Antarctic Epilithic Lichens as Niches for Black Meristematic Fungi

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Abstract: Sixteen epilithic lichen samples (13 species), collected from seven locations in Northern and Southern Victoria Land in Antarctica, were investigated for the presence of black fungi. Thirteen fungal strains isolated were studied by both morphological and molecular methods. Nuclear ribosomal 18S gene sequences were used together with the most similar published and unpublished sequences of fungi from other sources, to reconstruct an ML tree. Most of the studied fungi could be grouped together with described or still unnamed rock-inhabiting species in lichen dominated Antarctic cryptoendolithic communities. At the edge of life, epilithic lichens withdraw inside the airspaces of rocks to find conditions still compatible with life; this study provides evidence, for the first time, that the same microbes associated to epilithic thalli also have the same fate and chose endolithic life. These results support the concept of lichens being complex symbiotic systems, which offer attractive and sheltered habitats for other microbes.

Keywords: black meristematic fungi; Dothideomycetes; Eurotiomycetes; lichen-associated fungi; phylogeny
1. Introduction

Black meristematic fungi are known to be tolerant to extreme environmental conditions. The term black fungi embraces a polyphyletic group of fungi that share some phenotypic characters such as melanized cell walls and meristematic development, which seem to support survival and persistence in hostile environmental conditions. They are commonly isolated from environments that are almost devoid of other eukaryotic life-forms, including saltpans [1], acidic and contaminated sites [2–4], exposed rocks in dry and extremely hot or cold climates, ranging from hot deserts [5], the Mediterranean [6] to the Antarctic [7] and on monuments [8–12]. Owing to the stress pressure of the sites where they normally occur, black meristematic fungi are rarely found in complex microbial populations, rather they occur alone or in association with similar stress resistant organisms such as lichens [13,14] and cyanobacteria [15]. In the Antarctic, black meristematic fungi are recurrent members of endolithic microbial communities of ice free areas, including the lichen-dominated cryptoendolithic communities of the McMurdo Dry Valleys, one of the most inhospitable environments on Earth [16,17]. In these sites the limits for life are reached; since the conditions are too harsh to sustain epilithic settlement, mosses almost completely disappear and lichens grow protected in cracks and fissures or move inside the rocks, giving rise to well structured communities [16]. Together with lichens, other organisms can participate in these communities, in particular bacteria, cyanobacteria and non-lichenized fungi [18–20], but their biodiversity, their role and interactions are still scarcely investigated. Among these, the rock black fungi represent a peculiar group of colonizers [7,17].

Lichens are commonly described as a mutualistic symbiosis between fungi and “algae” (Chlorophyta or Cyanobacteria); however, recent studies revealed that they host a number of other microbes. Several culture-dependent and -independent studies have deepened our understanding of diverse populations of bacteria associated with lichens and their potential functional roles within the symbiosis [21–25]. Lichens also host numerous fungal species. The mycobiont is the dominant fungal species but other fungi may be present. These include lichenicolous fungi, which expresses symptoms [26,27] and endolichenic fungi, which grow without symptoms in the interior of lichens [28–31]. These studies have fueled the concept that, in addition to being symbiotic systems, where symbiotic partners may interact, lichens can also be considered miniature ecosystems [22,32]. However, despite a growing body of literature on organisms associated with lichens, we still have limited knowledge of the extent of eukaryotic diversity that may be associated with individual lichen thalli [31].

In this study we focused on extremotolerant black fungi associated with cold-loving lichens from the Antarctic, including the endemic species Lecanora fuscobrunnea Dodge & Baker. Antarctic lichens are still an unexplored niche for these organisms and we aimed to compare the black fungi diversity among different lichen species distributed in diverse ecosystems.

2. Experimental Section

Sampling sites and lichen identification: lichen thalli were collected using a sterile chisel and preserved in sterile plastic bags at −20 °C until processed for isolation of associated fungi. Lichens were identified using the key by Castello [33]. All data concerning the sampling sites and the identifications are reported in Table 1.
Table 1. Lichen species analyzed collection data and fungal strains isolated.

| Lichen species       | Location                  | Coordinates             | Sampling date | Fungal strains (CCFEE) |
|----------------------|----------------------------|-------------------------|---------------|------------------------|
| *Acarospora* sp.     | Ford Peak, NVL             | 75°41'26.3''S 160°26'25.3''E | 28/01/2004    | -                      |
| *Acarospora flavocordia* Castello & Nimis | Kay Island, NVL | 75°04'13.7''S 165°19'02.0''E | 30/01/2004    | 5324                   |
| *Buellia frigida* Darb. | Inexpressible Island, NVL | 75°52'23.2''S 163°42'16.5''E | 17/01/2004    | -                      |
| *Lecanora fuscobrunnea* Dodge & Baker | Edmonson Point, NVL | 74°19'43.7''S 165°08'00.7''E | 29/01/2004    | 5320 *                 |
| *Lecanora fuscobrunnea* Dodge & Baker | Convoy Range, Terra SVL | 76°54'33.0''S 160°50'00.0''E | 25/01/2004    | 5303                   |
| *Lecanora sp.*       | Inexpressible Island, NVL | 75°52'23.2''S 163°42'16.5''E | 17/01/2004    | 5319 *, 5323           |
| *Lecidea* sp.        | Starr Nunatak, NVL         | 75°53'55.7''S 162°35'31.3''E | 15/02/2004    | 5318                   |
| *Lecidea* sp.        | Starr Nunatak, Terra Vittoria del Nord | 75°53'55.7''S 162°35'31.3''E | 15/02/2004    | 5326                   |
| *Lecidea cancriformis* Dodge & Baker | Widowmaker Pass, NVL | 74°55'23.5''S 162°24'17.0''E | 12/02/2004    | 5321 **                |
| *Rhizocarpus* sp.    | Vegetation Island, NVL     | 74°47'05.2''S 163°38'40.3''E | 16/01/2004    | 5312                   |
| *Umbilicaria aprina* Nyl. | Kay Island, NVL         | 75°04'13.7''S 165°19'02.0''E | 30/01/2004    | -                      |
| *Umbilicaria decussata* (Vill.) Zahlbr. | Kay Island, NVL | 75°04'13.7''S 165°19'02.0''E | 02/02/2004    | -                      |
| *Umbilicaria decussata* (Vill.) Zahlbr. | Vegetation Island, NVL | 74°47'05.2''S 163°38'40.3''E | 16/01/2004    | 5317                   |
| *Usnea antarctica* Du Rietz | Kay Island, NVL | 75°04'13.7''S 165°19'02.0''E | 30/01/2004    | -                      |
| *Usnea antarctica* Du Rietz | Vegetation Island, NVL | 74°47'05.2''S 163°38'40.3''E | 16/01/2004    | 5313 *                 |
| *Xanthoria elegans* (Link) th. Fr. | Kay Island, NVL | 75°04'13.7''S 165°19'02.0''E | 30/01/2004    | 5314, 5322             |

CCFEE—Culture Collection of fungi From Extreme Environments; NVL—Northern Victoria Land; SVL—Southern Victoria Land. Identified strains: * Elasticomyces elasticus; ** Friedmannomyces endolithicus.

Isolation: in order to remove any potential contaminant before isolation, lichens were treated with H2O2 (8%) for 5 min; H2O2 was removed by washing with distilled sterile water for 5 min. The solution was filtered using 500 µm porosity filters. All fragments were collected and seeded on MEA (Malt Extract Agar, Oxoid, Ltd. Basingstoke, Hampshire, UK) in Petri Dishes and incubated at 5 °C and 15 °C. Plates were inspected weekly and as soon as new black colonies appeared they were transferred on fresh agar slant. Pure cultures were deposited in the CCFEE (Culture Collection of Fungi from Extreme Environments, DEB, Università degli Studi della Tuscia, Viterbo, Italy).

Morphology and temperature preferences: hyphal maturation was studied using light microscope. Slide cultures were seeded onto MEA, incubated for 10 w and mounted in lactic acid. Temperature
preferences were performed in triplicate on MEA, in Petri dishes in the range 0–30 °C ± 1, with 5 °C intervals. Colony diameters were recorded monthly.

Molecular analysis: DNA was extracted from 6-months-old mycelium grown on MEA at 10 °C, using Nucleospin Plant kit (Macherey-Nagel, Düren, Germany) following the protocol optimized for fungi. Target gene for our analysis was the nuclear ribosomal 18S and ITS genes. PCR reactions were performed using BioMix (BioLine, Luckenwalde, Germany) and primers NS1-NS24 and ITS1-ITS4 to amplify 18S and ITS respectively [34]. Reaction mixtures were prepared by adding 5 pmol of each primer and 40 ng of template DNA in a final volume of 25 μL. For amplification, a MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Munich, Germany) was used. The protocol used for amplification of the nuclear ribosomal 18S was as follows: 3 min at 95 °C for a first denaturation step, a denaturation step at 95 °C for 45 s, annealing at 52 °C for 30 s. Cycles were repeated 35 times, with a last extension at 72 °C for 5 min. ITS portion was amplified as previously described [7]. Products were purified using Nucleospin Extract kit (Macherey-Nagel, Düren, Germany). Sequencing reactions were performed according to the dideoxynucleotide method using the TF BigDye Terminator 1,1 RR kit (Applied Biosystems). Fragments were analyzed by Macrogen Inc. (Seoul, Korea). Sequence assembly was done using the software ChromasPro (version 1.32, Technelysium, Conor McCarthy School of Health Science, Griffith University, Southport, Queensland, Australia).

The alignment based on nuclear ribosomal 18S included 79 sequences of strains belonging to the class *Dothideomycetes* and *Eurotiomycetes* in the public domain chosen on the base of the Blastn results. Additional sequences of black fungi deposited in the database of the CCFEE (Culture Collection of Fungi from Extreme Environments, Università degli Studi della Tuscia, Viterbo, Italy) were analyzed (Table 2). Sequences were aligned iteratively with ClustalX [35], exported in Mega5 [36] for a manual improvement. The best-fit substitution model and Maximum Likelihood phylogenetic tree reconstruction was performed as previously described [17]. The robustness of the phylogenetic inference was estimated using the bootstrap method [37] with 1000 pseudoreplicates.

| Species                        | Strains no. | Source            | Location | SSU      |
|-------------------------------|-------------|-------------------|----------|----------|
| *Acidomyces acidophilum*      | C2          | acid mine drainage | CA, USA  | AY374300 |
| *Acidomyces acidophilum*      | A3-7        | acid mine drainage | CA, USA  | AY374299 |
| *Acidomyces acidophilum*      | B1          | acid mine drainage | CA, USA  | AY374298 |
| *Aureobasidium pullulans*     | 28v1        | -                 | -        | AY137505 |
| *Aureobasidium pullulans*     | 30v4        | -                 | -        | AY137507 |
| *Botryosphaeria ribis*        | CBS 121.26  | Ribes rubrum      | -        | U42477   |
| *Botryosphaeria ribis*        | CBS 115475  | Ribes             | -        | DQ678000 |
| *Capnobotryella renispora*    | CBS 214.90  | Abies             | Japan    | EF137360 |
| *Capnobotryella renispora*    | CBS 215.90  | Sphagnum          | Japan    | AY220613 |
| *Capnobotryella renispora*    | CBS 572.89  | Roof tile         | Sweeden  | AY220614 |
| *Capnobotryella renispora*    | UAMH 9870   | Sphagnum          | -        | AY220611 |
| *Capronia coronata*           | CBS 617.96  | Decorticated wood | New Zealand | AY232939 |
| *Capronia seminimansa*        | CBS 840.69  | Decaying timber   | Finland  | AY554291 |
| *Catenulostroma abietis*      | CBS 459.93  | Abies             | Germany  | DQ678040 |
| *Cladophialophora carrionii*  | CBS 260.83  | Skin lesion       | -        | AY554285 |
| Species                      | Strains no. | Source | Location      | SSU              |
|------------------------------|-------------|--------|---------------|------------------|
| *Cladophialophora* sp.       | CBS 985.96  | Brain  | USA           | AJ232953         |
| *Coccodinium bartschii*      | UME30232    | -      | -             | U77668           |
| *Coniosporium* sp.           | MA 4597     | Marble | Turkey        | A972863          |
| *Cyphellophora laciniata*    | MUCL 9569   | -      | -             | AY342010         |
| *Cryomyces antarcticus*      | CCFEE 514   | Rock   | Antarctica    | GU250319         |
| *Cryomyces antarcticus*      | CCFEE 515   | Rock   | Antarctica    | GU250320         |
| *Cryomyces antarcticus*      | CBS 116301T; CCFEE 534 | Sandstone | Antarctica | DQ028269 |
| *Cryomyces minteri*          | CBS 116302; CCFEE 5187 | Sandstone | Antarctica | DQ028270 |
| *Discosphaerina fagi*        | CBS 171.93  | *Populus* leaf | UK | AY016342        |
| *Elasticomyces elasticus*    | CBS 122538; CCFEE 5313 | Lichen | Antarctica | FJ415474 |
| *Elasticomyces elasticus*    | CBS 122539; CCFEE 5319 | Lichen | Antarctica | GU250332 |
| *Elasticomyces elasticus*    | CBS 122540; CCFEE 5320 | Lichen | Antarctica | GU250333 |
| *Elsinoe centrolobii*        | CBS 222.50  | *Centrolobium robustum* | Brazil | DQ678041 |
| *Exophiala salmonis*         | CBS 157.67  | *Salmo clarkii* | Canada | JN856020 |
| *Exophiala salmonis*         | FTOL-ID 671 | -      | -             | EF413608         |
| *Friedmanniomyces endolithicus* | CCFEE 670    | Rock   | Antarctica    | GU250322         |
| *Friedmanniomyces endolithicus* | CCFEE 5208    | Rock   | Antarctica    | Unpublished      |
| *Friedmanniomyces endolithicus* | CCFEE 5321    | Lichen | *Antarctic* | Unpublished      |
| *Fonsecaea pedrosoi*         | CBS 272.37  | -      | -             | AY554290         |
| *Guignardia mangiferae*      | IFO 33119   | *Rhododendron palchrum* | - | AB041247 |
| *Guignardia mangiferae*      | CBS 226.77  | *Paphiopedium callosum* | - | AB041248 |
| *Guignardia mangiferae*      | CBS 398.80  | Orchid | -             | AB041249         |
| *Hobsonia santessonii*       | -           | -      | -             | AF289658         |
| *Hortaea werneckii*          | dH10921     | Marble | -             | Y18700           |
| *Hortaea werneckii*          | CBS 107.67  | human *Tinea nigra* | - | Y18693 |
| *Knufia chersonesos*         | CBS 600.93; dH16058 | Marble | Greece | Y18702 |
| *Knufia chersonesos*         | CBS 726.95  | Marble | Italy         | Unpublished      |
| *Knufia perforans*           | CBS 885.95  | Marble | Delos, Greece | Y11714 |
| *Knufia perforans*           | CBS 665.80  | Marble | Delos, Greece | Y11712 |
| *Myocalicium victoriae*      | CBS 109863  | Soil   | Italy         | Unpublished      |
| *Myriangium duriae*          | CBS 260.36  | *Chrysomphalus* | Argentina | NG 013129 |
| *Pseudotaeniolina globosa*   | CBS 109889  | Rock   | Italy         | GU214576         |
| *Saxomyces alpinus*          | CCFEE 5466  | Rock   | Alps, Italy   | GU250350         |
| *Saxomyces alpinus*          | CCFEE 5469  | Rock   | Alps, Italy   | KC315860         |
| *Saxomyces alpinus*          | CCFEE 5470  | Rock   | Alps, Italy   | KC315861         |
| *Saxomyces pennisicus*       | CCFEE 5495  | Rock   | Alps, Italy   | KC315864         |
| *Recurvomyces mirabilis*     | CBS 119434; CCFEE 5264 | Rock | Antarctica | GU250329 |
| *Rhinocladiella atrovirens*  | CBS 688.76  | *Pinus* | Australia | AJ232937 |
| *Rock black fungus*          | CCFEE 451   | Rock   | Antarctic     | GU250314         |
| *Rock black fungus*          | CCFEE 457   | Rock   | Antarctic     | GU250317         |
| *Rock black fungus*          | CCFEE 507   | Rock   | Antarctic     | Unpublished      |
| *Rock black fungus*          | CCFEE 5176  | Rock   | Antarctic     | GU250325         |
| *Rock black fungus*          | CCFEE 5177  | Rock   | Antarctic     | Unpublished      |
| *Rock black fungus*          | CCFEE 5205  | Rock   | Antarctic     | GU250327         |
| *Rock black fungus*          | CCFEE 5207  | Rock   | Antarctic     | Unpublished      |
Table 2. Cont.

| Species                          | Strains no. | Source | Location | SSU    |
|---------------------------------|-------------|--------|----------|--------|
| Rock black fungus               | CCFEE 5267  | Rock   | Antarctic| Unpublished |
| Rock black fungus               | CCFEE 5284  | Rock   | Antarctic| GU250330 |
| Rock black fungus               | CCFEE 5303  | Rock   | Antarctic| GU250331 |
| Rock black fungus               | CCFEE 5329  | Rock   | Antarctic| Unpublished |
| *Teratosphaeria microspora*     | CBS 101951;  | Leaf   | South Africa| EU167572 |
| *Teratosphaeria molleriana*     | CPC 1214    | Eucalyptus globulus | Portugal | GU214606 |
| *Teratosphaeria molleriana*     | CPC 4577    | Eucalyptus | Australia| GU214582 |
| *Teratosphaeria molleriana*     | CPC 10397   | Eucalyptus globulus | Spain | GU214607 |
| *Teratosphaeria nubilosa*       | CPC 933     | Eucalyptus nitens | South Africa | GU214608 |
| *Teratosphaeria nubilosa*       | CPC 937     | Eucalyptus globulus | Australia | GU214609 |
| Unknown black fungus            | CCFEE 5304  | Lichen | Antarctic| Unpublished |
| Unknown black fungus            | CCFEE 5312  | Lichen | Antarctic| Unpublished |
| Unknown black fungus            | CCFEE 5314  | Lichen | Antarctic| Unpublished |
| Unknown black fungus            | CCFEE 5317  | Lichen | Antarctic| Unpublished |
| Unknown black fungus            | CCFEE 5318  | Lichen | Antarctic| Unpublished |
| Unknown black fungus            | CCFEE 5322  | Lichen | Antarctic| GU250334 |
| Unknown black fungus            | CCFEE 5323  | Lichen | Antarctic| Unpublished |
| Unknown black fungus            | CCFEE 5324  | Lichen | Antarctic| Unpublished |
| Unknown black fungus            | CCFEE 5326  | Lichen | Antarctic| Unpublished |

AFTOL—Assembling Fungal Tree Of Life project; CBS—Centraalbureau voor Schimmelcultures; CCFEE—Culture Collection of Fungi From Extreme Environments; CPC—Culture collection of P Crous, housed at the CBS; dH—de Hoog private collection housed at the CBS; IFO—Institute for Fermentation Culture Collection, Japan; MUCL—Belgian Co-ordinated Collections of micro-organisms; STE-U—University of Stellenbosch fungal culture collection, Stellenbosch, South Africa; UAMH—The University of Alberta Microfungus Collection and Herbarium, Edmonton, AB, Canada; UME—Herbarium Department of Ecology and Environmental Sciences (EMG) University of Umeå, Sweden.

Strains isolated in this study are reported in bold.

3. Results and Discussion

Data concerning lichen sample (Figure 1), collection sites and black fungi isolated are reported in Table 1. The epilithic vegetation is rather rare in the Dry Valleys, it is therefore not surprising that only one lichen sample, *Lecanora fuscobrunnea*, out of 16 studied, was collected in Southern Victoria Land. Black fungi (Figure 2) were recovered from 11 out of 16 lichens examined.

Temperature relations are given in Table 3. All the strains tested were able to grow at 0 °C and none of the strains grew at 30 °C. Strains 5303, 5314, 5317, 5321, 5324, 5326 had their optimal growth temperature at 15 °C and did not show any growth above that temperature. All these strains can therefore be classified as psychrophilic, as defined for yeasts and other eukaryotic microorganisms [38]. Strain 5323, with optimal temperature and upper limit for growth at 20 °C, also may be defined as psychrophilic. Peculiar temperature relations, highlighting a more eurythermic behavior, were observed for strains 5313, 5319, 5320 with optimum at 15 °C but 25 °C as upper limit, too high for a true psychrophilic fungus. A similar profile was observed for strain CCFEE 5318 but with an optimal temperature at 20 °C.
**Figure 1.** Some of the lichen thalli examined for black fungi.

**Figure 2.** Some of the black fungi isolated from the lichens examined. This is a selection made based on morphological and phylogenetic characteristics.
Table 3. Temperature relations.

| Isolates       | Temperature (°C) | 0    | 5    | 10   | 15   | 20   | 25   | 30   |
|----------------|------------------|------|------|------|------|------|------|------|
| CCFEE 5303     |                  | 6.8 ± 1.8 | 4.3 ± 2.5 | 11.3 ± 0.4 | 13.8 ± 1.8 | -    | -    | -    |
| CCFEE 5312     |                  | 6.9 ± 1.3  | 7.8 ± 1.1 | 11.8 ± 0.4 | 18.2 ± 1.9 | 5.2 ± 1.6 | -    | -    |
| CCFEE 5313     |                  | 16.4 ± 0.8 | 12.9 ± 0.6 | 25.8 ± 0.4 | 31.9 ± 3.7 | 30.8 ± 1.1 | 19.5 ± 3.5 | -    |
| CCFEE 5314     |                  | 4.4 ± 0.6  | 5.2 ± 1.6 | 7 ± 0   | 12.3 ± 2.5 | -    | -    | -    |
| CCFEE 5317     |                  | 4.8 ± 0.4  | 3.3 ± 0.4 | 8 ± 1.4 | 12 ± 0    | -    | -    | -    |
| CCFEE 5318     |                  | 7.3 ± 0.4  | 4.1 ± 1.8 | 13.3 ± 0.4 | 13 ± 0.4 | 13.5 ± 0.7 | 11.3 ± 3.2 | -    |
| CCFEE 5319     |                  | 15.5 ± 0.7 | 11.9 ± 0.1 | 22 ± 0 | 30 ± 0.7 | 24 ± 0.7 | 11.8 ± 1.1 | -    |
| CCFEE 5320     |                  | 14 ± 0.7   | 10.8 ± 1.1 | 23 ± 1.4 | 27.7 ± 0.9 | 22.5 ± 0.7 | 16.8 ± 1.8 | -    |
| CCFEE 5321     |                  | 3.5 ± 0.7  | 4.7 ± 0.9 | 8.8 ± 0 | 10.3 ± 1.8 | -    | -    | -    |
| CCFEE 5322     |                  | 10.3 ± 1.8 | 9.8 ± 1.1 | 14 ± 1.4 | 18.4 ± 2.3 | 4 ± 0 | -    | -    |
| CCFEE 5323     |                  | 7 ± 0      | 4.7 ± 0.9 | 7 ± 2.1 | 18 ± 0.7 | 20.5 ± 0.7 | -    | -    |
| CCFEE 5324     |                  | 3.5 ± 0.7  | 4.1 ± 1.8 | 5.3 ± 0.4 | 8.8 ± 0 | -    | -    | -    |
| CCFEE 5326     |                  | 2.5 ± 0    | 3.5 ± 0.7 | 4.1 ± 1.8 | 7 ± 0.7 | -    | -    | -    |

Growth are reported as diameter of the colonies (mm) after 3 months of incubation. Highest growth values are reported in bold.

Most of the ITS sequences obtained showed too low identities in the GenBank and were not used for the phylogenetic inference. Figure 3 shows the ML phylogenetic tree, generated using a GTR+IG model, which was selected using the Akaike’s information criterion with a Maximum likelihood approach. The alignment was based on 79 nuclear ribosomal 18S gene sequences and 1707 positions, including gaps, belonging to strains of both plant pathogenic and rock fungi, some of which were still unidentified. The tree includes two classes within the Ascomycota: Dothideomycetes (Orders Capnodiales, Dothideales, Myriangiales and Botryosphaeriales) and Eurotiomycetes (Order Chaetothyriales). The tree was rooted with Debaryomyces hansenii MUCL 29826.

The backbone remains uncertain, but orders in the class Dothideomycetes, are resolved although the 18S gene only was compared. The tree is in agreement with the most recent phylogenetic analyses with the Order Botryosphaeriales separated from Capnodiales [39,40]. Two sister clades are segregated in the Order Chaetothyriales: the group comprising most of the human opportunists of the family Herpotrichiellaceae as Cladophialophora carrionii (Trejos) de Hoog, Kwon-Chung & McGinnis, and the clade composed of mostly rock fungi, including the genus Knufia [41].

Seven of the strains here studied were grouped in the order Capnodiales placed in lineages purely constituted of fungi from rocks. Strains CCFEE 5312 and 5318 are included in a wide clade of rock fungi [40]; here only a selection of strains from the Antarctic is included, but the clade comprises rock fungi from the Mediterranean and Alps too, as well as the melanised micro-filamentous lichen Cystocoleus ebeneus (Dillwyn) Thwaites [42]. The strain CCFEE 5322 groups with rock black fungi exclusively from the Antarctic. The remaining strains in the Capnodiales belong to the rock fungal species Elasticomyces elasticus Zucconi & Selbmann and Friedmanniomyces endolithicus Onofri [43], the last one exclusively from the Antarctic continent [7,17]. The strain CCFEE 5304 as included in a well separated and supported clade of rock fungi from Antarctic rocks collected both in Northern and Southern Victoria land colonized with endolithic communities. This group remains without a clear assignment at any known fungal order. The remaining five strains were in the order Chaetothyriales.
(class Eurotiomycetes). Strains CCFEE 5326 and 5317 grouped together in a separated position with high bootstrap value and do not show clear relations with any described or undescribed species in the tree the ITS sequences were only 88% similar with the closest deposited in GenBank: this is not uncommon for black fungi from locations where genetic and geographic isolation, coupled with environmental pressure, promoted adaptive radiation [7]. Yet, their long branches indicate that these strains are distantly related to each other. Strains CCFEE 5314, 5323 and 5324 cluster with a rock Antarctic fungus, CCFEE 457, isolated from sandstone collected in the Dry Valleys; this group of Antarctic black fungi is sister of a clade represented by the recently formalized genus Knufia [41], including species mainly isolated from monuments.

**Figure 3.** SSU ML tree indicating the phylogenetic position of the black meristematic fungi isolated from lichens (reported in bold in the tree). The strains reported as Rock black fungus are still unidentified rock fungi deposited in the Culture Collection of Fungi From Extreme Environments. Bootstrap values are the results of 1,000 pseudoreplicates. Values below 70 are not shown.
All the strains examined here are not related to groups that contain known lichenicolous species. Rather, they show strict phylogenetic relations with fungi occurring on and in Antarctic rocks. Likewise, the rock black fungi included in this study were found to belong to two classes of Ascomycota: Dothideomycetes and Eurotiomycetes, in this last case specifically in the order Chaetothyriales (Figure 3). Dothideomycetous rock black fungi prefer natural, non-contaminated environments, while chaetotryiomycetous rock black fungi are recurrent particularly in areas influenced by human activities, rich in pollutants [44] probably as consequence of their ability to metabolize aromatic compounds [45].

Lichens can host a wide range of associated fungi with varied ecologies, specificities, and biological traits [26]. Some fast-growing lichenicolous species (e.g., *Athelia*, *Marchandiomyces*), with often low host specificity, can rapidly eradicate lichen vegetation, whereas many others grow slowly without expressing any or only local pathogenic symptoms on their specific hosts, apparently as a long-term result of evolutionary adaptation [46]. These lichenicolous fungi are not found to express their phenotypes without their hosts. Some groups of black fungi have also been observed to colonize a wide range of lichens, as lichenicolous fungi. Some species in the genus *Lichenothelia*, a cosmopolitan genus of rock-inhabiting melanised fungi in the superclass dothideomyceta [47] have been found in association with algae or with lichen thalli, where they produce fertile structures with asci and ascospores. However, species reported in this study were not related to *Lichenothelia* nor with any of the groups comprising known lichenicolous fungi. Moreover, they do not produce visible symptoms on thalli. Several melanized fungi were isolated from lichens from Armenia and the Alps with obscure discolorations [14] belonging to the genera *Mycosphaerella*, *Rhinocladiella*, *Capnobotryella* (class Dothideomycetes) and *Coniosporium*, in this last case related to *Knufia perforans* (Sterflinger) Tsuneda (class Eurotiomycetes). The strains CCFEE 5314, 5323, 5324 isolated during this study may be related to this last species, but the above mentioned isolates were not included in our tree since the SSU sequences are not available. Comparing the ITS sequences of our isolates we found that they were 10% distant from the sequences FJ265756 (*Coniosporium* sp. h6) and FJ265754 (*Coniosporium* sp. c-SH-2009a) isolated form *Caloplaca saxicola* (Hoffm.) Nordin and *Protoparmeliopsis muralis* (Scherb.) M. Choisy respectively, both from Armenia.

Association of black fungi with primary producers could be interpreted from a nutrition-ecological point of view. Oligotrophy is important adaptation for life on rocks and these fungi may often rely only on the sparse, airborne nutrients available, as pollutants in urban environments [45]. In natural environments, with low anthropogenic impact and scarce nutrient availability, they could more conveniently obtain nutrients, by living in association with lichens and other microbial primary producers, such as algae and cyanobacteria, which reside in the endolithic microbial communities of the highest mountain peaks and Antarctica [7,17,48].

Interestingly, some rock-inhabiting species were previously observed to develop lichen-like structures in axenic cultures with phototrophic algae [49,50]. This ability to develop symbiotic interactions with unicellular free-living algae might have allowed some rock-inhabiting fungal lineages to evolve lichenisation and a common link between rock-inhabiting meristematic and lichen-forming lifestyles of ascomycetous fungi has been recently hypothesized [47]. Some studies suggest that some rock-inhabiting fungi constitute early diverging lineages for lichenized fungal groups as Verrucariales and Arthoniomycetes [51].
4. Conclusions

This study represents the first contribution regarding black fungi associated with lichen thalli in the Antarctic. All strains isolated were either closely related or conspecific with black fungi previously found associated with Antarctic endolithic microbial communities. These are mostly cryptoendolithic lichen dominated communities, on the borderlines of what life can tolerate [16]. Even from cosmopolitan lichen species, we isolated endemic Antarctic fungi as *F. endolithicus*. Data obtained in this study give new insights into the biology of lichens: they are particularly well adapted to survive in extreme conditions and the ability to vary microbial communities associated according with the location may give further advantage in adaptation and survival of the whole community.

It is still unclear whether or not black fungi may supply benefits to epilithic lichens as well. It was suggested previously [7] that black fungi may play a role in in hydration or protection of photobionts by dissipating excessive sunlight. Cryptoendolitic lichens are melanized fungi that form a black barrier just above the photobionts stratification [14] making this a plausible scenario. The presence of black fungi may therefore play a crucial role to allow survival in these highly stressful conditions.

Apparently, at the cold edge of life, lichens, together with their associated microbes could find a solution to survive inside the rock by taking advantage of the suite of traits from each microbial partner in order to improve stress resistance and allow the whole community to survive in new conditions.

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References

1. Plemenitaš, A.; Gunde-Cimerman, N. Cellular responses in the halophilic black yeast *Hortaea werneckii* to high environmental salinity. In *Adaptation to Life at High Salt Concentrations in Archaea, Bacteria, and Eukarya*; Gunde-Cimerman, N., Oren, A., Plemenitaš, A., Eds.; Springer: Dordrecht, The Netherlands, 2005; pp. 455–470.
2. Baker, B.J.; Lutz, M.A.; Dawson, S.C.; Bond, P.L.; Banfield, J.F. Metabolically active eukaryotic communities in extremely acidic mine drainage. *Appl. Environ. Microbiol.* **2004**, 70, 6264–6271.
3. Selbmann, L.; Egidi, E.; Isola, D.; Onofri, S.; Zucconi, Z.; de Hoog, G.S.; Chinaglia, S.; Testa, L.; Tosi, S.; Balestrazzi, A.; *et al.* Biodiversity, evolution and adaptation of fungi in extreme environments. *Plant Biosyst.* **2012**, 147, doi:10.1080/11263504.2012.753134.
4. Isola, D.; Selbmann, L.; de Hoog, G.S.; Fenice, M.; Onofri, S.; Prenafeta-Boldú, F.X.; Zucconi, L. Isolation and screening of black fungi as degraders of volatile aromatic hydrocarbons. *Mycopathologia* **2013**, doi:10.1007/s11046-013-9635-2.
5. Staley, J.T.; Palmer, F.; Adams, J.B. Microcolonial fungi: Common inhabitants on desert rocks? *Science* **1982**, 215, 1093–1095.
6. Ruibal, C.; Gonzalo, P.; Bills, G.F. Isolation and characterization of melanized fungi from limestone formations in Mallorca. *Mycol. Progress* **2005**, 4, 23–38.
7. Selbmann, L.; de Hoog, G.S.; Mazzaglia, A.; Friedmann, E.I.; Onofri, S. Fungi at the edge of life: Cryptoendolithic black fungi from Antarctic desert. *Stud. Mycol.* 2005, 51, 1–32.

8. Sert, H.B.; Sümbül, H.; Sterflinger, K. Microcolonial fungi from antique marbles in Perge/Side/Termessos (Antalya/Turkey). *Antonie Leeuwenhoek* 2007, 91, 217–227.

9. Sert, H.B.; Sümbül, H.; Sterflinger, K. *Sarcinomyces sideticae*, a new black yeast from historical marble monuments in Side (Antalya, Turkey). *Bot. J. Linnean Soc.* 2007, 154, 373–380.

10. Sert, H.B.; Sümbül, H.; Sterflinger, K. A new species of *Capnobotryella* from monument surfaces. *Mycol. Res.* 2007, 111, 1235–1241.

11. Marvasi, M.; Donnarumma, F.; Frandi, A.; Mastromei, G.; Sterflinger, K.; Tiano, P.; Perito, B. Black microcolonial fungi as deteriogens of two famous marble statues in Florence, Italy. *Int. Biodeterior. Biodegrad.* 2012, 68, 36–44.

12. Zucconi, L.; Gagliardi, M.; Isola, D.; Onofri, S.; Andaloro, M.C.; Pelosi, C.; Pogliani, P.; Selbmann, L. Biodeteriorigenous agents dwelling the wall paintings of the Holy Saviour’s Cave (Vallerano, Italy). *Int. Biodeterior. Biodegrad.* 2012, 70, 40–46.

13. Onofri, S.; Selbmann, L.; Zucconi, L.; de Hoog, G.S.; de los Rios, A.; Ruisi, S.; Grube, M. Fungal associations at the cold edge of life. In *Algae and Cyanobacteria in Extreme Environments*; Seckbach, J., Ed.; Springer: Dordrecht, The Netherlands, 2007; pp. 735–757.

14. Harutyunyan, S.; Muggia, L.; Grube, M. Black fungi in lichens from seasonally arid habitats. *Stud. Mycol.* 2008, 61, 83–90.

15. Sterflinger, K. Black Yeast and Meristematic Fungi: Ecology, Diversity and Identification. In *The Yeast Handbook. Biodiversity and Ecophysiology of Yeasts*; Péter, G., Rosa, C., Eds.; Springer: Berlin, Germany, 2006; pp. 501–514.

16. Nienow, J.A.; Friedmann, E.I. Terrestrial Litophytic (Rock) Communities. In *Antarctic Microbiology*; Friedmann, E.I., Ed.; Wiley-Liss: New York, NY, USA, 1993; pp. 343–412.

17. Selbmann, L.; de Hoog, G.S.; Gerrits van den Ende, A.H.G.; Ruibal, C.; de Leo, F.; Zucconi, L.; Isola, D.; Ruisi, S.; Onofri, S. Drought meets acid: Three new genera in a Dothidealean clade of extremotolerant fungi. *Stud. Mycol.* 2008, 61, 1–20.

18. Friedmann, E.I. Endolithic microorganisms in the Antarctic Cold Desert. *Science* 1982, 215, 1045–1053.

19. De la Torre, J.R.; Goebel, B.M.; Friedmann, E.I.; Pace, N.R. Microbial diversity of cryptoendolithic communities from the McMurdo Dry Valleys, Antarctica. *Appl. Environ. Microbiol.* 2003, 215, 3858–3867.

20. Selbmann, L.; Zucconi, L.; Ruisi, S.; Grube, M.; Cardinale, M.; Onofri, S. Culturable bacteria associated with Antarctic lichens: Affiliation and psychrotolerance. *Polar Biol.* 2010, 33, 71–83.

21. Cardinale, M.; Vieira de Castro, J., Jr.; Müller, H.; Berg, G.; Grube, M. *In situ* analysis of the bacterial community associated with the reindeer lichen *Cladonia arbuscula* reveals predominance of Alphaproteobacteria. *FEMS Microbiol. Ecol.* 2008, 66, 63–71.

22. Grube, M.; Cardinale, M.; Vieira de Castro, J.; Müller, H.; Berg, G. Species-specific structural and functional diversity of bacterial communities in lichen symbioses. *ISME J.* 2009, 3, 1105–1115.

23. Hodkinson, B.; Lutzoni, F. A microbiotic survey of lichen-associated bacteria reveals a new lineage from the Rhizobiales. *Symbiosis* 2009, 49, 163–180.
24. Bates, S.T.; Cropsey, G.W.G.; Caporaso, J.G.; Knight, R.; Fierer, N. Bacterial communities associated with the lichen symbiosis. *Appl. Environ. Microbiol.* **2011**, *77*, 1309–1314.

25. Grube, M.; Berg, G. Microbial consortia of bacteria and fungi with focus on the lichen symbiosis. *Fungal Biol. Rev.* **2009**, *23*, 72–85.

26. Lawrey, J.D.; Diederich, P. Lichenicolous fungi: Interactions, evolution, and biodiversity. *Bryologist* **2003**, *106*, 80–120.

27. Lawrey, J.D.; Binder, M.; Diederich, P.; Molina, M.C.; Sikaroodi, M.; Ertz, D. Phylogenetic diversity of lichen-associated homobasidiomycetes. *Mol. Phylogenetics Evol.* **2007**, *44*, 778–789.

28. Girlanda, M.; Isocrono, D.; Bianco, C.; Luppi-Mosca, A.M. Two foliose lichens as microfungal ecological niches. *Mycologia* **1997**, *89*, 531–536.

29. Suryanarayanan, T.S.; Thirunavukkarasu, N.; Hariharan, G.N.; Balaji, P. Occurrence of non-obligate microfungi inside lichen thalli. *Sydowia* **2005**, *57*, 120–130.

30. U’Ren, J.; Lutzoni, F.; Miadlikowska, J.; Arnold, A.E. Community analysis reveals close affinities between endophytic and endolithic fungi in mosses and lichens. *Microb. Ecol.* **2010**, *60*, 340–353.

31. Bates, S.T.; Berg-Lyons, D.; Lauber, C.L.; Walters, W.A.; Knight, R.; Fierer, N. A preliminary survey of lichen associated eukaryotes using Pyrosequencing. *Lichenologist* **2012**, *44*, 137–146.

32. Farrar, J.F. The Lichen as an Ecosystem: Observation and Experiment. In *Lichenology: Progress and Problems*; Brown, D.H., Hawksworth, D.L., Bailey, R.H., Eds.; Academic Press: London, UK, 1976; pp. 385–406.

33. Castello, M. Lichens of the Terra Nova Bay area, Northern Victoria Land (Continental Antarctica). *Stud. Geobot.* **2003**, *22*, 3–54.

34. White, T.J.; Bruns, T.; Lee, S.B.; Taylor, J.W. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In *PCR Protocols, a Guide to Methods and Applications*; Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., Eds.; Academic Press: San Diego, CA, USA, 1990; pp. 315–322.

35. Thompson, J.D.; Gibson, T.J.; Plewniak, F.; Jeanmougin, F.; Higgins, D.G. The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **1997**, *24*, 4876–4882.

36. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **2011**, *28*, 2731–2739.

37. Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **1985**, *40*, 783–791.

38. Van Uden, N. Temperature profiles of yeasts. *Adv. Microbiol. Physiol.* **1984**, *25*, 195–251.

39. Schoch, C.L.; Shoemaker, R.A.; Seifert, K.A.; Hambleton, S.; Spatafora, J.W.; Crous, P.W. A multigene phylogeny of the *Dothideomycetes* using four nuclear loci. *Mycologia* **2006**, *98*, 1041–1052.

40. Ruibal, C.; Gueidan, C.; Selbmann, L.; Gorbushina, A.A.; Crous, P.W.; Groenewald, J.Z.; Muggia, L.; Grube, M.; Isola, D.; Schoch, C.L.; *et al.* Phylogeny of rock-inhabiting fungi related to *Dothideomycetes*. *Stud. Mycol.* **2009**, *64*, 123–133.
41. Tsuneda, A.; Hambleton, S.; Currah, R.S. The anamorph genus *Knufia* and its phylogenetically allied species in *Coniosporium, Sarcinomyces*, and *Phaeococcomyces*. *Botany* 2011, 89, 523–536.

42. Muggia, L.; Hafellner, J.; Wirtz, N.; Hawksworth, D.L.; Grube, M. The sterile microfilamentous lichenized fungi *Cystocoleus ebeneus* and *Racodium rupestre* are relatives of plant pathogens and clinically important dothidealean fungi. *Mycol. Res.* 2008, 112, 50–56.

43. Onofri, S.; Pagano, S.; Zucconi, L.; Tosi, S. *Friedmanniomyces endolithicus* (Fungi, Hyphomycetes), anam.-gen. and sp.nov., from continental Antarctica. *Nova Hedwig.* 1999, 68, 175–181.

44. Onofri, S.; Anastasi, A.; Del Frate, G.; di Piazza, S.; Garnero, N.; Guglielminetti, M.; Isola, D.; Panno, L.; Ripa, C.; Selbmann, L.; *et al.* Biodiversity of rock, beach and water fungi in Italy. *Plant Biosyst.* 2011, 45, 978–987.

45. Prenafeta-Boldú, F.X.; Summerbell, R.C.; de Hoog, G.S. Fungi growing on aromatic hydrocarbons: Biotechnology’s unexpected encounter with biohazard. *FEMS Microbiol. Rev.* 2006, 30, 109–130.

46. Diederich, P.; Lawrey, J.D. New lichenicolous, muscilicious, corticolous and lignicolous taxa of *Burgoa* s. l. and *Marchandiomycetes* s. l. (anamorphic Basidiomycota), a new genus for *Omphalina foliacea*, and a catalogue and a key to the non-lichenized, bulbiliferous basidiomycetes, *Mycol. Progress* 2007, 6, 61–80.

47. Muggia, L.; Gueidan, C.; Knudsen, K.; Perlmutter, G.; Grube, M. The lichen connections of black fungi. *Mycopathologia* 2012, doi:10.1007/s11046-012-9598-8.

48. Selbmann, L.; Isola, D.; Egidi, E.; Zucconi, L.; Gueidan, C.; de Hoog, G.S.; Onofri, S. Rock inhabiting fungi: *Saxomyces* gen. nov. and four new species from the Alps. *Fungal Diver.* 2013, in press.

49. Gorbushina, A.A.; Beck, A.; Shulte, A. Microcolonial rock inhabiting fungi and lichen photobionts: Evidence for mutualistic interactions. *Mycol. Res.* 2005, 109, 1288–1296.

50. Brunauer, G.; Blaha, J.; Hager, A.; Turk, R.; Stocker-Worgotter, E.; Grube, M. An isolated lichenicolous fungus forms lichenoid structures when co-cultured with various coccoid algae. *Symbiosis* 2007, 44, 127–136.

51. Gueidan, C.; Ruibal, C.; de Hoog, G.S.; Gorbushina, A.; Untereiner, W.A.; Lutzoni, F. A rock-inhabiting ancestor for mutualistic and pathogen-rich fungal lineages. *Stud. Mycol.* 2008, 61, 111–119.

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