria, the Tower of the Elephant, Porta Cristina and the columns of St Saturnino Church.

Some of the artworks and historical buildings sampled in this study are shown in Fig. 1.

Sample collection and isolation

Biological samples were taken from vertical surfaces of the stone monuments using sterile needles (Wollenzien et al. 1995) and sterile swabs moistened with 50 µl of distilled water. Samples were plated on dichloran-rose bengal agar (DRBC, Oxoid, Basingstoke, Hampshire, U.K.) supplemented with chloramphenicol 100 ppm to prevent bacterial growth; plates were incubated at 20 °C and inspected every day. As soon as they appeared, black colonies were transferred onto fresh Malt Agar 4% (MA) slants and incubated at 20 °C.

Holotypes were deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands; ex-types are preserved in the Culture Collection of Fungi from Extreme Environments (CCFEE) in Viterbo, Italy

Morphology

Morphology was studied using slide cultures. Inoculation was performed onto 2 cm^2 water agar blocks, incubated for 2 weeks at 20 ºC in Petri dishes with wet sterile paper filter to avoid drying out of the medium; agar blocks with inoculated fungi were then transferred onto slides with lactophenol and observed with light microscope. Digital images of the samples were acquired using Digital Nikon Coolpix 5000 and digitally optimized with the software Micromax Arkon (v. 8.12.05). Colony descriptions are based on cultures grown on Malt Agar 4% (MA) incubated at 20 °C for 3 weeks.

DNA extraction, PCR amplification, and DNA sequencing

DNA was extracted from mycelium grown on MA slants at 20 °C, using Nucleospin Plant kit (Macherey-Nagel, Düren, Germany), following the protocol optimized for fungi. PCR reactions were performed using BioMix (BioLine, Luckenwalde, Germany). In each 25 µL reaction solution, 5 pmol of each primer and 40 ng of template DNA were added. Amplifications were carried out using MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Munich, Germany). Three nuclear (ITS, nucLSU, nucSSU) and one protein encoding (RPB2) genes were amplified, according to Egidi et al. (2014). Fragments were analysed by Macrogen Inc. (Seoul, Korea). Sequence assembly was done using the software CHROMASPRO v. 1.32 (Technelysium, Southport, Queensland, Australia). Sequence data were deposited in GenBank (Tab. 1).

GenBank comparison, alignment and tree reconstruction

Sequences were compared in the GenBank using BLASTN search (Altschul et al. 1997). The sequences in the GenBank that could be confidently aligned were chosen for multilocus analyses. In order to define the phylogenetic position of the isolates in the order Capnodiales (class Dothideomycetes) and in the order Chaetothyriales (class Eurotiomycetes), two multilocus datasets were generated. For the former, nucLSU, ITS and RPB2 gene targets were chosen for constructing the alignment, while nucLSU and ITS were used for the latter. Sequences were aligned iteratively with MUSCLE (Edgar 2004) in MEGA6 (Tamura et al. 2013) and final alignment was improved manually. One-gene alignments were concatenated using FABOX (http://users-birc.au.dk/biopv/php/fabox/alignment_joiner.php, Villesen 2007) to combine sequences for two bi- and three-locus datasets. Optimized alignments were exported and the best-fit substitution model was determined using MODELTEST MrAIC 1.4.3 (Nylander 2004) as implemented in PHYML (Guindon and Gascuel 2003). Phylogenetic trees were reconstructed by Maximum Likelihood, using TREEFINDER (Jobb et al. 2004) and the resulting tree was visualized using TREEVIEW v. 1.6.6 (Page 1996). The robustness of the phylogenetic inference was estimated using the bootstrap method (Felsenstein 1985) with 1,000 pseudoreplicates generated and analysed with TREEFINDER.

Results

Molecular analyses

Blast identification

Twenty-seven strains isolated from the artworks in Cagliari (21 from the cemetery of Bonaria, 6 from monuments in the city) and two isolated from the “Cortile della Pigna”, Vatican City State, were identified using BLASTN search (Tab 2). Among Capnodiales, strains CCFEE 5660 and 5737 from monuments in Cagliari and CCFEE 5924 and 5927 from monuments in the “Cortile della Pigna” (Vatican City State) were identified as *Coniosporium uncinatum*, a species recurrent on monuments (Sterflinger et al. 1997), while strains CCFEE 5723, 5735 and 5919 from the cemetery of Bonaria and CCFEE 5565 from the city of Cagliari were identified as *Hortaea thailandica*.

Strains CCFEE 5703 and 5706 identified as *Phaeotheca triangularis* were isolated from the cemetery of Bonaria only as well as *Verrucocladosporium dirinae* (strains CCFEE 5707 and 5708). The strains CCFEE 5704 and 5922 belonged to the recently described species *Devriesia bulbillosa* and *Vermiconia flagrans*, respectively (Egidi et al. 2014), the latter, and the two strains of *C. uncinatum*, were the only fungi isolated from stoneworks of the Vatican belonging to known species. *Aureobasidium pullulans* (CCFEE 5736), *Austroafricana parva* (CCFEE 5742), *Pringsheimia smilacis* (CCFEE 5778), and *Teratosphaeria capensis* were found in monuments of Cagliari and the cemetery of Bonaria. *Knufia petricola* (Tab 2; CCFEE 5702, 5705, 5709, 5740, 5741, 5776, 5777, and 5937), isolated from many monuments in Cagliari and the cemetery of Bonaria, was one of the most frequent RIF species in this study.

Multi-locus phylogeny

Molecular phylogeny was performed to define the phylogenetic position of the strains with low identities in the GenBank. The two-gene dataset included 82 strains belonging to the order Chaetothyriales (Tab. 1). Introns were delimited manually and excluded from the alignment, so the combined dataset included 2,172 positions (ITS: 1–759; nucLSU: 760–2,172) including gaps. Fig. 2 shows the multilocus phylogenetic tree generated using a GTR+G (4) model, selected using the Akaike's information criterion with a Maximum-likelihood approach. The base frequencies were as follows: T=0.2371581, C=0.2366716, A=0.24823, G=0.27794, TC=0.4013773, TA=0.1540797; TG=0.080884; CA=0.1273652; CG=0.099919; AG=0.1363733. The tree was rooted with *Cladophialophora humicola* CBS 117536, Epibryaceae (Gueidan et al. 2014).

The topology of our tree is in accordance with the most recent phylogenetic studies on the Chaetothyriales (Chomnunti et al. 2012, Guiedan et al. 2014).

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The genus *Knufia* was ~~clearly~~ well ~~defined and~~ supported in the tree (Fig. 2); within the genus, two groups were discernable. The first comprised 10 strains isolated from all localities sampled in this study, and the second, with five strains originated exclusively from monuments of the city of Cagliari, were assigned to two separate species, *K. marmoricola* and *K. karalitana*, respectively. A further strain, CCFEE 5939, isolated from the St Peter colonnade, Vatican City State, was in a separated position sister to *K. perforans* and was assigned to the new species *K. vaticanii*. Within the genus *Knufia* there was a further supported clade (99.8% bootstrap) comprising 4 strains isolated exclusively from the cemetery of Bonaria. These strains were described here as the new species *K. mediterranea*, having a very low intraspecific ITS variability (0.5%). A well-separated cluster of eight strains isolated from monuments of the Vatican only, supported with 100% bootstrap, was in a basal position of the family Trichomeriaceae and here described as new genus and species *Lithophila guttulata*, with 2.4% ITS variability within the group. Finally, strains CCFEE 5792 and 5899 from the cemetery of Bonaria pooled together in the genus *Exophiala*, family Herpotrichiellaceae and were here described as a new species, *E. bonariae*.

The three-gene dataset included 60 strains belonging to the order Capnodiales. After exclusion of ambiguous regions and introns, the combined dataset included 2,098 positions (ITS: 1–545; *nuc*LSU: 546–1,781; RPB2: 1,782–2098), including gaps. Fig. 3 shows the multilocus phylogenetic tree generated using a GTR+G (4) model, which was selected using the Akaike's information criterion with a Maximum-likelihood approach. The base frequencies were as follows: T=0.2267053, C=0.2481356, A=0.2457273, G=0.2794318, TC=0.365155, TA=0.1365375; TG=0.09423267; CA=0.1193942; CG=0.1099502; AG=0.1747303. The tree was rooted with strains belonging to the family Cladosporiaceae as closest outgroup (Quaedvlieg et al. 2014): *Cladosporium langeronii* R1 (ITS accession number: HQ115727; Gorfer et al. 2011); *Cladosporium sphaerospermum* (ITS accession number: L25433; Curtis et al. 1994); *Davidiella tassiana* ATCC 26362 (Park et al. 2004).

The topology of the tree is in accordance with the most recent phylogenetic studies allocating species in the Teratosphaeriaceae and related families (Quaedvlieg et al. 2014).

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Strain CCFEE 5935, isolated from the cemetery of Bonaria, formed a separate, well-supported group together with three rock-inhabiting strains found on limestone at Mallorca, Spain. The group is sister to the recently described genus *Extremus* in a poorly resolved position in the tree and is here indicated as a new genus, *Saxophila*. The last two capnodialean species, both from the cemetery of Bonaria, formed a well-supported group in the family Neodevriesiaceae (Quaedvlieg et al. 2014) and were here indicated as the new species *Devriesia sardiniae*.

Taxonomy

Descriptions based on 3-week-old cultures grown on MA at 20 °C.

Devriesia sardiniae Isola & de Hoog, **sp. nov**. MycoBank MB811669, Fig. 4 A-I

Etymology: named after Sardinia, the island where the strain was isolated.

Diagnosis: Colonies growing slowly, up to 10 mm diam mm, cerebriform, often deeply immersed into the agar, olivaceous brown, with a velvety mouse-grey aerial short hyphae and well-defined black and glossy margin; black in reverse.

Mycelium consisting of subhyaline, closely septate, branched hyphae, (3.5–5.0 µm) with occasional anastomoses; hyphae gradually widening and darkening, forming brown, closely septate, constricted at septa, thick-walled, simple (6.5–7.0 × 4.5–4.8 µm) and 1-septate (12.0 × 6.5 µm) cells. Moniliform hyphae present. Meristematic conidia terminal, lateral and intercalary, with transverse, longitudinal and oblique septa, dark brown, thick-walled, rugose, globose to pyriform, measuring 8.5–16.5 × 7.5–13.5 µm.

Holotypus: CBS 139724=CCFEE 6202, isolated from a marble cross (Frau-Carta funerary monument) in the cemetery of Bonaria (Cagliari, Italy). Culture preserved in liquid nitrogen and in dried condition.

Exophiala bonariae Isola & Zucconi, **sp. nov**. MycoBank MB811668, Fig. 4 J-Q
Etymology: named after the cemetery of Bonaria, where the type strain was isolated.

Diagnosis: Colonies growing slowly, up to 20 mm, convex, circular, dark brown, with a velvety mouse-grey aerial short hyphae, pigment diffuse around the colony; dark brown in reverse.
Mycelium composed of short and brown hyphae in the central area, from which originate diffuse, slender, hairy and thin-walled hyphae, rarely branched, seldom spirally twisted, occasionally circinate, pale brown, with rare anastomoses. Conidiophores poorly differentiated, subhayline. Conidiogenous cells phialidic, occasionally slender up to 2.4 × 3.5 µm, hyaline towards the apex. Conidia numerous, hyaline, simple, thin-walled, mostly cylindrical, 5.5–6.5 × 2.2–2.8 µm, often grouped near the apex of the conidiogenous cells.

Holotypus: CBS 139957=CCFEE 5792, named after the cemetery of Bonaria where the strain was isolated. Culture preserved in liquid nitrogen and in dried condition.

Knufia karalitana Isola & Onofri, **sp. nov**. MycoBank MB811670, Fig. 4 R-X
Etymology: named after the ancient name of Cagliari (*Karalis*), the city where the strain was isolated.

Diagnosis: Colonies growing very slowly, up to 8 mm, black, raised,
often deeply immersed into the agar, with short, scarce, velvety, greyish-brown aerial hyphae; margin irregular; black in reverse.
Hyphae moniliform, occasionally toruloid, brown. Conidiophores micronematous. Conidia blastic or endogenous; blastic conidia terminal and intercalary, dark brown, formed by differentiation of hyphal cells which enlarge, forming mostly spherical (6.5–10.0 µm), thick-walled, rugose 1-celled,

sometimes bicellular and ellipsoidal conidia (10–12 × 6.5–9.0 µm) or evolving in multicellular roundish, thick-walled, rugose bodies (10.0–12.0 µm) containing endoconidia.

Notes: conidia and hyphal fragments may secede both rhexolytically and occasionally schizolytically, and enteroblastic growth may occur by budding of hyphal cells or blastic conidia.

Holotypus: CBS 139720=CCFEE 5656, Cagliari (Italy), isolated from marble lion in front of the Cathedral of Santa Maria. Culture preserved in liquid nitrogen and in dried condition.

Knufia marmoricola Onofri & Zucconi, **sp. nov**. MycoBank MB811671, Fig. 5 A-H
Etymology: named after the substratum, from which the type strain was isolated.

Diagnosis: Colonies growing very slowly, up to 8 mm, black, raised centrally, flat and glossy near the periphery, even growing in depth, velvety with grayish-brown aerial short hyphae and black crenate margin; black in reverse.
Hyphae moniliform, branched, cylindrical, smooth, 5.0–5.5 µm wide, straight to slightly curved, enteroblastically proliferating, with anastomoses, pale brown at first, darker when mature. Conidia blastic or endogenous; blastic conidiadark brown, mostly spherical 5.5 - 8 µm in diam, thick-walled, smooth, formed by differentiation of terminal and intercalary hyphal cells; endoconidia unicellular, globose (4.4–5.0 µm diam), developing within hyphal cells, which enlarge up to form roundish, thick- and smooth walled, mostly spherical, bodies, 7.5–15.5 µm wide.

Holotypus: CBS XXX=CCFEE 5895, isolated from travertine of St Peter colonnade (Vatican City State). Culture preserved in liquid nitrogen and in dried condition.

Knufia mediterranea Selbmann & Zucconi, **sp. nov**. MycoBank MB811672, Fig. 5 I-P
Etymology: named after the Mediterranean basin, the site from which the strain was isolated.

Diagnosis: Colonies slow growing up to 12 mm, black, cerebriform moist, glossy, later slightly crunchy with some greenish gray, velvety aerial mycelium and sharp, irregularly lobate margin; black in reverse.
Mycelium composed of scant filamentous hyphae and mostly moniliform, enteroblastically proliferating, catenate cells. Moniliform hyphae subhyaline to pale brown, branched, consisting of broadly ellipsoidal to cylindrical 0 -1 septate cells, apically and laterally budding to form dense and

darker aggregates, with flat scars due to rhexolythic secession. Cells later becoming darker, roundish, most frequently cylindrical and 1-septate, slightly constricted at septa, 9.0–14.0 × 7.0–8.0 µm.

Holotypus: CBS 139721=CCFEE 5738, isolated from Francesca Warzee funerary marble monument, cemetery of Bonaria (Cagliari, Italy). Culture preserved in liquid nitrogen and in dried condition.

Knufia vaticanii Zucconi & Onofri, **sp. nov**. MycoBank MB811673, Fig. 5 Q-X.
Etymology: named after the Vatican City State, where the type strain was isolated.

Diagnosis: Colonies growing very slowly, up to 10 mm, brownish black, cerebriform, covered by a dense, velvety, greyish-brown aerial mycelium, with sharp lobate margin; black in reverse. Mycelium initially composed of pale brown, branched, finely guttulate, filamentous hyphae, formed by small simple and roundish to 1-septate and cylindrical, catenate cells, enteroblastically proliferating, gradually developing into torulose hyphae producing swollen arthroconidia. Arthroconidia terminal and intercalary, dark brown, thick-walled, globose when simple (8.5–10.0 µm diam), cylindrical when bicellular (10.0–12.0 × 6.5–10.0 µm), globose to ovoid when multicellular, with longitudinal and oblique septa (13.0–16.5 × 9.0–17.0 µm).

Notes: The presence of small droplets in mycelial cells is limited to early growth stages. Each cell of which arthroconidia are formed may germinate and produce filamentous hyphae; cracks in outer cell wall layers are sometimes present, similarly to multicellular conidia of *Cryomyces antarcticus* and *Knufia petricola*.

Holotypus: CBS 139722=CCFEE 5939, isolated from travertine St Peter colonnade (Vatican City State). Culture preserved in liquid nitrogen and in dried condition.

Lithophila Selbmann & Isola **gen. nov**. MycoBank MB811675
Etymology: named after rock substrate where it was isolated from.

Diagnosis: Colonies growing slowly, cauliflower-shaped, dark brown, velvety with irregularly lobate margin in obverse; black in reverse. Hyphae branched, composed of irregularly shaped, simple to pluriseptate, catenate cells. Teleomorph unknown.

Type species: *Lithophila guttulata* Selbmann & Isola

Lithophila guttulata Selbmann & Isola, **sp. nov**. MycoBank MB811676, Fig. 6 A-G
Etymology: named after its guttulate cells.

Diagnosis: Colonies growing slowly, up to 11 mm, dark brown, cauliflower-shaped, finely velvety, with sharp and irregularly lobate margin; black in reverse.
Hyphae pale brown, diffusely guttulate, partly filamentous, partly composed of irregularly shaped, catenate cells produced by enteroblastic proliferation. Globose cells frequently produced intercalarly, terminarly or laterally 7.0–10.5 µm at first, gradually turning dark, thick, 1-multicellular widening up to 15.5 µm, sometimes with endoconidia.

Notes: globose, dark cells generally enlarge to form multicellular bodies which probably function as resistance structures.

Holotypus: CBS 139723=CCFEE 5907, isolated from marble stone (cat. 37106) exposed in the Vatican Museums – "Cortile della Pigna" (Vatican City State). Culture preserved in liquid nitrogen and in dried condition.

Saxophila Selbmann & de Hoog, **gen. nov**. MycoBank MB811677
Etymology: named after the rock substrate where it was isolated.

Diagnosis: Colonies growing slowly, dark in reverse, brown and velvety, with undulate margin in obverse. Hyphae curved, branched, repeatedly septate. Conidiophores micronematous. Conidia thallic-arthric. Teleomorph unknown.

Type species: *Saxophila tyrrhenica* Selbmann & de Hoog.

Saxophila tyrrhenica Selbmann & de Hoog, **sp. nov**. MycoBank MB811678, Fig. 6 H-O
Etymology: named after the Tyrrhenian basin, the site from which the strain was isolated.

Diagnosis: Colonies slow growing, up to 10 mm, brown and velvety in the obverse, raised centrally, flat near the periphery, with undulate margin; dark in reverse.

Hyphae smooth, straight, curved to geniculate, pale brown, branched, with occasional anastomoses, holoblastically proliferating, consisting of spherical cells when simple, cylindrical when 1-septate, eventually disarticulating. Conidiophores micronematous, conidia arthric. Globose to ellipsoidal, brown, multicellular bodies (10.0–12.0 × 15.0–20.0 µm) terminally or intercalarly produced in late stages. Teleomorph unknown

Holotypus: type culture CBS 139725=CCFEE 5935, isolated from little marble angels of an anonymous funerary monument in the cemetery of Bonaria (Cagliari, Italy). Culture preserved in liquid nitrogen and in dried condition.

Vermiconia calcicola de Hoog & Onofri, **sp. nov**. MycoBank MB811679 Fig. 6 P-V
Etymology: named after the substrate where the type strain was isolated.

Diagnosis: Colonies growing very slowly, up to 4.5 mm, black, cerebriform, raised centrally, growing deeply into the agar, velvety with short, brownish-green aerial hyphae, with dark brown flat, sharp and lobate margin; black in reverse.
Mycelium composed of filamentous to moniliform, branched hyphae, subhyaline to pale brown, with cells of irregular shape, with frequent anastomoses; abundant oil drops present in young hyphae. Conidiophores micronematous. Arthroconidia terminal and intercalary, dark brown, thick-walled, smooth, cylindrical, mostly 1-2 septate, 6.5–12.0 × 5.5–7.5 µm, sometimes also subglobose, with transverse, longitudinal and oblique septa, mostly in terminal position, up to 14 µm wide.

Holotypus: CBS XXX=CCFEE 5770, isolated from Giuseppina Ara funerary marble monument in the cemetery of Bonaria (Cagliari, Italy). Culture preserved in liquid nitrogen and in dried condition.

Discussion

Exposed rocks harbor an unexpected biodiversity of black fungi. Dozens of species were isolated from natural rocks in Spain (Ruibal 2004, Ruibal et al. 2005, 2008) and even from very inhospitable environments such as hot dry deserts of Arizona and Negev (Staley et al. 1982) and very cold areas of the Alps, Andes and Antarctica (Selbmann et al. 2005, 2008, 2014c, Isola 2010). Under strong environmental pressure and extreme isolation, speciation processes are promoted. New species are constantly being reported, even from the easily accessible Mediterranean area (Egidi et al. 2014).

Biodeterioration of the stone cultural heritage in Mediterranean countries has long been an area of concern. Rock-inhabiting fungi have been repeatedly isolated from stone monuments in Greece, Turkey (e.g. Gorbushina et al. 1993, Sterflinger et al. 1997, Bogomolova and Minter 2003, Sert et al. 2007a, b, c, Sert and Sterflinger 2010) and, to a less extent, Georgia (Caneva et al. 2013) and Italy (De Leo et al. 2003, Marvasi et al. 2010, Onofri et al. 2011). Some RIF colonizing monuments in urban environments are prone to additional external stresses than sun exposition, oligotrophy, and prolonged dryness, due to air pollution, while others may have a competitive advantage by their ability to assimilate toxic hydrocarbons (Prenafeta et al. 2006). The present study focuses on RIF from artworks of historical sites in Italy which had not been investigated before.

All RIF isolated in this study belonged to the orders Capnodiales and Chaetothyriales, two orders that are known to be overrepresented in rock-inhabiting black fungi (Gueidan et al. 2008, Ruibal et al. 2008, 2009, Egidi et al. 2014). All families included in the tree shown in Fig. 2 were well-resolved and the topology was in accordance with the most recent phylogenetic studies on the order Chaetothyriales (Réblová et al 2013, Gueidan et al. 2014). Most of the new species described here were included in the family Trichomeriaceae which was recently classified in the order Chaetothyriales (Chomnunti et al. 2012). The family includes genera of diverse ecologies: *Trichomerium* comprises epiphytic fungi, and *Metulocladosporiella* species are plant pathogens (Crous et al. 2006). The two species of the recently described genus *Bradymyces* (Hubka et al. 2014), *B. oncorhynchi* and *B. alpinus* have quite different ecologies: the first was found in a hyperhaemic focus near the spleen of rainbow trout (*Oncorhynchus mykiss*) and the second shows a saxicolous habitus, having been isolated from Alpine rocks. The genus *Knufia* (Tsuneda et al. 2011) includes ecologically diverse species ranging from lichenicolous taxa as *K. peltigerae*, human opportunistics colonizing human skin as *K. epidermidis* (Li et al. 2008, Li & Chen 2010), insect-associated species as *K. aspidioti* (He et al. 2013), and plant pathogens as *K. cryptophialidica* and *K. endospora* (Hutchison et al. 1995, Tsuneda and Currah 2004). In the same family, the saxicolous species *Phaeococcomyces chersonesos* and *Sarcinomyces petricola* were proven to be conspecific (Tsuneda et al. 2011) and were included in the genus *Knufia* as *K. petricola*, one of the most frequent species on stone monuments. The genus *Knufia* is also widely represented in our study and has been found in all sites investigated. The previously described species *K. petricola* was encountered in the monuments of Cagliari and the cemetery of Bonaria. Multilocus phylogeny aided to define a number of new *Knufia* species. *Knufia karalitana* and *K. mediterranea* were found in the Sardinian sites exclusively, and the latter species was restricted to the monuments of the cemetery of Bonaria. This location is characterized by the vicinity of the sea and the local preponderance of some species may be enhanced by the presence of salt. *Knufia marmoricola* in contrast had a wider distribution and occurred in all sampling sites. The new chaetothyrialean fungus *Lithophila guttulata* has a remarkably high intraspecific variability (2.4% in ITS), despite its restricted distribution on monuments of the Vatican only. Intraspecific variability is known to be rather high in RIF, mostly linked to geographic distance but sometimes high (up to 1.7 %) even between strains isolated from the same piece of rock (Egidi et al. 2014).

It has been hypothesized that the molecular diversity observed in RIF may have been promoted by the stressful conditions characterizing their natural niches. Irrespective the causes of their emergence, stress-induced mutations participate in adaptive evolution (Bjedov et al. 2003, Kang et al. 2006). This is particularly pronounced in these fungi where sexual recombination has never been observed. ~~This stress-increasing mutational rate in RIF might therefore be regarded as a genetic strategy to improve survival rates under stress conditions.~~

Two further isolates in the order Chaetothyriales were here described as new *Exophiala* species, *E. bonariae*. The genus belongs to the family Herpotrichiellaceae that includes many opportunists on humans and cold-blooded vertebrates (Badali et al. 2009, de Hoog et al. 2011), and only few stone-inhabiting fungi have been encountered. The ability of fungi in this family to metabolize hydrocarbons, particularly alkylbenzenes, may explain their success in colonizing antropogenic, polluted environments (Isola et al. 2013). This unique physiology may also play a role in human pathogenicity involving the central nervous system explained by the structural analogy of pollutants to neurotransmitters (Prenafeta-Boldú et al. 2006). Under this perspective an investigation of this ability in *E. bonariae* would be overdue.

The topology of the tree displayed in Fig. 3 is in accordance with the most recent phylogenies (Quaedvlieg et al. 2014). The two families Extremaceae and Neodevriesiaceae are well-separated and supported with high bootstrap values. One of the clades is represented by saxicolous species only and almost exclusively includes rock-inhabiting fungi, exceptionally intermingled with strains from soil forest or plants (Quaedvlieg et al. 2014). Most of the fungi belonging to the order Capnodiales studied here are included in this family and cluster in the genus *Vermiconia.* This genus (Egidi et al. 2014) exclusively comprises saxicolous species and has a worldwide distribution on rocks in Mallorca, Spain, at high altitude in the Alps and in the Antarctic desert. Strain CCFEE 5922 has been assigned to *V. flagrans*, deviating only 0.5% from this species, while the remaining 10 strains of a separate cluster were introduced as new species, *V. calcicola,* which proved to be ubiquitous in all sampling sites.

In the same family Extremaceae, a new genus and species, here named *Saxophila tyrrhenica*, is represented by four strains. One of these, CCFEE 5935 was isolated from a monument of the cemetery of Bonaria, while the remaining strains were isolated in earlier studies from natural rocks in Spain. Despite its rather wide distribution, this species shows a rather low genetic variability: strains TRN66, TRN 136 and CCFEE 5935 differed maximally 0.42% in ITS, while strain TRN65 is slightly more distant. The maximum genetic distance between the four strains was 2.1%, which is still well within the range of species variability for RIF. A strain indicated as "*Capnobotryella* sp. MA3619" was found in an external position to this group and was 12% different in ITS to the type species of *S. tyrrhenica*; this strain might, therefore, be reassigned to a different species within the genus *Saxophila*.

The family Neodevriesiaceae mostly includes plant-associated fungi and only few examples of rock-inhabiting fungi. Many of the strains isolated were located in the genus *Devriesia*, a polyphyletic genus accommodating a group of heat-resistant, cladosporium-like (Seifert et al. 2004) and plant-associated (Crous et al. 2009) fungi. Several *Devriesia*-like species were isolated from leaf litter or leaf spots, and placed in this genus based on molecular phylogenies. The genus was previously accommodated in the family Teratosphaeriaceae (Egidi et al. 2014) from which the families Extremaceae and Neodevriesiaceae have recently been segregated (Quaedvlieg et al. 2014). The general ecological trend in this group of fungi (i.e., mainly plant-association) is confirmed in the present study. Two of the RIF isolated, CCFEE 6202 and 6210, clustered in a ~~sufficiently supported~~ separate position ~~to justify the~~and were assignment to a new species, *D. sardiniae*.

Thirty of the strains isolated belonged to known species in the orders Chaetothyriales and Capnodiales (Tab. 2). All the species of the latter order were not typical RIF but rather plant-associated species: for instance fungi recovered in the monumental cemetery of Bonaria, *Aureobasidium pullulans*, *Pringshemia smilacis*, *Hortaea thailandica*, and *Verrucocladosporium dirinae*, are known to have plant- and lichen-associated life styles (Zalar et al. 2008, Crous et al. 2009). The strains were isolated from shaded monuments with mild microclimates due to the proximity of vegetation. Biological deterioration was mainly characterized by the presence of black patinas rather than biopitting or rock exfoliation, which are typical destruction patterns of rock fungi. The presence of plant-associated fungal species on rock may thus be regarded as relatively coincidental. Another external factor influencing the composition of fungal assemblages on monument surfaces is salinity. *Phaeotheca triangularis*, which combines halophily with a preference for oligotrophic conditions (Butinar et al. 2005) was found on a monument of the cemetery of Bonaria located close to the sea (less than 400 meters) and impacted by salty aerosols. All above mentioned species were not found anywhere in the sampling site of the Vatican, where vegetation is absent and the nearest beach is about 40 km away. Two of the taxa described here, *K. vaticanii* and *L. guttulata*, were found in the Vatican only and are probably able to cope with air pollution. *Knufia petricola* remains one of the recurrent species on stone monuments in the Mediterranean area (Wollenzien et al. 1997, Bogomolova and Minter 2003, Onofri et al. 2011).

In contrast to most reports on RIF, in the present study the order Chaetothyriales (class Eurotiomycetes) is predominant with 56.7% of the fungi isolated over the Capnodiales (class Dothideomycetes). In general it can be observed that in natural, unpolluted but harsher environments the tendency is mostly the opposite, with dothideomycetous rock fungi predominating. In the Antarctic lithic communities, for example, they constitute almost 70% of the black fungi isolated while chaetothyrialean fungi 7.4% only (Selbmann et al. 2014b). The different distribution of RIF in such diverse niches may be due to the general ecological traits of the fungi in these two groups. In the Dothideomycetes a diffuse adaptation to extremophily is noted and many true extremophiles cluster in this group (Gostinčar et al. 2010)~~,~~ such as the halophilic species *Hortaea werneckii* and the highly acidophilic fungus *Acidomyces acidophilus* (Selbmann et al. 2008), as well as the psychrophilic black yeasts in the genera *Cryomyces*, *Friedmanniomyces*, and *Saxomyces*. In contrast, Chaetothyriales have evolved a larger spectrum of assimilative abilities, including toxic organic compounds. This ecological versatility, rather than extremotolerance, is hypothesized to have driven diversification in this group, including the shift to opportunism (Gueidan et al. 2008). Vatican City State, being located in the middle of Rome, is the most polluted site investigated; here almost exclusively chaetothyrialean RIF were found and only 6 out of 23 strains were members of the Capnodiales, isolated exclusively on monuments in the "Cortile della Pigna", not directly exposed to the traffic jam as the St Peter colonnade.

Extreme survival and alkylbenzene degrading abilities are suggested to be the main characteristics allowing RIF to colonize stone monuments in urban environments. RIF have adopted surprising means of dispersal, since most of them lack conidia. They actively penetrate and break up the substratum by a mechanical and chemical actions by the production of siderophores (Favero-Longo et al. 2011); powdered rock, on which RIF strictly adhere by the production of extracellular polymeric substances, ~~and~~ are ~~probably~~ then dispersed as dry dust and transported by winds. This bio-deteriorating ability is one of the main forces in the natural rock weathering process (Nienow and Friedmann 1993, Brunner et al. 2011), which strongly interferes with the integrity of stone monuments. Their resistance to physical and chemical factors make the fungi extremely difficult to eradicate. More data on the biological and ecological traits of these organisms and on their biodiversity will help to deepen knowledge and may allow to develop tools to control their presence on stone monuments and to protect our cultural heritage.

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Captions

Fig. 1. Sites of sampling. A-J, Cagliari (Italy): (A) view of the monumental cemetery of Bonaria from Bonaria Hill; (B) funerary marble monument devoted Francesca Warzee; (C, D) little angels, unknown funerary marble monument and a particular of the patina covering a wing from which *Saxophila tyrrenica* CCFEE 5935 was isolated; (E, F) funerary marble monument devoted to Giuseppina Ara dei Conti Ciarella and particular of the angel's left wing from which *Vermiconia calcicola* CCFEE 5770 was isolated; (G) Autumn, one of the allegoric marble sculptures representing 'The Four Seasons' in the balustrade of the Boyl Palace; (H, I) lion sculpture in front of the Cathedral of Santa Maria and a particular of a deteriorated spot from which *Knufia karalitana* CCFEE 5656 was isolated. K-N, Vatican City State: (K, M) Aerial view of the Cortile della Pigna (K) with the Albani's Family coat of arm (L), a black fungal colonization in fissures and cracks of a marble capital (M) and a particular of the colonized travertine of the Saint Peter colonnade (N). Scale bars = 1 cm

Fig. 2. ML 2-genes multilocus phylogeny showing the phylogenetic placement of the new taxa described in the order Chaetothyriales (Class Eurotiomycetes). The tree, based on 82 sequences and 2,172 nucleotide positions, has been generated using a GTR+IG model calculated using ML in the software MrAIC. Bootstrap values above 85%, calculated from 1,000 resampled data sets, are shown.

Fig. 3. ML 3-genes multilocus phylogeny showing the phylogenetic positions of the taxa described in the order Capnodiales (class Dothideomycetes). The tree, based on 60 sequences and 2,098 nucleotide positions, has been generated using a GTR+IG model calculated using ML in the software MrAIC. Bootstrap values above 85%, calculated from 1,000 resampled data sets, are shown.

Fig 4. A-H *Devriesia karalitana* CBS 139724=CCFEE 6202: (A) colony appearance; (B, E-H) meristematic development of dark brown, thick-walled conidia with longitudinal and oblique septa (white arrowheads), and germinating meristematic conidia (E); (D) wider and darker hyphae; (C, I) subhyaline, closely septate, branched young hyphae and anastomoses (black arrowheads). J-Q *Exophiala bonariae* CBS XXXX = CCFEE 5792: (J) colony appearance; (K-Q) hyphae, conidiophores and conidia; (M) spirally twisted hypha; (P, Q) cylindrical, hyaline and simple conidia, numerous at the conidiophore apex. R-X *Knufia karalitana* CBS XXXX = CCFEE 5656: (R) colony appearance; (S) acropetal moniliform hyphae; (T, U, W) chains of globose and subglobose blastic conidia; (V, X) thick walled rugose cells and terminal and intercalar multicellular bodies containing endoconidia (arrowhead). Scale bars 20 μm.

Fig 5. A-H *Knufia marmoricola* CBS 139726= CCFEE 5895: (A) colony appearance; (B, E, F) moniliform branched hyphae and multicellular bodies; (C) pale brown and mature chains of blastic conidia; (D, G) enlarged, thick walled and brown bodies containing endoconidia (G, arrowhead); (H) endoconidia released by rupture of a roundish body. I-P *Knufia mediterranea* CBS XXXX = CCFEE 5738: (I) colony appearance; (J-L) pale brown moniliform hyphae with composed of ellipsoidal to cylindrical cells, budding apically and laterally; (M) dense aggregates of roundish dark cells containing endoconidia (arrowhead); (N) globose thick walled cell with endoconidia; (O, P) enteroblastic proliferations and budding cells. Q-X *Knufia vaticanii* CBS XXXX = CCFEE 5939: (Q) colony appearance; (R, W) filamentous hyphae formed by small roundish or cylindrical catenate cells, finely guttulate; (S-V) torulose hyphae, producing terminal and intercalary multicellular arthroconidia; (X) torulose hyphae and arthroconidia (arrowhead).

Fig 6. A-G *Litophila guttulata* CBS XXXX = CCFEE 5907: (A) colony appearance; (B) network of hyphae of different morphologies; (C-G) branched, catenate and finely guttulate hyphae, with intercalar and apical globose cells; (D) apical globose cell. H-O *Saxophila tyrrenica* CBS XXXX =CCFEE 5935: (H) colony appearance; (I-O) filamentous, sometime geniculate, hyphae, composed of simple to 1-septate cells; (K) septate hyphae and anastomoses (arrowheads); M-O terminal or intercalar multicellular bodies (arrowheads). P-V *Vermiconia calcicola* CBS XXXX=CCFEE 5770: (P) colony appearance; (Q-V) moniliform branched hyphae; (R, S, U) terminal and intercalary cylindrical arthroconidia and occasional subglobose pluriseptate conidia in terminal position (S). Scale bars 20 μm.

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Extremotolerant rock inhabiting black fungi from Italian monumental sites

D. Isola - L. Zucconi - S. Onofri - G. Caneva - G.S. de Hoog - L. Selbmann

L. Selbmann (✉) - D. Isola - L. Zucconi - S. Onofri
DEB, Università degli Studi della Tuscia, Largo dell'Università, Viterbo, Italy
e-mail: selbmann@unitus.it

G. Caneva
Department of Sciences, Università Roma Tre, Viale Marconi 446, 00146 Roma, Italy

G.S. de Hoog
CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

Abstract A wide sampling for isolating highly destructive and extremotolerant black fungi was performed from Italian monuments in selected historical sites which include the "Cortile della Pigna", Vatican Museum and the St Peter colonnade (Vatican City State), the monumental cemetery of Bonaria (Cagliari), and other monuments in the city of Cagliari. Thirty out of seventy-four strains isolated were identified basing on ITS Blastn comparison. Based on multilocus phylogeny and morphological data, one new genus and species *Lithophila guttulata*, five new species *Knufia marmoricola*, *K. vaticanii*, *K. karalitana*, *K. mediterranea* and *Exophiala bonariae*, order Chaetothyriales and one new genus and species, *Saxophila tyrrhenica*, and two new species *Vermiconia calcicola* and *Devriesia sardiniae*, order Capnodiales, were proposed. Ecological considerations are put forward.

Taxonomic novelties: *Devriesia sardiniae* Isola & de Hoog **sp. nov.**; *Exophiala bonariae* Isola & Zucconi **sp. nov.**; *Knufia karalitana* Isola & Onofri **sp. nov.**; *Knufia marmoricola* Onofri & Zucconi **sp. nov.**; *Knufia mediterranea* Selbmann & Zucconi **sp. nov.**; *Knufia vaticanii* Zucconi & Onofri **sp. nov.**; *Lithophila* Selbmann & Isola **gen. nov.**; *Lithophila guttulata* Selbmann & Isola **sp. nov.**; *Saxophila* Selbmann & de Hoog **gen. nov.**; *Saxophila tyrrhenica* Selbmann & de Hoog **sp. nov.**; *Vermiconia calcicola* de Hoog & Onofri **sp. nov.**

Key words
Extremotolerance – Monuments – Microcolonial fungi – Multilocus phylogeny

Introduction

'Black yeasts and relatives', 'meristematic fungi', or 'microcolonial fungi' are frequently used terms to indicate a group of fungi having highly melanized cell walls, producing daughter cells by yeast-like, or monopolar or multilateral budding, and being able to shift from a mycelial to a meristematic growth by longitudinal and transversal septum formation. These fungi are phylogenetically heterogeneous, including entities that belong to different orders of Ascomycota (de Hoog and McGinnis 1987). Meristematic growth is infrequent in the fungal kingdom and can be viewed as a specific response to external stress. Conversion towards isodiametric expansion can be induced in hyphal or yeast-like thalli of herpotrichiellaceous fungi by environmental factors such as acidification of the culture medium (Mendoza et al. 1993). Similar observations have been made in Dothideomycetes (de Hoog et al. 1999). In some cases, this can become a stable character (Matsumoto et al. 1986) as for black yeasts living under permanent stress (Selbmann et al. 2005, 2008). Also permanently meristematic mutants are known in species that normally grow as filamentous fungi (Xi et al. 2009). Black yeasts are typically extremotolerant or extremophilic, being able to tolerate stress factors such as high and low temperatures, drought, osmotic stress, high solar and UV radiation or prolonged period of water deficiency (Sterflinger 2006, Selbmann et al. 2008, 2011, 2013, 2014a, Zakharova et al. 2013); some of them may even survive simulated and real space conditions (Onofri et al. 2008, 2012). Their ecology reflects these abilities and they occur in environments mostly precluded to others as saltpans (Plemenitaš and Gunde-Cimerman 2005), acidic and hydrocarbon-contaminated sites (Baker et al. 2004, Seyedmousavi et al. 2011, Selbmann et al. 2013, Isola et al. 2013) and exposed rocks. Rocks are characterized by multiple stress factors as oligotrophic conditions, high or low and changing temperatures, UV-radiation, osmotic stress and are, therefore, very hard to colonize. Nevertheless, rock is one of the most recurrent substrates for specialized melanized fungi, and these lithobionts, also known as 'rock inhabiting fungi' (RIF), are the most extremotolerant among black yeasts (Selbmann et al. 2014b). Originally they have been found in the rocks of the hot Arizona desert (USA) and of the extremely cold Antarctic deserts (Friedmann 1982, Staley et al. 1982), but were later commonly isolated in the Mediterranean area from exposed natural rocks (Ruibal et al. 2005) and monuments (Sert et al. 2007a, b, c, Marvasi et al. 2012, Onofri et al. 2014) and other temperate bioclimates (Wollenzein et al. 1995, Salvadori 2000, Ascaso et al. 2008, Caneva et al. 2013). When live on monuments, RIF must cope with sun exposition, prolonged periods of drought and nutrient deficiency, as well as with the additional stress of pollution, since monuments are often located in urban environments. RIF are actually one of the most destructive and scarcely investigated groups of microorganisms causing deterioration of monuments exposed to outdoor conditions (Isola et al. 2013b, Onofri et al. 2014). They cause esthetical damage as a consequence of melanin production and mechanical destruction leading to exfoliation of stone artworks and biopitting (Sterflinger and Krumbein 1997, Lombardozzi et al. 2012). They represent a serious challenge for restorers because of the strong destructive potential and the high resistance to many types of chemical treatment (Isola et al. 2013b).

Because of their role in deterioration of monuments, a great deal of interest is being paid to species growing exclusively on rock and causing visible biodeterioration patterns. Only in relatively few cases the fungal communities on rock surfaces were completely inventoried and still fewer species have been described and characterized phylogenetically. Earlier studies concerned marble monuments from historical sites in Greece (Sterflinger and Krumbein 1997, Wollenzien et al. 1995, 1997), more recently from Ukraine and Turkey (Bogomolova and Minter 2003, Sert et al. 2007a, b, c) and, to a less extent, Italy (De Leo et al. 1999, 2003, Zucconi et al. 2012).

Due to its long and varied history, Italy hosts the largest number of UNESCO World Heritage Sites; high art and monuments are distributed everywhere along the country. Despite this huge cultural heritage and the risk that black fungi may represent, a deep and accurate sampling in historical areas has never been conducted. In the frame of a collaboration for the preservation of art-works in some Italian historical sites, the Vatican, the monumental cemetery of Bonaria (Cagliari) and some monuments in the city of Cagliari, have been sampled for isolating biodeteriogens. The aim of the present study was to document the biodiversity of extremotolerant RIF on stone monuments of these sites; here rock colonizers must cope with additional strong stresses of high levels of pollution in the Vatican and high salt concentrations due to the proximity of the sea in the other sites. New genera and species were defined by multilocus phylogeny.

Materials and methods

Studied areas

Vatican City State

The Vatican City State is a walled enclave located within the city of Rome, being comprised in the transitional Mediterranean region; it is characterized by medium Meso-Mediterranean thermo type with hot and dry summers, mild winter temperatures and rainfalls that are concentrated in autumn and winter (Blasi, 2001).

It is the smallest country in the world and, being the center of catholic religion, is characterized by invaluable monuments and historical buildings. Over centuries, the Vatican has had official artists conceiving masterpieces in the name of the Catholic Church and these nowadays are exposed in the Vatican Museum together with roman stone-works of art and others coming from ancient Egypt, Middle East and Etruria.

Two famous areas were sampled in the Vatican City State; the first sampling concerned 8 ancient Roman stone artworks: a sarcophagus, four capitals, the Albani family's coat of arms in exedra designed by Pirro Ligorio, and two memorial funeral stones exposed along the perimeter of the "Cortile della Pigna"; this is one of the three courtyards of the Vatican Museum, named after the four meters high bronze pine cone placed on a double staircase, dated I-II century a. C., found in the middle age at the Thermae of Agrippa. The second sampling was performed at St Peter square from the Bernini's colonnade. Known worldwide, the square was built by Bernini for Pope Alexander VII in 1656-67. The colonnade consists of 284 majestic Doric columns and 88 pillars of white, porous travertine carved from "Piani di San Clemente", Tivoli, Rome.

Cagliari - Cemetery of Bonaria and historical buildings sampled

The city of Cagliari is situated in the south of Sardinia and occupies the flat spaces between the hills and the sea to the south and south-east, in an articulate landscape including beaches, lagoons and mountains surrounding the east part of the city. Cagliari is characterized by a thermo-Mediterranean bioclimate, with a low pluviometry, hot, dry and long summers and very mild winters (Grapow and Blasi 1998). The humidity may be high due to the proximity of the sea, ranging between 65% and 95% in winter and between 55% and 85% in summer. Dominant winds blow from south (Sirocco, Libeccio) and north-west (Mistral) transporting red dust from Sahara and marine aerosols, that constitute abundant natural pollutant across the urban area.

<u>The monumental cemetery of Bonaria</u> rises on the homonymous hill less than 400 meters from the sea; it is located in the proximity of a necropolis remains in an area populated since the Punic, Roman and early Christian times. The cemetery, inaugurated on January 1829, is included in the Association of Significant Cemeteries in Europe (ASCE) and preserves a huge number of funeral monuments created by many artists. Beyond the artistic value of stone-works, the cemetery of Bonaria also represents the open-air memory of many political, cultural, literary, scientific, military, religious and commercial people that made the history of Cagliari at the turn of 1800-1900. As other monumental cemeteries, it is reach of vegetation; among the botanical species present, cypresses are dominant with the species *Cupressus sempervirens*, *C. cashmeriana*, and *C. macrocarpa*. The vegetation also includes species of palms such as *Phoenix canariensis, P. dactilifera*, *Dracaena indivisa*, evergreen-oaks, Japanese maples (*Acer palmatum*), carobs (*Ceratonia siliqua)*, pines (*Pinus pinea*, *P. halepensis*), roses, hibiscus (*Hybiscus mutabilis*), pittosporum (*Pittosporum tobira*), oleanders (*Nerium oleander*), mastic trees (*Pistacia lentiscus*), Judas' trees (*Cercis siliquastrum*), and *Clerodendrum trichotomum*.

<u>Boyl Palace</u> is one of the most representative noble buildings of the district of "Castello" in Cagliari. It was erected in 1840 by Carlo Boyl Pilo, Marquis of Putifigari. The palace has a marble

balustrade, decorated with four statues representing the seasons and, in a central position the coat of arms.

The Cagliari Cathedral, or the Cathedral of Santa Maria, was built in the 13^{th} century in Pisan-Romanesque style, obtaining cathedral status in 1258. In the 17th and 18th centuries it was renovated along Baroque lines and the current façade was constructed in the 1930s in Neo-Romanesque style. The other monuments sampled in Cagliari were Bastion Saint Remy, the lions outside of the Cathedral of Santa Maria, the Tower of the Elephant, Porta Cristina and the columns of St Saturnino Church.

Some of the artworks and historical buildings sampled in this study are shown in Fig. 1.

Sample collection and isolation

Biological samples were taken from vertical surfaces of the stone monuments using sterile needles (Wollenzien et al. 1995) and sterile swabs moistened with 50 µl of distilled water. Samples were plated on dichloran-rose bengal agar (DRBC, Oxoid, Basingstoke, Hampshire, U.K.) supplemented with chloramphenicol 100 ppm to prevent bacterial growth; plates were incubated at 20 °C and inspected every day. As soon as they appeared, black colonies were transferred onto fresh Malt Agar 4% (MA) slants and incubated at 20 °C.

Holotypes were deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands; ex-types are preserved in the Culture Collection of Fungi from Extreme Environments (CCFEE) in Viterbo, Italy

Morphology

Morphology was studied using slide cultures. Inoculation was performed onto 2 cm^2 water agar blocks, incubated for 2 weeks at 20 ºC in Petri dishes with wet sterile paper filter to avoid drying out of the medium; agar blocks with inoculated fungi were then transferred onto slides with lactophenol and observed with light microscope. Digital images of the samples were acquired using Digital Nikon Coolpix 5000 and digitally optimized with the software Micromax Arkon (v. 8.12.05). Colony descriptions are based on cultures grown on Malt Agar 4% (MA) incubated at 20 °C for 3 weeks.

DNA extraction, PCR amplification, and DNA sequencing

DNA was extracted from mycelium grown on MA slants at 20 °C, using Nucleospin Plant kit (Macherey-Nagel, Düren, Germany), following the protocol optimized for fungi. PCR reactions were performed using BioMix (BioLine, Luckenwalde, Germany). In each 25 µL reaction solution, 5 pmol of each primer and 40 ng of template DNA were added. Amplifications were carried out using MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Munich, Germany). Three nuclear (ITS, nucLSU, nucSSU) and one protein encoding (RPB2) genes were amplified, according to Egidi et al. (2014). Fragments were analysed by Macrogen Inc. (Seoul, Korea). Sequence assembly was done using the software CHROMASPRO v. 1.32 (Technelysium, Southport, Queensland, Australia). Sequence data were deposited in GenBank (Tab. 1).

GenBank comparison, alignment and tree reconstruction

Sequences were compared in the GenBank using BLASTN search (Altschul et al. 1997). The sequences in the GenBank that could be confidently aligned were chosen for multilocus analyses. In order to define the phylogenetic position of the isolates in the order Capnodiales (class Dothideomycetes) and in the order Chaetothyriales (class Eurotiomycetes), two multilocus datasets were generated. For the former, nucLSU, ITS and RPB2 gene targets were chosen for constructing the alignment, while nucLSU and ITS were used for the latter. Sequences were aligned iteratively with MUSCLE (Edgar 2004) in MEGA6 (Tamura et al. 2013) and final alignment was improved manually. One-gene alignments were concatenated using FABOX (http://users-birc.au.dk/biopv/php/fabox/alignment_joiner.php, Villesen 2007) to combine sequences for two bi- and three-locus datasets. Optimized alignments were exported and the best-fit substitution model was determined using MODELTEST MrAIC 1.4.3 (Nylander 2004) as implemented in PHYML (Guindon and Gascuel 2003). Phylogenetic trees were reconstructed by Maximum Likelihood, using TREEFINDER (Jobb et al. 2004) and the resulting tree was visualized using TREEVIEW v. 1.6.6 (Page 1996). The robustness of the phylogenetic inference was estimated using the bootstrap method (Felsenstein 1985) with 1,000 pseudoreplicates generated and analysed with TREEFINDER.

Results

Molecular analyses

Blast identification

Twenty-seven strains isolated from the artworks in Cagliari (21 from the cemetery of Bonaria, 6 from monuments in the city) and two isolated from the “Cortile della Pigna”, Vatican City State, were identified using BLASTN search (Tab 2). Among Capnodiales, strains CCFEE 5660 and 5737 from monuments in Cagliari and CCFEE 5924 and 5927 from monuments in the “Cortile della Pigna” (Vatican City State) were identified as *Coniosporium uncinatum*, a species recurrent on monuments (Sterflinger et al. 1997), while strains CCFEE 5723, 5735 and 5919 from the cemetery of Bonaria and CCFEE 5565 from the city of Cagliari were identified as *Hortaea thailandica*.

Strains CCFEE 5703 and 5706 identified as *Phaeotheca triangularis* were isolated from the cemetery of Bonaria only as well as *Verrucocladosporium dirinae* (strains CCFEE 5707 and 5708). The strains CCFEE 5704 and 5922 belonged to the recently described species *Devriesia bulbillosa* and *Vermiconia flagrans*, respectively (Egidi et al. 2014), the latter, and the two strains of *C. uncinatum*, were the only fungi isolated from stoneworks of the Vatican belonging to known species. *Aureobasidium pullulans* (CCFEE 5736), *Austroafricana parva* (CCFEE 5742), *Pringsheimia smilacis* (CCFEE 5778), and *Teratosphaeria capensis* were found in monuments of Cagliari and the cemetery of Bonaria. *Knufia petricola* (Tab 2; CCFEE 5702, 5705, 5709, 5740, 5741, 5776, 5777, and 5937), isolated from many monuments in Cagliari and the cemetery of Bonaria, was one of the most frequent RIF species in this study.

Multi-locus phylogeny

Molecular phylogeny was performed to define the phylogenetic position of the strains with low identities in the GenBank. The two-gene dataset included 82 strains belonging to the order Chaetothyriales (Tab. 1). Introns were delimited manually and excluded from the alignment, so the combined dataset included 2,172 positions (ITS: 1–759; nucLSU: 760–2,172) including gaps. Fig. 2 shows the multilocus phylogenetic tree generated using a GTR+G (4) model, selected using the Akaike’s information criterion with a Maximum-likelihood approach. The base frequencies were as follows: T=0.2371581, C=0.2366716, A=0.24823, G=0.27794, TC=0.4013773, TA=0.1540797; TG=0.080884; CA=0.1273652; CG=0.099919; AG=0.1363733. The tree was rooted with *Cladophialophora humicola* CBS 117536, Epibryaceae (Gueidan et al. 2014).

The topology of our tree is in accordance with the most recent phylogenetic studies on the Chaetothyriales (Chomnunti et al. 2012, Guiedan et al. 2014).

The genus *Knufia* was well supported in the tree (Fig. 2); within the genus, two groups were discernable. The first comprised 10 strains isolated from all localities sampled in this study, and the second, with five strains originated exclusively from monuments of the city of Cagliari, were assigned to two separate species, *K. marmoricola* and *K. karalitana*, respectively. A further strain, CCFEE 5939, isolated from the St Peter colonnade, Vatican City State, was in a separated position sister to *K. perforans* and was assigned to the new species *K. vaticanii*. Within the genus *Knufia* there was a further supported clade (99.8% bootstrap) comprising 4 strains isolated exclusively from the cemetery of Bonaria. These strains were described here as the new species *K. mediterranea*, having a very low intraspecific ITS variability (0.5%). A well-separated cluster of eight strains isolated from monuments of the Vatican only, supported with 100% bootstrap, was in a basal position of the family Trichomeriaceae and here described as new genus and species

Lithophila guttulata, with 2.4% ITS variability within the group. Finally, strains CCFEE 5792 and 5899 from the cemetery of Bonaria pooled together in the genus *Exophiala*, family Herpotrichiellaceae and were here described as a new species, *E. bonariae*.

The three-gene dataset included 60 strains belonging to the order Capnodiales. After exclusion of ambiguous regions and introns, the combined dataset included 2,098 positions (ITS: 1–545; *nuc*LSU: 546–1,781; RPB2: 1,782–2098), including gaps. Fig. 3 shows the multilocus phylogenetic tree generated using a GTR+G (4) model, which was selected using the Akaike's information criterion with a Maximum-likelihood approach. The base frequencies were as follows: T=0.2267053, C=0.2481356, A=0.2457273, G=0.2794318, TC=0.365155, TA=0.1365375; TG=0.09423267; CA=0.1193942; CG=0.1099502; AG=0.1747303. The tree was rooted with strains belonging to the family Cladosporiaceae as closest outgroup (Quaedvlieg et al. 2014): *Cladosporium langeronii* R1 (ITS accession number: HQ115727; Gorfer et al. 2011); *Cladosporium sphaerospermum* (ITS accession number: L25433; Curtis et al. 1994); *Davidiella tassiana* ATCC 26362 (Park et al. 2004).

The topology of the tree is in accordance with the most recent phylogenetic studies allocating species in the Teratosphaeriaceae and related families (Quaedvlieg et al. 2014).Fifteen of the strains studied pooled within the genus *Vermiconia* (100% bootstrap) (Tab. 1). Ten of these, pooling in a single cluster, showed 4.2% of variability in the ITS sequences; this value is within the range of species genetic variability determined for rock fungi (Egidi et al. 2014) and the strains were therefore assigned to the new species *V. calcicola*. Two rock strains, MA 4902 and MA 3615, isolated from the Turkish historical site of Perge, Antalya and from a monument in Vienna, Austria, respectively and indicated as *Capnobotryella* species, cluster in the same group and might be assigned to the same species. Strain CCFEE 5922 was in an external position with respect the new species; it was assigned to *V. flagrans* having 2 polymorphisms only in the ITS sequence compared to the type strain CBS 118283.

Strain CCFEE 5935, isolated from the cemetery of Bonaria, formed a separate, well-supported group together with three rock-inhabiting strains found on limestone at Mallorca, Spain. The group is sister to the recently described genus *Extremus* in a poorly resolved position in the tree and is here indicated as a new genus, *Saxophila*. The last two capnodialean species, both from the cemetery of Bonaria, formed a well-supported group in the family Neodevriesiaceae (Quaedvlieg et al. 2014) and were here indicated as the new species *Devriesia sardiniae*.

Taxonomy

Descriptions based on 3-week-old cultures grown on MA at 20 °C.

Devriesia sardiniae Isola & de Hoog, **sp. nov**. MycoBank MB811669, Fig. 4 A-I

Etymology: named after Sardinia, the island where the strain was isolated.

Diagnosis: Colonies growing slowly, up to 10 mm diam mm, cerebriform, often deeply immersed into the agar, olivaceous brown, with a velvety mouse-grey aerial short hyphae and well-defined black and glossy margin; black in reverse.

Mycelium consisting of subhyaline, closely septate, branched hyphae, (3.5–5.0 µm) with occasional anastomoses; hyphae gradually widening and darkening, forming brown, closely septate, constricted at septa, thick-walled, simple (6.5–7.0 × 4.5–4.8 µm) and 1-septate (12.0 × 6.5 µm) cells. Moniliform hyphae present. Meristematic conidia terminal, lateral and intercalary, with transverse, longitudinal and oblique septa, dark brown, thick-walled, rugose, globose to pyriform, measuring 8.5–16.5 × 7.5–13.5 µm.

Holotypus: CBS 139724=CCFEE 6202, isolated from a marble cross (Frau-Carta funerary monument) in the cemetery of Bonaria (Cagliari, Italy). Culture preserved in liquid nitrogen and in dried condition.

Exophiala bonariae Isola & Zucconi, **sp. nov**. MycoBank MB811668, Fig. 4 J-Q

Etymology: named after the cemetery of Bonaria, where the type strain was isolated.

Diagnosis: Colonies growing slowly, up to 20 mm, convex, circular, dark brown, with a velvety mouse-grey aerial short hyphae, pigment diffuse around the colony; dark brown in reverse.

Mycelium composed of short and brown hyphae in the central area, from which originate diffuse, slender, hairy and thin-walled hyphae, rarely branched, seldom spirally twisted, occasionally circinate, pale brown, with rare anastomoses. Conidiophores poorly differentiated, subhayline. Conidiogenous cells phialidic, occasionally slender up to 2.4 × 3.5 µm, hyaline towards the apex. Conidia numerous, hyaline, simple, thin-walled, mostly cylindrical, 5.5–6.5 × 2.2–2.8 µm, often grouped near the apex of the conidiogenous cells.

Holotypus: CBS 139957=CCFEE 5792, named after the cemetery of Bonaria where the strain was isolated. Culture preserved in liquid nitrogen and in dried condition.

Knufia karalitana Isola & Onofri, **sp. nov**. MycoBank MB811670, Fig. 4 R-X

Etymology: named after the ancient name of Cagliari (*Karalis*), the city where the strain was isolated.

Diagnosis: Colonies growing very slowly, up to 8 mm, black, raised,
often deeply immersed into the agar, with short, scarce, velvety, greyish-brown aerial hyphae; margin irregular; black in reverse.
Hyphae moniliform, occasionally toruloid, brown. Conidiophores micronematous. Conidia blastic or endogenous; blastic conidia terminal and intercalary, dark brown, formed by differentiation of hyphal cells which enlarge, forming mostly spherical (6.5–10.0 µm), thick-walled, rugose 1-celled, sometimes bicellular and ellipsoidal conidia (10–12 × 6.5–9.0 µm) or evolving in multicellular roundish, thick-walled, rugose bodies (10.0–12.0 µm) containing endoconidia.

Notes: conidia and hyphal fragments may secede both rhexolytically and occasionally schizolytically, and enteroblastic growth may occur by budding of hyphal cells or blastic conidia.

Holotypus: CBS 139720=CCFEE 5656, Cagliari (Italy), isolated from marble lion in front of the Cathedral of Santa Maria. Culture preserved in liquid nitrogen and in dried condition.

Knufia marmoricola Onofri & Zucconi, **sp. nov**. MycoBank MB811671, Fig. 5 A-H
Etymology: named after the substratum, from which the type strain was isolated.

Diagnosis: Colonies growing very slowly, up to 8 mm, black, raised centrally, flat and glossy near the periphery, even growing in depth, velvety with grayish-brown aerial short hyphae and black crenate margin; black in reverse.
Hyphae moniliform, branched, cylindrical, smooth, 5.0–5.5 µm wide, straight to slightly curved, enteroblastically proliferating, with anastomoses, pale brown at first, darker when mature. Conidia blastic or endogenous; blastic conidiadark brown, mostly spherical 5.5 - 8 µm in diam, thick-walled, smooth, formed by differentiation of terminal and intercalary hyphal cells; endoconidia unicellular, globose (4.4–5.0 µm diam), developing within hyphal cells, which enlarge up to form roundish, thick- and smooth walled, mostly spherical, bodies, 7.5–15.5 µm wide.

Holotypus: CBS XXX=CCFEE 5895, isolated from travertine of St Peter colonnade (Vatican City State). Culture preserved in liquid nitrogen and in dried condition.

Knufia mediterranea Selbmann & Zucconi, **sp. nov**. MycoBank MB811672, Fig. 5 I-P
Etymology: named after the Mediterranean basin, the site from which the strain was isolated.

Diagnosis: Colonies slow growing up to 12 mm, black, cerebriform moist, glossy, later slightly crunchy with some greenish gray, velvety aerial mycelium and sharp, irregularly lobate margin; black in reverse.
Mycelium composed of scant filamentous hyphae and mostly moniliform, enteroblastically proliferating, catenate cells. Moniliform hyphae subhyaline to pale brown, branched, consisting of broadly ellipsoidal to cylindrical 0 -1 septate cells, apically and laterally budding to form dense and darker aggregates, with flat scars due to rhexolythic secession. Cells later becoming darker, roundish, most frequently cylindrical and 1-septate, slightly constricted at septa, 9.0–14.0 × 7.0–8.0 µm.

Holotypus: CBS 139721=CCFEE 5738, isolated from Francesca Warzee funerary marble monument, cemetery of Bonaria (Cagliari, Italy). Culture preserved in liquid nitrogen and in dried condition.

Knufia vaticanii Zucconi & Onofri, **sp. nov**. MycoBank MB811673, Fig. 5 Q-X.
Etymology: named after the Vatican City State, where the type strain was isolated.

Diagnosis: Colonies growing very slowly, up to 10 mm, brownish black, cerebriform, covered by a dense, velvety, greyish-brown aerial mycelium, with sharp lobate margin; black in reverse. Mycelium initially composed of pale brown, branched, finely guttulate, filamentous hyphae, formed by small simple and roundish to 1-septate and cylindrical, catenate cells, enteroblastically proliferating, gradually developing into torulose hyphae producing swollen arthroconidia. Arthroconidia terminal and intercalary, dark brown, thick-walled, globose when simple (8.5–10.0 µm diam), cylindrical when bicellular (10.0–12.0 × 6.5–10.0 µm), globose to ovoid when multicellular, with longitudinal and oblique septa (13.0–16.5 × 9.0–17.0 µm).

Notes: The presence of small droplets in mycelial cells is limited to early growth stages. Each cell of which arthroconidia are formed may germinate and produce filamentous hyphae; cracks in outer cell wall layers are sometimes present, similarly to multicellular conidia of *Cryomyces antarcticus* and *Knufia petricola*.

Holotypus: CBS 139722=CCFEE 5939, isolated from travertine St Peter colonnade (Vatican City State). Culture preserved in liquid nitrogen and in dried condition.

Lithophila Selbmann & Isola **gen. nov**. MycoBank MB811675
Etymology: named after rock substrate where it was isolated from.

Diagnosis: Colonies growing slowly, cauliflower-shaped, dark brown, velvety with irregularly lobate margin in obverse; black in reverse. Hyphae branched, composed of irregularly shaped, simple to pluriseptate, catenate cells. Teleomorph unknown.

Type species: *Lithophila guttulata* Selbmann & Isola

Lithophila guttulata Selbmann & Isola, **sp. nov**. MycoBank MB811676, Fig. 6 A-G
Etymology: named after its guttulate cells.

Diagnosis: Colonies growing slowly, up to 11 mm, dark brown, cauliflower-shaped, finely velvety, with sharp and irregularly lobate margin; black in reverse.
Hyphae pale brown, diffusely guttulate, partly filamentous, partly composed of irregularly shaped, catenate cells produced by enteroblastic proliferation. Globose cells frequently produced intercalarly, terminarly or laterally 7.0–10.5 µm at first, gradually turning dark, thick, 1-multicellular widening up to 15.5 µm, sometimes with endoconidia.

Notes: globose, dark cells generally enlarge to form multicellular bodies which probably function as resistance structures.

Holotypus: CBS 139723=CCFEE 5907, isolated from marble stone (cat. 37106) exposed in the Vatican Museums – "Cortile della Pigna" (Vatican City State). Culture preserved in liquid nitrogen and in dried condition.

Saxophila Selbmann & de Hoog, **gen. nov**. MycoBank MB811677
Etymology: named after the rock substrate where it was isolated.

Diagnosis: Colonies growing slowly, dark in reverse, brown and velvety, with undulate margin in obverse. Hyphae curved, branched, repeatedly septate. Conidiophores micronematous. Conidia thallic-arthric. Teleomorph unknown.

Type species: *Saxophila tyrrhenica* Selbmann & de Hoog.

Saxophila tyrrhenica Selbmann & de Hoog, **sp. nov**. MycoBank MB811678, Fig. 6 H-O
Etymology: named after the Tyrrhenian basin, the site from which the strain was isolated.

Diagnosis: Colonies slow growing, up to 10 mm, brown and velvety in the obverse, raised centrally, flat near the periphery, with undulate margin; dark in reverse.
Hyphae smooth, straight, curved to geniculate, pale brown, branched, with occasional anastomoses, holoblastically proliferating, consisting of spherical cells when simple, cylindrical when 1-septate, eventually disarticulating. Conidiophores micronematous, conidia arthric. Globose to ellipsoidal, brown, multicellular bodies (10.0–12.0 × 15.0–20.0 µm) terminally or intercalarly produced in late stages. Teleomorph unknown

Holotypus: type culture CBS 139725=CCFEE 5935, isolated from little marble angels of an anonymous funerary monument in the cemetery of Bonaria (Cagliari, Italy). Culture preserved in liquid nitrogen and in dried condition.

Vermiconia calcicola de Hoog & Onofri, **sp. nov**. MycoBank MB811679 Fig. 6 P-V
Etymology: named after the substrate where the type strain was isolated.

Diagnosis: Colonies growing very slowly, up to 4.5 mm, black, cerebriform, raised centrally, growing deeply into the agar, velvety with short, brownish-green aerial hyphae, with dark brown flat, sharp and lobate margin; black in reverse.
Mycelium composed of filamentous to moniliform, branched hyphae, subhyaline to pale brown, with cells of irregular shape, with frequent anastomoses; abundant oil drops present in young hyphae. Conidiophores micronematous. Arthroconidia terminal and intercalary, dark brown, thick-walled, smooth, cylindrical, mostly 1-2 septate, 6.5–12.0 × 5.5–7.5 µm, sometimes also subglobose, with transverse, longitudinal and oblique septa, mostly in terminal position, up to 14 µm wide.

Holotypus: CBS XXX=CCFEE 5770, isolated from Giuseppina Ara funerary marble monument in the cemetery of Bonaria (Cagliari, Italy). Culture preserved in liquid nitrogen and in dried condition.

Discussion

Exposed rocks harbor an unexpected biodiversity of black fungi. Dozens of species were isolated from natural rocks in Spain (Ruibal 2004, Ruibal et al. 2005, 2008) and even from very inhospitable environments such as hot dry deserts of Arizona and Negev (Staley et al. 1982) and very cold areas of the Alps, Andes and Antarctica (Selbmann et al. 2005, 2008, 2014c, Isola 2010). Under strong environmental pressure and extreme isolation, speciation processes are promoted. New species are constantly being reported, even from the easily accessible Mediterranean area (Egidi et al. 2014).

Biodeterioration of the stone cultural heritage in Mediterranean countries has long been an area of concern. Rock-inhabiting fungi have been repeatedly isolated from stone monuments in Greece, Turkey (e.g. Gorbushina et al. 1993, Sterflinger et al. 1997, Bogomolova and Minter 2003, Sert et al. 2007a, b, c, Sert and Sterflinger 2010) and, to a less extent, Georgia (Caneva et al. 2013) and Italy (De Leo et al. 2003, Marvasi et al. 2010, Onofri et al. 2011). Some RIF colonizing monuments in urban environments are prone to additional external stresses than sun exposition, oligotrophy, and prolonged dryness, due to air pollution, while others may have a competitive advantage by their ability to assimilate toxic hydrocarbons (Prenafeta et al. 2006). The present study focuses on RIF from artworks of historical sites in Italy which had not been investigated before.

All RIF isolated in this study belonged to the orders Capnodiales and Chaetothyriales, two orders that are known to be overrepresented in rock-inhabiting black fungi (Gueidan et al. 2008, Ruibal et al. 2008, 2009, Egidi et al. 2014). All families included in the tree shown in Fig. 2 were well-resolved and the topology was in accordance with the most recent phylogenetic studies on the order Chaetothyriales (Réblová et al 2013, Gueidan et al. 2014). Most of the new species described here were included in the family Trichomeriaceae which was recently classified in the order Chaetothyriales (Chomnunti et al. 2012). The family includes genera of diverse ecologies: *Trichomerium* comprises epiphytic fungi, and *Metulocladosporiella* species are plant pathogens (Crous et al. 2006). The two species of the recently described genus *Bradymyces* (Hubka et al. 2014), *B. oncorhynchi* and *B. alpinus* have quite different ecologies: the first was found in a hyperhaemic focus near the spleen of rainbow trout (*Oncorhynchus mykiss*) and the second shows a saxicolous habitus, having been isolated from Alpine rocks. The genus *Knufia* (Tsuneda et al. 2011) includes ecologically diverse species ranging from lichenicolous taxa as *K. peltigerae*, human opportunistics colonizing human skin as *K. epidermidis* (Li et al. 2008, Li & Chen 2010), insect-associated species as *K. aspidioti* (He et al. 2013), and plant pathogens as *K. cryptophialidica* and *K. endospora* (Hutchison et al. 1995, Tsuneda and Currah 2004). In the same family, the saxicolous species *Phaeococcomyces chersonesos* and *Sarcinomyces petricola* were proven to be conspecific (Tsuneda et al. 2011) and were included in the genus *Knufia* as *K. petricola*, one of the most frequent species on stone monuments. The genus *Knufia* is also widely represented in our study and has been found in all sites investigated. The previously described species *K. petricola* was encountered in the monuments of Cagliari and the cemetery of Bonaria. Multilocus phylogeny aided to define a number of new *Knufia* species. *Knufia karalitana* and *K. mediterranea* were found in the Sardinian sites exclusively, and the latter species was restricted to the monuments of the cemetery of Bonaria. This location is characterized by the vicinity of the sea and the local preponderance of some species may be enhanced by the presence of salt. *Knufia marmoricola* in contrast had a wider distribution and occurred in all sampling sites. The new chaetothyrialean fungus *Lithophila guttulata* has a remarkably high intraspecific variability (2.4% in ITS), despite its restricted distribution on monuments of the Vatican only. Intraspecific variability is known to be rather high in RIF, mostly linked to geographic distance but sometimes high (up to 1.7 %) even between strains isolated from the same piece of rock (Egidi et al. 2014).

It has been hypothesized that the molecular diversity observed in RIF may have been promoted by the stressful conditions characterizing their natural niches. Irrespective the causes of their emergence, stress-induced mutations participate in adaptive evolution (Bjedov et al. 2003, Kang et al. 2006). This is particularly pronounced in these fungi where sexual recombination has never been observed.

Two further isolates in the order Chaetothyriales were here described as new *Exophiala* species, *E. bonariae*. The genus belongs to the family Herpotrichiellaceae that includes many opportunists on humans and cold-blooded vertebrates (Badali et al. 2009, de Hoog et al. 2011), and only few stone-inhabiting fungi have been encountered. The ability of fungi in this family to metabolize hydrocarbons, particularly alkylbenzenes, may explain their success in colonizing antropogenic, polluted environments (Isola et al. 2013). This unique physiology may also play a role in human pathogenicity involving the central nervous system explained by the structural analogy of pollutants to neurotransmitters (Prenafeta-Boldú et al. 2006). Under this perspective an investigation of this ability in *E. bonariae* would be overdue.

The topology of the tree displayed in Fig. 3 is in accordance with the most recent phylogenies (Quaedvlieg et al. 2014). The two families Extremaceae and Neodevriesiaceae are well-separated and supported with high bootstrap values. One of the clades is represented by saxicolous species only and almost exclusively includes rock-inhabiting fungi, exceptionally intermingled with strains from soil forest or plants (Quaedvlieg et al. 2014). Most of the fungi belonging to the order Capnodiales studied here are included in this family and cluster in the genus *Vermiconia.* This genus (Egidi et al. 2014) exclusively comprises saxicolous species and has a worldwide distribution on rocks in Mallorca, Spain, at high altitude in the Alps and in the Antarctic desert. Strain CCFEE 5922 has been assigned to *V. flagrans,* deviating only 0.5% from this species, while the remaining 10 strains of a separate cluster were introduced as new species, *V. calcicola,* which proved to be ubiquitous in all sampling sites.

In the same family Extremaceae, a new genus and species, here named *Saxophila tyrrhenica*, is represented by four strains. One of these, CCFEE 5935 was isolated from a monument of the cemetery of Bonaria, while the remaining strains were isolated in earlier studies from natural rocks in Spain. Despite its rather wide distribution, this species shows a rather low genetic variability: strains TRN66, TRN 136 and CCFEE 5935 differed maximally 0.42% in ITS, while strain TRN65 is slightly more distant. The maximum genetic distance between the four strains was 2.1%, which is still well within the range of species variability for RIF. A strain indicated as "*Capnobotryella* sp. MA3619" was found in an external position to this group and was 12% different in ITS to the type species of *S. tyrrhenica*; this strain might, therefore, be reassigned to a different species within the genus *Saxophila*.

The family Neodevriesiaceae mostly includes plant-associated fungi and only few examples of rock-inhabiting fungi. Many of the strains isolated were located in the genus *Devriesia*, a polyphyletic genus accommodating a group of heat-resistant, cladosporium-like (Seifert et al. 2004) and plant-associated (Crous et al. 2009) fungi. Several *Devriesia*-like species were isolated from leaf litter or leaf spots, and placed in this genus based on molecular phylogenies. The genus was previously accommodated in the family Teratosphaeriaceae (Egidi et al. 2014) from which the families Extremaceae and Neodevriesiaceae have recently been segregated (Quaedvlieg et al. 2014). The general ecological trend in this group of fungi (i.e., mainly plant-association) is confirmed in the present study. Two of the RIF isolated, CCFEE 6202 and 6210, clustered in a separate position and were assignment to a new species, *D. sardiniae*.

Thirty of the strains isolated belonged to known species in the orders Chaetothyriales and Capnodiales (Tab. 2). All the species of the latter order were not typical RIF but rather plant-associated species: for instance fungi recovered in the monumental cemetery of Bonaria, *Aureobasidium pullulans*, *Pringshemia smilacis*, *Hortaea thailandica*, and *Verrucocladosporium dirinae*, are known to have plant- and lichen-associated life styles (Zalar et al. 2008, Crous et al. 2009). The strains were isolated from shaded monuments with mild microclimates due to the proximity of vegetation. Biological deterioration was mainly characterized by the presence of black patinas rather than biopitting or rock exfoliation, which are typical destruction patterns of rock fungi. The presence of plant-associated fungal species on rock may thus be regarded as relatively coincidental. Another external factor influencing the composition of fungal assemblages on monument surfaces is salinity. *Phaeotheca triangularis*, which combines halophily with a preference for oligotrophic conditions (Butinar et al. 2005) was found on a monument of the cemetery of Bonaria located close to the sea (less than 400 meters) and impacted by salty aerosols. All above mentioned species were not found anywhere in the sampling site of the Vatican, where vegetation is absent and the nearest beach is about 40 km away. Two of the taxa described here, *K. vaticanii* and *L. guttulata*, were found in the Vatican only and are probably able to cope with air pollution. *Knufia petricola* remains one of the recurrent species on stone monuments in the Mediterranean area (Wollenzien et al. 1997, Bogomolova and Minter 2003, Onofri et al. 2011).

In contrast to most reports on RIF, in the present study the order Chaetothyriales (class Eurotiomycetes) is predominant with 56.7% of the fungi isolated over the Capnodiales (class Dothideomycetes). In general it can be observed that in natural, unpolluted but harsher environments the tendency is mostly the opposite, with dothideomycetous rock fungi predominating. In the Antarctic lithic communities, for example, they constitute almost 70% of the black fungi isolated while chaetothyrialean fungi 7.4% only (Selbmann et al. 2014b). The different distribution of RIF in such diverse niches may be due to the general ecological traits of the fungi in these two groups. In the Dothideomycetes a diffuse adaptation to extremophily is noted and many true extremophiles cluster in this group (Gostinčar et al. 2010) such as the halophilic species *Hortaea werneckii* and the highly acidophilic fungus *Acidomyces acidophilus* (Selbmann et al. 2008), as well as the psychrophilic black yeasts in the genera *Cryomyces*, *Friedmanniomyces*, and *Saxomyces*. In contrast, Chaetothyriales have evolved a larger spectrum of assimilative abilities, including toxic organic compounds. This ecological versatility, rather than extremotolerance, is hypothesized to have driven diversification in this group, including the shift to opportunism (Gueidan et al. 2008). Vatican City State, being located in the middle of Rome, is the most polluted site investigated; here almost exclusively chaetothyrialean RIF were found and only 6 out of 23 strains were members of the Capnodiales, isolated exclusively on monuments in the “Cortile della Pigna”, not directly exposed to the traffic jam as the St Peter colonnade.

Extreme survival and alkylbenzene degrading abilities are suggested to be the main characteristics allowing RIF to colonize stone monuments in urban environments. RIF have adopted surprising means of dispersal, since most of them lack conidia. They actively penetrate and break up the substratum by a mechanical and chemical actions by the production of siderophores (Favero-Longo et al. 2011); powdered rock, on which RIF strictly adhere by the production of extracellular polymeric substances, are then dispersed as dry dust and transported by winds. This bio-deteriorating ability is one of the main forces in the natural rock weathering process (Nienow and Friedmann 1993, Brunner et al. 2011), which strongly interferes with the integrity of stone monuments. Their resistance to physical and chemical factors make the fungi extremely difficult to eradicate. More data on the biological and ecological traits of these organisms and on their biodiversity will help to deepen knowledge and may allow to develop tools to control their presence on stone monuments and to protect our cultural heritage.

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Captions

Fig. 1. Sites of sampling. A-J, Cagliari (Italy): (A) view of the monumental cemetery of Bonaria from Bonaria Hill; (B) funerary marble monument devoted Francesca Warzee; (C, D) little angels, unknown funerary marble monument and a particular of the patina covering a wing from which *Saxophila tyrrenica* CCFEE 5935 was isolated; (E, F) funerary marble monument devoted to Giuseppina Ara dei Conti Ciarella and particular of the angel's left wing from which *Vermiconia calcicola* CCFEE 5770 was isolated; (G) Autumn, one of the allegoric marble sculptures representing 'The Four Seasons' in the balustrade of the Boyl Palace; (H, I) lion sculpture in front of the Cathedral of Santa Maria and a particular of a deteriorated spot from which *Knufia karalitana* CCFEE 5656 was isolated. K-N, Vatican City State: (K, M) Aerial view of the Cortile della Pigna (K) with the Albani's Family coat of arm (L), a black fungal colonization in fissures and cracks of a marble capital (M) and a particular of the colonized travertine of the Saint Peter colonnade (N). Scale bars = 1 cm

Fig. 2. ML 2-genes multilocus phylogeny showing the phylogenetic placement of the new taxa described in the order Chaetothyriales (Class Eurotiomycetes). The tree, based on 82 sequences and 2,172 nucleotide positions, has been generated using a GTR+IG model calculated using ML in the

software MrAIC. Bootstrap values above 85%, calculated from 1,000 resampled data sets, are shown.

Fig. 3. ML 3-genes multilocus phylogeny showing the phylogenetic positions of the taxa described in the order Capnodiales (class Dothideomycetes). The tree, based on 60 sequences and 2,098 nucleotide positions, has been generated using a GTR+IG model calculated using ML in the software MrAIC. Bootstrap values above 85%, calculated from 1,000 resampled data sets, are shown.

Fig 4. A-H *Devriesia karalitana* CBS 139724=CCFEE 6202: (A) colony appearance; (B, E-H) meristematic development of dark brown, thick-walled conidia with longitudinal and oblique septa (white arrowheads), and germinating meristematic conidia (E); (D) wider and darker hyphae; (C, I) subhyaline, closely septate, branched young hyphae and anastomoses (black arrowheads). J-Q *Exophiala bonariae* CBS XXXX = CCFEE 5792: (J) colony appearance; (K-Q) hyphae, conidiophores and conidia; (M) spirally twisted hypha; (P, Q) cylindrical, hyaline and simple conidia, numerous at the conidiophore apex. R-X *Knufia karalitana* CBS XXXX = CCFEE 5656: (R) colony appearance; (S) acropetal moniliform hyphae; (T, U, W) chains of globose and subglobose blastic conidia; (V, X) thick walled rugose cells and terminal and intercalar multicellular bodies containing endoconidia (arrowhead). Scale bars 20 μm.

Fig 5. A-H *Knufia marmoricola* CBS 139726= CCFEE 5895: (A) colony appearance; (B, E, F) moniliform branched hyphae and multicellular bodies; (C) pale brown and mature chains of blastic conidia; (D, G) enlarged, thick walled and brown bodies containing endoconidia (G, arrowhead); (H) endoconidia released by rupture of a roundish body. I-P *Knufia mediterranea* CBS XXXX = CCFEE 5738: (I) colony appearance; (J-L) pale brown moniliform hyphae with composed of ellipsoidal to cylindrical cells, budding apically and laterally; (M) dense aggregates of roundish dark cells containing endoconidia (arrowhead); (N) globose thick walled cell with endoconidia; (O, P) enteroblastic proliferations and budding cells. Q-X *Knufia vaticanii* CBS XXXX = CCFEE 5939: (Q) colony appearance; (R, W) filamentous hyphae formed by small roundish or cylindrical catenate cells, finely guttulate; (S-V) torulose hyphae, producing terminal and intercalary multicellular arthroconidia; (X) torulose hyphae and arthroconidia (arrowhead).

Fig 6. A-G *Litophila guttulata* CBS XXXX = CCFEE 5907: (A) colony appearance; (B) network of hyphae of different morphologies; (C-G) branched, catenate and finely guttulate hyphae, with intercalar and apical globose cells; (D) apical globose cell. H-O *Saxophila tyrrenica* CBS XXXX =CCFEE 5935: (H) colony appearance; (I-O) filamentous, sometime geniculate, hyphae, composed of simple to 1-septate cells; (K) septate hyphae and anastomoses (arrowheads); M-O terminal or intercalar multicellular bodies (arrowheads). P-V *Vermiconia calcicola* CBS XXXX=CCFEE 5770: (P) colony appearance; (Q-V) moniliform branched hyphae; (R, S, U) terminal and intercalary cylindrical arthroconidia and occasional subglobose pluriseptate conidia in terminal position (S). Scale bars 20 μm.

Table 1. Fungal strains isolated

Taxon	Strains no.	Source	Location	ITS	*nuc*LSU	RPB2
Chaetothyriales						
Bradymyces alpinus	CCFEE5493 T	rock 3200 m a.s.l., Alps	Italy	HG793052	GU250396	-
Bradymyces alpinus	CCFEE5478	rock 3200 m a.s.l., Alps	Italy	HG974249	-	-
Bradymyces oncorhynchi	CCF 4369 T	Spleen of *Oncorhynchus mykiss*	Czech Republic	HG426062	HG426063	-
Capronia mansonii	CBS 101.67	*Populus tremula*	Sweden	AF050247	AY004338	-
Capronia semiimmersa	MUCL40572	Rotten wood	-	FJ358226	AF050283	-
Ceramothyrium thailandicum	MFLUCC10-0008	*Lagerstroemia* sp.	Thailand		HQ895835	-
Cladophialophora australiensis	CBS112793	Sport drink	Australia	EU035402	-	-
Cladophialophora minourae	CBS556.83	Decaying wood	Japan	AY251087	FJ358235	-
Cyphellophora laciniata	CBS 190.61 T	Man skin	Switzerland	EU035416	FJ358239	-
Cyphellophora olivacea	CBS 122.74	Moist wallpaper	Germany	KC455247	KC455260	-
Cyphellophora sessilis	CBS 243.85 T	Resin of *Picea abies*	Netherlands	EU514700	---	-
Cyphellophora sessilis	CBS 238.93	Styrene	Netherlands	AY857541	KC455264	-
Cyphellophora suttoni	CBS 449.91 T	Dog´s ear subcutaneous lesion	USA	KC455243	KC455256	-
Cyphellophora vermispora	CBS 228.86 T	Root of *Triticum aestivum*	Germany	KC455244	KC455257	-
Exophiala bergeri	CBS 353.52 T	Chromomycosis, man	Canada	EF551462	FJ358240	-
Exophiala bonariae	CBS 139957 T CCFEE 5792	Marble, Zelina Ferra born Castaldi Millelire funerary monument, CB	Italy	**JX681046**	**KR781083**	-
Exophiala bonariae	CCFEE 5899	Marble, A. Viganigo funerary monument,CB	Italy	**KP791795**	**KR781082**	-
Exophiala dermatitidis	CBS 207.35 T	Facial chromomycosis	Japan	AF050269	DQ823100	-
Exophiala equina	CBS 116009	Lung tissue of *Galapagos tortoise*, zoopark	Chicago, USA	JF747095	KF155181	-
Exophiala jeanselmei	CBS 507.90 T	Man	Uruguay	AF050271	AF050271	-
Exophiala lecanii-corni	CBS 123.33	*Lecanium corni*	---	AY857528	FJ358243	-
Exophiala opportunistica	CBS 109811 T	Drinking water	Germany	JF747123	KF928501	-
Exophiala pisciphila	CBS 537.73 T	Channel catfish	Alabama	AF050272	AF361052	-
Exophiala psychrophila	CBS 191.87 T	Fish	Norway	JF747135	-	-
Exophiala salmonis	CBS 157.67 T	Cerebral mycetoma	Canada	AF050274	AY213702	-
Fonsecaea pedrosoi	CBS 271.37	Human, chromoblastomycosis	Argentina	AB114127	AB114127	-
Knufia aspidioti	BJ01A29	*Aspidioti* sp. (scale insect)	China	KF589944	-	-

Knufia aspidioti	BJ01A10	*Aspidioti* sp. (scale insect)	China	KF589943	-	-
Knufia cryptophialidica	DAOM 216555 T	*Populus tremuloides*	-	JN04050	JN040500	-
Knufia endospora	UAMH 10396 T	*Populus tremuloides*	-	JN040510	-	-
Knufia epidermidis	CBS 120353 T	Foot infection	China	EU730589	FJ355954	-
Knufia karalitana	CBS 139720 T CCFEE 5656	Marble, Cathedral S. Maria, Cagliari	Italy	**KP791781**	**KR781069**	-
Knufia karalitana	CCFEE 5732	Pietraforte (sandstone), Boyl Palace, Cagliari	Italy	**KP791782**	**KR781070**	-
Knufia karalitana	CCFEE 5921	Marble, CB	Italy	**KP791784**	**KR781072**	-
Knufia karalitana	CCFEE 5929	Marble, Saint Remy bastion, Cagliari	Italy	**KP791783**	**KR781071**	-
Knufia karalitana	CCFEE 6001	Marble tombstone, CB	Italy	**KP791785**	**KR781073**	-
Knufia marmoricola	CCFEE 5716	Marble, Cathedral S. Maria, Cagliari	Italy	**KP791786**	**KR781074**	-
Knufia marmoricola	CCFEE 5721	Pietraforte (sandstone), Boyl Palace, Cagliari	Italy	**KP791787**	**KR781075**	-
Knufia marmoricola	CCFEE 5725	Pietraforte (sandstone), Boyl Palace, Cagliari	Italy	**KP791788**	**KR781076**	-
Knufia marmoricola	CCFEE 5726	Pietraforte (sandstone), Boyl Palace, Cagliari	Italy	**KP791789**	Unpublished	-
Knufia marmoricola	CCFEE 5892	Travertine, St Peter’s colonnade	Vatican City State	**KP791778**	**KR781066**	-
Knufia marmoricola	CBS 139726 T CCFEE 5895	Travertine, St Peter’s colonnade	Vatican City State	**KP791775**	**KR781063**	-
Knufia marmoricola	CCFEE 5902	Marble capital, CP	Vatican City State	**KP791776**	**KR781064**	-
Knufia marmoricola	CCFEE 5923	Travertine, Albani family coat of arms, CP	Vatican City State	**KP791777**	**KR781065**	-
Knufia marmoricola	CCFEE 5886	Marble, (catalogue n. 22635), CP	Vatican City State	**KP791779**	**KR781067**	**KT247878**
Knufia marmoricola	CCFEE 6201	Marble, Frau-Carta funerary monument, CB	Italy	**KP791790**	**KR781077**	-
Knufia mediterranea	CBS 139721 T CCFEE 5738	Marble, F. Warzee funerary monument, CB	Italy	**KP791791**	**KR781078**	-
Knufia mediterranea	CCFEE 5768	Marble, G. Todde funerary monument, CB	Italy	**KP791792**	**KR781079**	-
Knufia mediterranea	CCFEE 6205	Marble, F. Warzee funerary monument, CB	Italy	**KP791794**	**KR781081**	-
Knufia mediterranea	CCFEE 6211	Marble, Frau-Carta funerary monument, CB	Italy	**KP791793**	**KR781080**	-
Knufia peltigerae	UAMH 11090	thalli of *Peltigera rufescens*	Luxembourg	HQ709322	HQ613813	-

Knufia perforans	CBS 885.95 T	Marble	Greece	AJ244230	FJ358237	-
Knufia petricola	CBS 123.872= A95	Marble statue, Messina	Italy	KC978734	FJ358269	-
Knufia petricola	CBS 726.95 T	Marble statue, Messina	Italy	KC978746	KC978741	-
Knufia petricola	CBS 725.95	Calcaremite obelisque, Corfù	Greece	KC978745	KC978740	-
Knufia petricola	IMI 38917 =CBS 726.95	Marble angel, Messina	Italy	AJ507323	AJ507323	-
Knufia tsunedae	CPC 22931 T	Soil	Spain	HG003669	HG003672	-
Knufia vaticanii	CBS 139722 T CCFEE 5939	Travertine, St Peter's colonnade	Vatican City State	**KP791780**	**KR781068**	-
Knufia sp.	CCFEE 5323	Lichen thallus	Antarctica	FJ392866	GU250378	-
Knufia sp.	CCFEE 5314	Lichen thallus	Antarctica	FJ392865	-	-
Knufia sp.	CPC 12171	*Prunus* sp.	Canada	EU035420	EU035420	-
Knufia sp.	CPC 12172	*Prunus* sp.	Canada	EU035421	EU035421	-
Knufia sp.	CPC 12173	*Prunus* sp.	Canada	EU035422	EU035422	-
Lithophila guttulata	CCFEE 5884	Marble, (catalogue n. 22635), CP	Vatican City State	**KP791768**	**KR781056**	-
Lithophila guttulata	CCFEE 5885	Marble, (catalogue n. 22635), CP	Vatican City State	**KP791774**	**KR781062**	-
Lithophila guttulata	CCFEE 5894	Marble, St Peter's colonnade	Vatican City State	**KP791769**	**KR781057**	-
Lithophila guttulata	CBS 139723 T CCFEE 5907	Marble, (catalogue n. 37106), CP	Vatican City State	**KP791773**	**KR781061**	-
Lithophila guttulata	CCFEE 5908	Marble, (catalogue n. 37106), CP	Vatican City State	**KP791770**	**KR781058**	-
Lithophila guttulata	CCFEE 5909	Marble, (catalogue n. 37106), CP	Vatican City State	**KP791771**	**KR781059**	-
Lithophila guttulata	CCFEE 5910	Marble, (catalogue n. 37106), CP	Vatican City State	**KP791772**	**KR781060**	-
Lithophila guttulata	CCFEE 5928	Marble, capital, CP	Vatican City State	**KP791767**	**KR781055**	-
Metulocladosporiella musicola	CBS113873	*Musa sapientum*	Mozambique	DQ008135	DQ008159	-
Metulocladosporiella musicola	CBS 110960 T	*Musa acuminata*	South Africa	DQ008127	DQ008153	-
Metulocladosporiella musicola	CBS113864	*Musa* sp.	Uganda	DQ008133	DQ008157	-
Metulocladosporiella musicola	CBS113865	*Musa sapientum*	Uganda	DQ008134	DQ008158	-
Phaeosaccardinula ficus	MFLUCC10-0009	*Ficus* sp.	Thailand	HQ895837	HQ895837	-
Rhinocladiella atrovirens	MUCL 9905=CBS 265.49	Honey	France	AF050289	AF050289	-
Rhinocladiella fasciculata	CBS132.86	Decayed wood	India	EU041807	EU041864	-
Trichomerium deniqulatum	MFLUCC 10-0884 T	Living leaf of *Psidium guajava*	Thailand	JX3136554	JX313660	-
Trichomerium foliicola	MFLUCC 10-0078 T	Living leaf of *Murray apaniculata*	Thailand	JX313655	JX313661	-

Trichomerium foliicola	MFLUCC 10-0054	*Mangifera indica*	Thailand	JX313651	JX313657	-
Vonarxia vagans	CBS 123533 T	*Eugenia uniflora*	Brazil	FJ839636	FJ839672	-
Capnodiales						
Capnobotryella antalyensis	MA 4615	Marble, Perge, Antalya	Turkey	AJ972858	-	-
Capnobotryella antalyensis	MA 4624	Marble, Perge, Antalya	Turkey	AJ972850	-	-
Capnobotryella antalyensis	MA 4766	Marble, Perge, Antalya	Turkey	AJ972851	-	-
Capnobotryella erdogani	MA 4625	Marble, Perge, Antalya	Turkey	AJ972857	-	-
Capnobotryella sp.	MA3615	Rock, Vienna	Austria	AM746203	-	-
Capnobotryella sp.	MA3619	Rock, Vienna	Austria	AM746201	-	-
Capnobotryella sp.	MA4902	Marble, Perge, Antalya	Turkey	AJ972847	-	-
Devriesia adstricta	CBS 118292 T=TRN96	Limestone, Mallorca	Spain	AY559346	KF310022	KF310103
Devriesia agapanthi	CBS 132689 T=CPC 19833	leaves of *Agapanthus africanus*	South Africa	JX069875	JX069859	KJ564346
Devriesia bulbillosa	CBS 118285 T=TRN81	Limestone, Mallorca	Spain	AY559341	KF310029	KF310102
Devriesia bulbillosa	TRN152	Limestone, Mallorca	Spain	AY559377	GU323976	-
Devriesia bulbillosa	CCFEE 5704	Marble, I. Ruda Roych funerary monument, CB	Italy	XXX	**KR781054**	XXX
Devriesia compacta	CBS 118294=TRN111	Limestone, Mallorca	Spain	AY559351	GU323967	KF310095
Devriesia fraseriae	CBS 128217	leaves of *Melaleuca* sp.	Australia	HQ599602	-	-
Devriesia lagerstroemiae	CBS 125422 T=CPC 14403	*Lagerstroemia indica*	USA	-	KF902149	-
Devriesia modesta	CCFEE 5671	Wallpaintings, Cave, Vallerano	Italy	Unpublished	Unpublished	Unpublished
Devriesia modesta	CBS 137182 T=CCFEE 5672	Wallpaintings, Cave, Vallerano	Italy	KF309984	KF310026	KF310093
Devriesia modesta	CCFEE 5673	Wallpaintings, Cave, Vallerano	Italy	Unpublished	Unpublished	Unpublished
Devriesia modesta	CCFEE 5675	Wallpaintings, Cave, Vallerano	Italy	Unpublished	Unpublished	Unpublished
Devriesia modesta	CCFEE 5676	Wallpaintings, Cave, Vallerano	Italy	Unpublished	Unpublished	Unpublished
Devriesia modesta	CCFEE 5682	Wallpaintings, Cave, Vallerano	Italy	Unpublished	Unpublished	Unpublished
Devriesia sardiniae	CCFEE 6202 T	Marble Cross, - Frau-Carta funerary monument, CB	Italy	**KP791765**	**KR781052**	**KT247887**
Devriesia sardiniae	CCFEE 6210	Marble angel, - Frau-Carta funerary monument, CB	Italy	**KP791766**	**KR781053**	**KT247888**
Devriesia simplex	CBS 137183T=CCFEE	Wallpaintings, Cave, Vallerano	Italy	KF309985	KF310027	KF310104

	5681					
Devriesia stirlingiae	CBS 133581 T=CPC 19948E	leaves of*Stirlingia latifolia*	Australia	KC005778	KC005799	-
Devriesia strelitziae	CBS 122379 T=X1037	leaves of*Strelitzia nicolai*	South Africa	EU436763	GU301810	-
Devriesia sp.	CCFEE 5674	Wallpaintings, Cave, Vallerano	Italy	Unpublished	Unpublished	Unpublished
Devriesia sp.	TRN87	Limestone, Mallorca	Spain	AY559343	GU323966	-
Devriesia sp.	TRN153	Limestone, Mallorca	Spain	AY559378	GU323977	
Extremus antarcticus	CBS 136103 T=CCFEE 451	Sandstone, Linnaeus Terrace	Antarctica	KF309978	GU250360	KF310085
Extremus antarcticus	CBS 136104=CCFEE 5207	Rock, Inexpressible Island	Antarctica	KF309980	KF310021	KF310087
Extremus antarcticus	CCFEE 5312	Vegetation Island	Antarctica	KF309979	KF310020	KF310086
Neodevriesia xanthorrhoeae	CBS 128219 T=CPC 17720	leaves of *Xanthorrhoea australis*	Australia	HQ599605	HQ599606	-
Petrophila incerta	CBS 118287=TRN77	Limestone, Mallorca	Spain	AY559337	GU323963	KF310101
Petrophila incerta	CBS 118305=TRN62	Limestone, Mallorca	Spain	AY559328	GU323961	KF310084
Petrophila incerta	CBS 118608 T=TRN139b	Limestone, Mallorca	Spain	AY559372	KF310030	KF310091
Pseudoteratosphaeria ohnowa	CBS 112896 T=CPC 1004	*Eucalyptus grandis*	South Africa	KF901620	KF901946	KF902348
Pseudoteratosphaeria secundaria	CBS 118507 T=CPC 11551	*Eucalyptus* sp.	Brazil	KF901621	KF901947	KF902354
Saxophila tyrrhenica	CBS 139725 T CCFEE 5935	Marble angels, CB	Italy	**KP791764**	**KR781051**	*XXX*
Saxophila tyrrhenica	TRN65	Limestone, Mallorca	Spain	AY559329	-	-
Saxophila tyrrhenica	TRN66	Limestone, Mallorca	Spain	AY559330	GU323962	-
Saxophila tyrrhenica	TRN136	Limestone, Mallorca	Spain	AY559368	-	-
Teratosphaeria knoxdaviesii	CBS 122898	*Protea* sp.	South Africa	EU707865	EU707865	-
Teratosphaeria tinara	CBS 124583 T=MUCC 666	*Corymbia* sp.	Australia	KF901599	KF901923	KF442599
Teratosphaeria toledana	CBS 113313 T=CMW 14457	*Eucalyptus* sp.	Spain	KF901734	KF902081	KF902492
Vermiconia antarctica	CBS 136107=CCFEE 5488	Rock, Battleship Promontory	Antarctica	KF309982	Unpublished	KF310089
Vermiconia antarctica	CBS 136108=CCFEE 5489	Rock, Battleship Promontory	Antarctica	KF309983	KF310023	KF310090
Vermiconia calcicola	CCFEE 5718	Marble lion, Cathedral	Italy	**KP791762**	**KR781049**	**KT247885**

Vermiconia calcicola	CCFEE 5714	Marble, F. Warzee funerary monument, CB	Italy	**KP791760**	**KR781047**	**KT247883**
Vermiconia calcicola	CCFEE 5769	Marble, G. Ara funerary monument, CB	Italy	**KP791758**	**KR781045**	**KT247881**
Vermiconia calcicola	CBS 140080 T CCFEE 5770	Marble, G. Ara funerary monument, CB	Italy	**KP791759**	**KR781046**	**KT247882**
Vermiconia calcicola	CCFEE 5780	Marble, G. Ara funerary monument, CB	Italy	**KP791761**	**KR781048**	**KT247884**
Vermiconia calcicola	CCFEE 5887	Marble (catalogue n. 37106), CP	Vatican City State	**KP791755**	**KR781042**	*XXX*
Vermiconia calcicola	CCFEE 5896	Travertine, St Peter’s colonnade	Vatican City State	**KP791754**	**KR781041**	**KT247877**
Vermiconia calcicola	CCFEE 5898	Marble (catalogue n. 22635), CP	Vatican City State	**KP791757**	**KR781044**	**KT247880**
Vermiconia calcicola	CCFEE 5905	Capital, CP	Vatican City State	**KP791756**	**KR781043**	*XXX*
Vermiconia calcicola	CCFEE 5947	Marble, R. Bolla funerary monument, CB	Italy	**KP791763**	**KR781050**	**KT247886**
Vermiconia flagrans	CCFEE 5922	Travertine, Albani’s family coat of arms, CP	Vatican City State	**KP791753**	**KR781040**	**KT247879**
Vermiconia flagrans	CBS 118283=TRN124	Limestone, Mallorca	Spain	AY559359	GU323971	KF310097
Vermiconia foris	CBS 136106=CCFEE 5459	Punta Indren, Monte Rosa	Italy	KF309981	GU250390	KF310088

CBS: CBS Fungal Biodiversity Centre, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CCF: Culture Collection of Fungi, Dept of Botany, Charles University, Prague, Czech Republic; CCFEE: Culture Collection of Fungi from Extreme Environments, Dept. of Ecological and Biological Sciences, University of Tuscia, Viterbo, Italy; CMW: Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI) of the University of Pretoria, Pretoria, South Africa; CPC: Collection Pedro Crous, housedat CBS; DAOM: Plant Research Institute, Department of Agriculture (Mycology), Ottawa, Canada; MFLUCC: Mae Fah Luang University Culture Collection, Thailand; MUCC: Murdoch University Culture Collection, Murdoch, Australia; TRN: T. Ruibal personal collection; UAMH: University of Alberta Microfungus Collection and Herbarium, Canada. CB = cemetery of Bonaria (Cagliari); CP = Cortile della Pigna (Vatican City). New species in bold with accession numbers of GenBank submissions.

Table 2. Additional strains identified by ITS Blast comparison

Taxon	Strains no.	Source	Location
Chaetothyriales			
Knufia petricola	CCFEE 5702, 5705	Marble, I. Ruda Roych funerary monument, CB	Italy
Knufia petricola	CCFEE 5709	Marble, G. Todde funerary monument, CB	Italy
Knufia petricola	CCFEE 5730	Marble, Porta Cristina, Cagliari	Italy
Knufia petricola	CCFEE 5740, 5741, 5777	Marble, F. Warzee funerary monument, CB	Italy
Knufia petricola	CCFEE 5776	Marble, G. Ara dei Conti Ciarella funerary monument, CB	Italy
Knufia petricola	CCFEE 5920	Marble tombstone, CB	Italy
Knufia petricola	CCFEE 5923	Travertine, Albani's family coat of arms, CP	Vatican City State
Knufia petricola	CCFEE 5931	Pietraforte (sanstone), Saint Remy bastion, Cagliari	Italy
Knufia petricola	CCFEE 5937	Marble angels, CB	Italy
Capnodiales			
Aureobasidium pullulans	CCFEE 5736	Marble, G. Ara dei Conti Ciarella funerary monument, CB	Italy
Austroafricana parva	CCFEE 5742	Marble column, S. Saturnino, Cagliari	Italy
Coniosporium uncinatum	CCFEE 5660	Marble, Lion out of the Cathedral of Santa Maria, Cagliari	Italy
Coniosporium uncinatum	CCFEE 5737	Marble, F. Warzee funerary monument, CB	Italy
Coniosporium uncinatum	CCFEE 5924	Sarcophagus, CP	Vatican City State
Coniosporium uncinatum	CCFEE 5927	Marble capital (catalogue n.5148), CP	Vatican City State
Hortaea thailandica	CCFEE 5565	Pietra Forte (sandstone), Tower of the Elephant, Cagliari	Italy
Hortaea thailandica	CCFEE 5723, 5735	Marble, A. Viganigo funerary monument, CB	Italy
Hortaea thailandica	CCFEE 5919	Marble tombstone, CB	Italy
Phaeotheca triangularis	CCFEE 5703, 5706	Marble, I. Ruda Roych funerary monument, CB	Italy
Pringsheimia smilacis	CCFEE 5778	Marble, Z. Ferra born Castaldi Millelire funerary monument, CB	Italy
Pseudotaeniolina globosa	CCFEE 5734	Marble, Boyl Palace statue, Cagliari	Italy
Teratosphaeria capensis	CCFEE 6200	Marble cross, Frau Carta funerary monument, CB	Italy
Vermiconia flagrans	CCFEE 5922	Albani's family coat of arms, CP	Vatican City State
Verrucocladosporium dirinae	CCFEE 5707	Marble, I. Ruda Roych funerary monument, CB	Italy
Verrucocladosporium dirinae	CCFEE 5708	Marble, I. Ruda Roych funerary monument, CB	Italy

CCFEE – Culture Collection of Fungi From Extreme Environments; CB = cemetery of Bonaria (Cagliari); CP = Cortile della Pigna (Vatican City).

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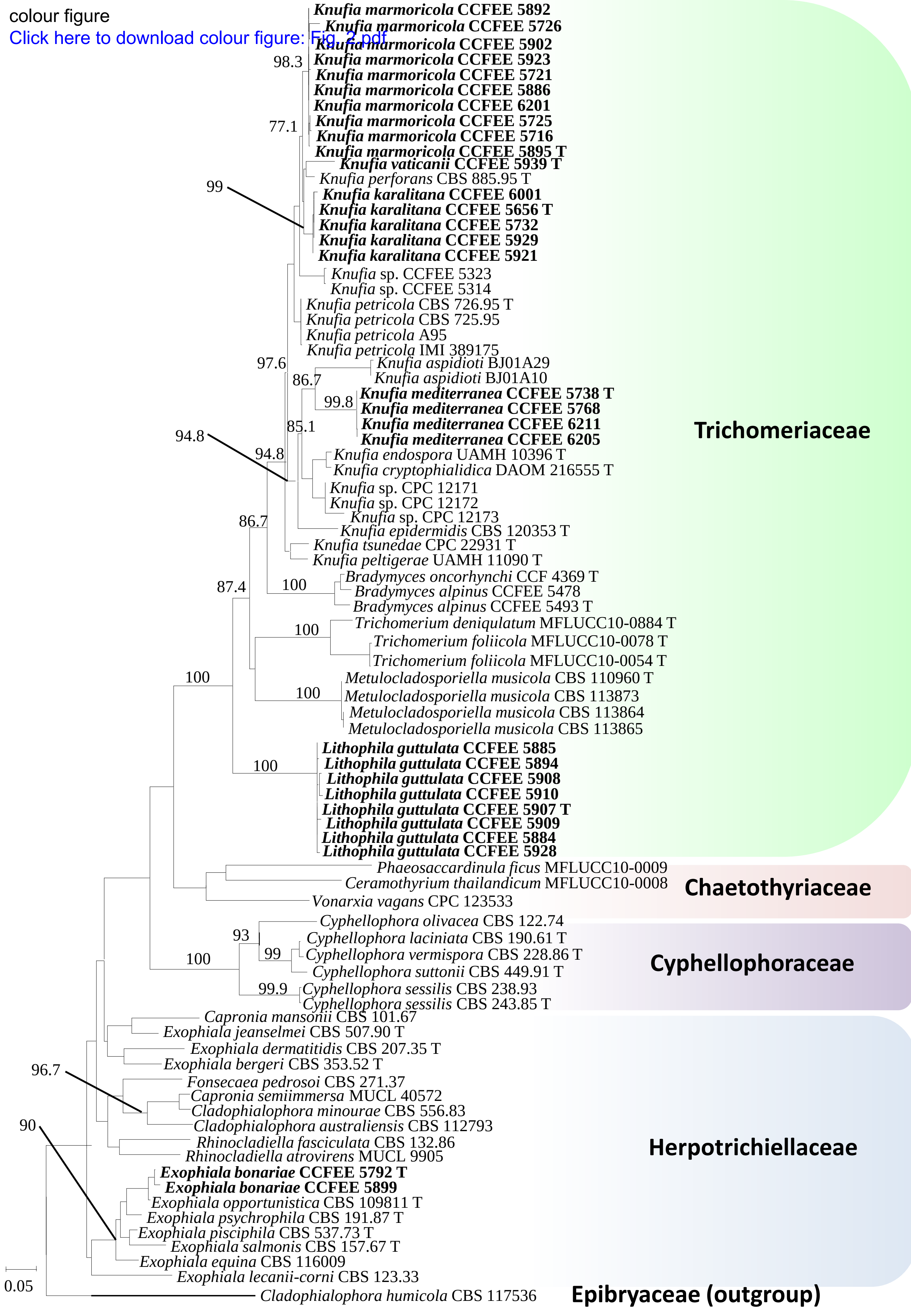

Figure 3

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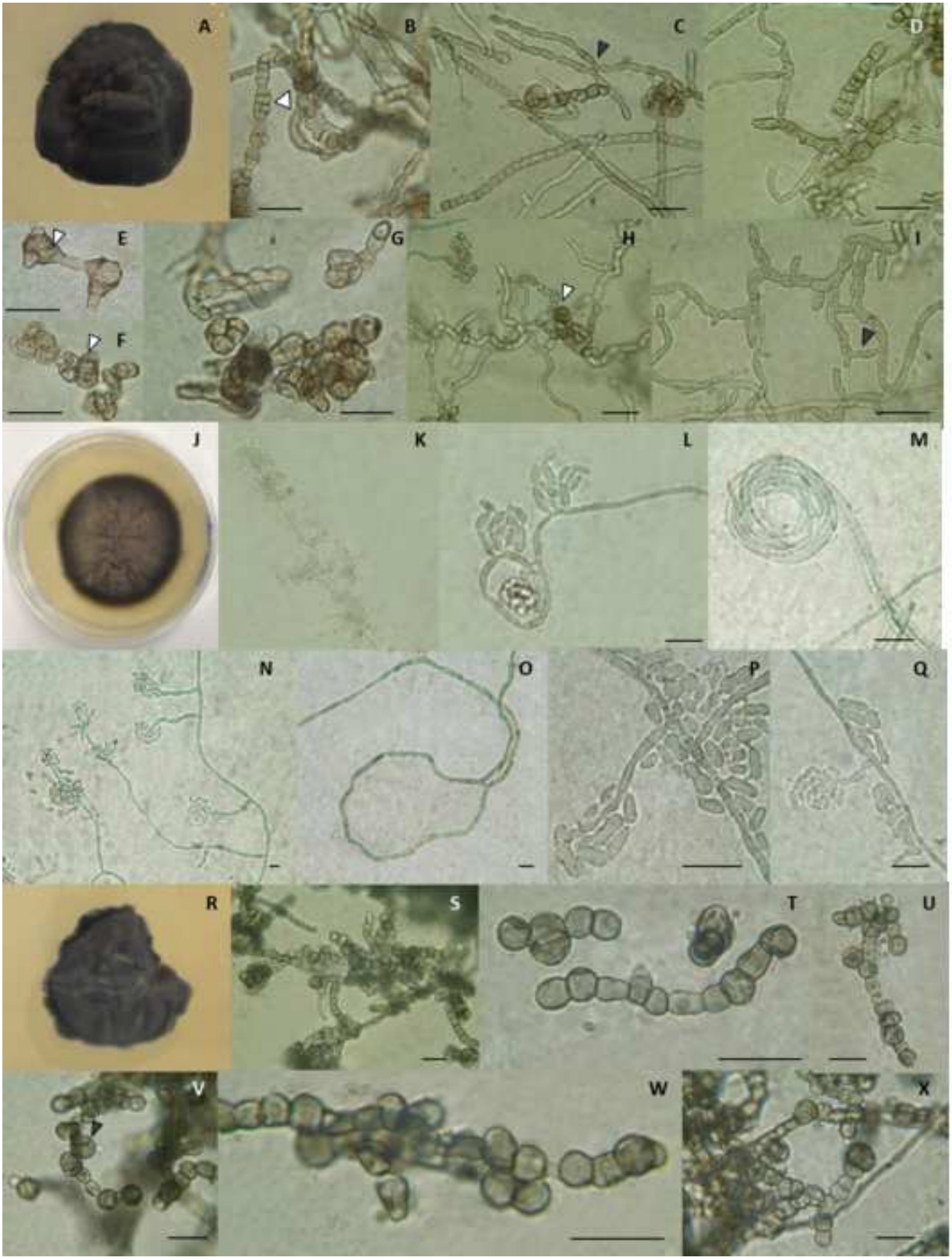

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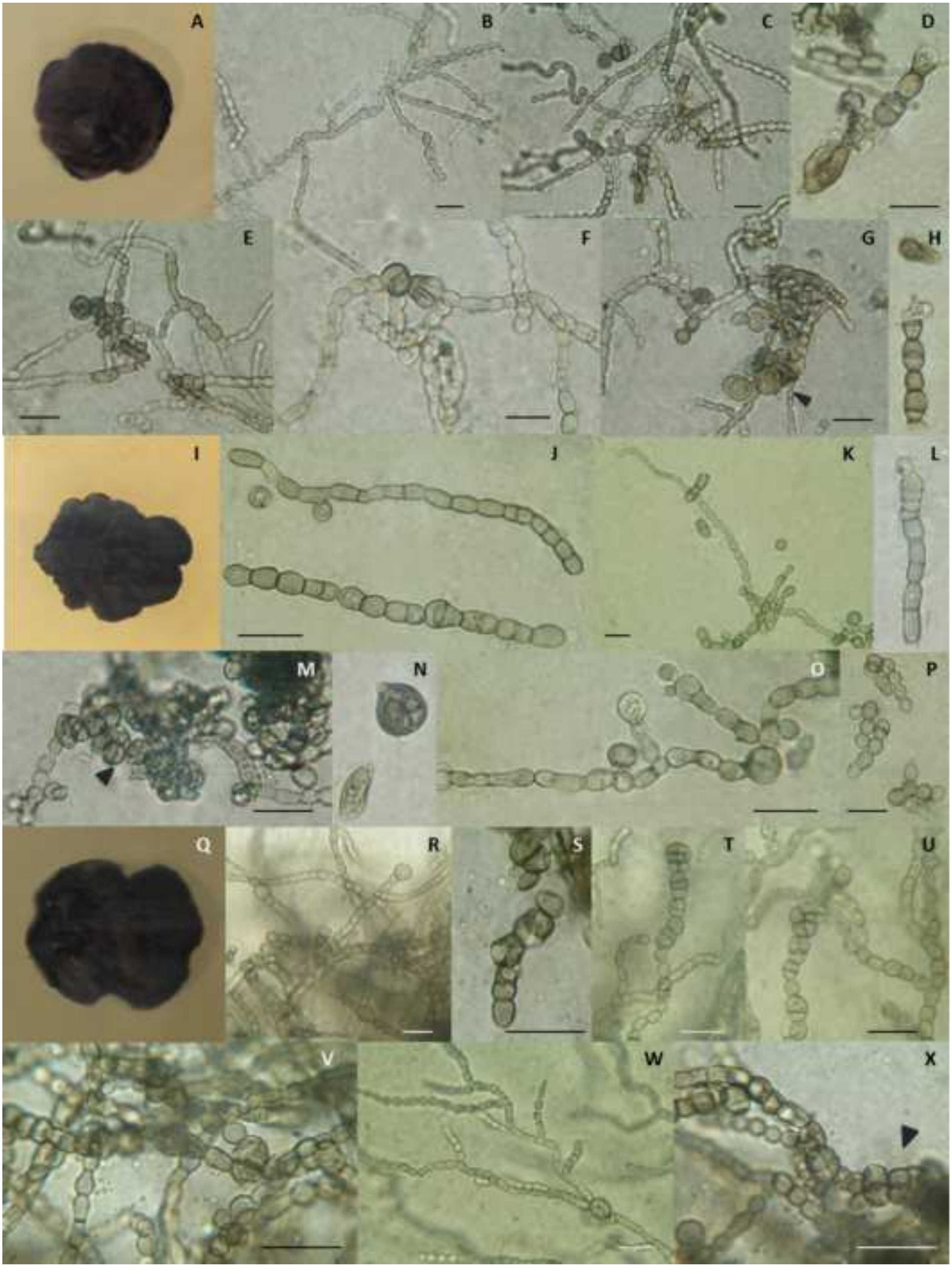

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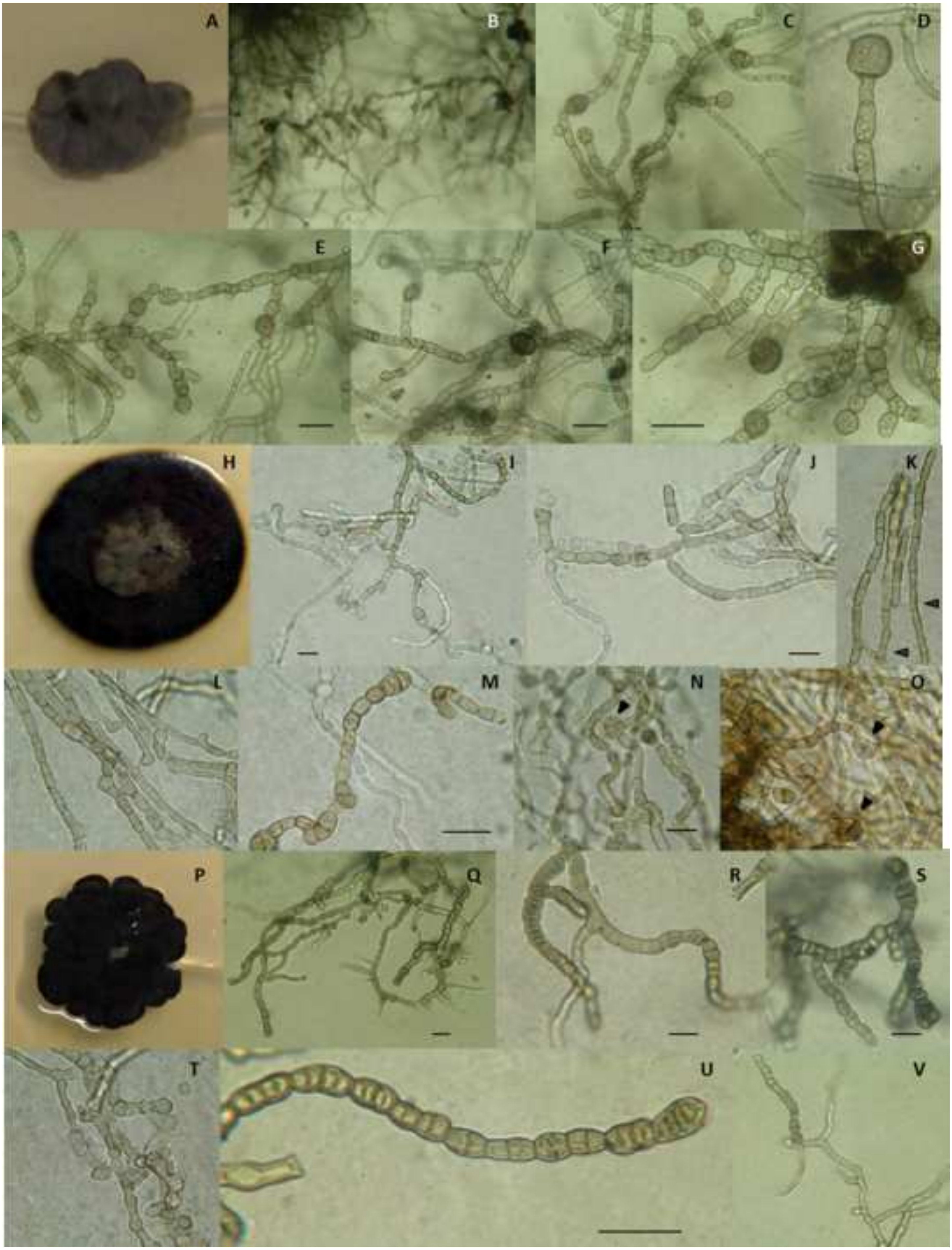

Answers to reviewers