

High diversity and morphological convergence among melanised fungi from rock formations in the Central Mountain System of Spain

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Key words

biodiversity
black fungi
Capnodiales
Chaetothyriales
Dothideomycetes
extremotolerance

Abstract Melanised fungi were isolated from rock surfaces in the Central Mountain System of Spain. Two hundred sixty six isolates were recovered from four geologically and topographically distinct sites. Microsatellite-primed PCR techniques were used to group isolates into genotypes assumed to represent species. One hundred and sixty three genotypes were characterised from the four sites. Only five genotypes were common to two or more sites. Morphological and molecular data were used to characterise and identify representative strains, but morphology rarely provided a definitive identification due to the scarce differentiation of the fungal structures or the apparent novelty of the isolates. Vegetative states of fungi prevailed in culture and in many cases could not be reliably distinguished without sequence data. Morphological characters that were widespread among the isolates included scarce micronematous conidial states, endoconidia, mycelia with dark olive-green or black hyphae, and mycelia with torulose, isodiametric or moniliform hyphae whose cells develop one or more transverse and/or oblique septa. In many of the strains, mature hyphae disarticulated, suggesting asexual reproduction by a thallic micronematous conidiogenesis or by simple fragmentation. Sequencing of the internal transcribed spacers (ITS1, ITS2) and 5.8S rDNA gene were employed to investigate the phylogenetic affinities of the isolates. According to ITS sequence alignments, the majority of the isolates could be grouped among four main orders of Pezizomycotina: Pleosporales, Dothideales, Capnodiales, and Chaetothyriales. Ubiquitous known soil and epiphytic fungi species were generally absent from the rock surfaces. In part, the mycota of the rock surfaces shared similar elements with melanised fungi from plant surfaces and fungi described from rock formations in Europe and Antarctica. The possibility that some of the fungi were lichen mycobionts or lichen parasites could not be ruled out.

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INTRODUCTION

Rock substrata have been explored and analysed with increased microbiological attention because of their extreme variations in environmental factors that select strongly for colonisation by stress-tolerant fungi (Staley et al. 1982, Urzú et al. 1993, Sterflinger & Krumbein 1995, Wollenzien et al. 1995, Sterflinger & Krumbein 1997, Sterflinger 2000, Sterflinger & Prillinger 2001, Bogomolova & Minter 2003, de Leo et al. 2003). Rock surfaces have been recognised as a reservoir for highly melanised, slow-growing filamentous and yeast-like fungi of ascomycetous affinities. Darkly pigmented fungi from geological materials have been named by a variety of terms, e.g., black fungi, black yeasts, or microcolonial fungi (Staley et al. 1982), in reference to their prominent shared morphological characteristics.

The interests in melanised fungi are multiple and varied. From a clinical point of view, the anamorphs of *Capronia* (Herpotrichiellaceae), and many species belonging to the *Exophiala-Ramichloridium-Rhinochloidiella-Cladophialophora* complex, constitute a group of medically significant opportunists (de Hoog

et al. 1994, 1998, 2000b). Another recent trend has been to focus on these organisms as a model for astrobiological studies of eukaryotes (Gorbushina 2003). Finally, the life histories of melanised fungi from rock surfaces may intersect with other saprobic, lichenicolous, epiphytic or even phytopathogenic fungi (Untereiner & Malloch 1999, Crous et al. 2000, 2007a).

Observations of novel melanised fungi from man-made rock surfaces in arid and semi-arid regions, especially those of the Mediterranean basin, led us to test whether these fungi were a widespread component of natural rock surfaces. If such fungi were present, are they abundant and taxonomically distinct? Alternatively, rock surfaces may be primarily colonised by fungi deposited from soils, the phyllosphere, or from nascent lichen mycobionts. These questions were important for determining whether rock surfaces could harbour organisms producing secondary metabolites of relevance for the pharmaceutical industry. The fungi associated with rock surfaces present a series of characteristics that makes them a potentially attractive group for a microbial screening programme, e.g., their apparently ubiquitous habitats, their relative ease of collecting and cultivation in culture, and the fact that at least some species are related to other ascomycetous fungi known to possess multiple secondary metabolite biosynthetic pathways (Kroken et al. 2003). Dark pigmentation of the strains indicates that secondary metabolite biosynthetic pathways, e.g., those involved in melanogenesis, are operational.

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Fungi isolated from rocks share macro- and micromorphological characteristics, but their phylogenetic origins appear to be diverse (Sterflinger et al. 1999). A previous study demonstrated that limestone from Mallorca was colonised by a diverse and complex assemblage of Pezizomycotina, with many representatives among the classes Dothideomycetes and Chaetothyriomycetes (Ruibal et al. 2005). However, many more of the fungi belonged to undetermined orders and families, or corresponded poorly to known families of Pezizomycotina.

In order to confirm and expand our preliminary results (Ruibal et al. 2005), we analysed the number and kinds of melanised fungi associated with the surfaces of four different types of rock in the Central Mountain System of Spain. Our first step was to explore practical methods of providing large numbers of unique fungal isolates for use in natural products discovery and to measure the biodiversity of the fungal community in order to understand the logistics of handling large numbers of fungal strains. We enumerated the different melanised fungi that were recovered from granite, black slate, limestone, and quartzite formations using a combined macromorphology and microsatellite-primed PCR technique to select unique strains, grouping isolates into genotypes assumed to represent species. A preliminary evaluation of their phylogenetic diversity based on ITS1-5.8S-ITS2 rDNA sequences is presented. Phylogenetic relationships among the isolates and possible novel groups were explored by constructing a general sequence-based framework with known genera of similar fungi. The phylogenetic framework was correlated with cultural features, attempting to recognise morphologically defined genera and species.

MATERIALS AND METHODS

Study area, environmental and geological features

The Spanish Central System is an intraplate mountain range located in the central part of the Iberian Peninsula and consists, for the most part, of plutonic and metamorphic rocks belonging to the Hercynian basement and a locally preserved thin cover of Upper Cretaceous sedimentary rocks. Therefore, the area is lithologically and structurally complex and comprises a number of formations such as the Sierras of Guadarrama, Somosierra and Ayllón (García & Aparicio 1987).

The four sites were located in the province of Madrid, within an area of c. 200 km², with distances between sites from 8 to 20 km. The zone receives c. 600–700 mm rainfall distributed irregularly during the year. Two main seasons predominate, a dry and hot summer, with temperatures fluctuating between 40 and 5 °C, and a more humid and cold winter, with temperatures ranging between 15 and -10 °C. The number of sunny days averages about 240/yr.

La Cabrera study site (40°52'30"N, 3°38'W) (Fig. 1) was located near the village of the same name, at c. 1600 m above sea level (asl). It is a pure granite formation of Plutonic origin, comprised of mixed fine to middle grained leucogranites of hercynic age (Bellido Mulas & Rodríguez Fernández 1991). The scarce vegetation consisted mostly of shrubs (dominated by *Cistus ladanifer*) and pasture. The site was sampled in August 2001. The Atazar site (40°57'N, 3°25'W), sampled in October 2001, was located c. 7 km northeast of the village of the same name, at c. 1300 m asl. The zone consisted of metamorphic, black slate from the Silurian period (Pérez González & Portero 1990).

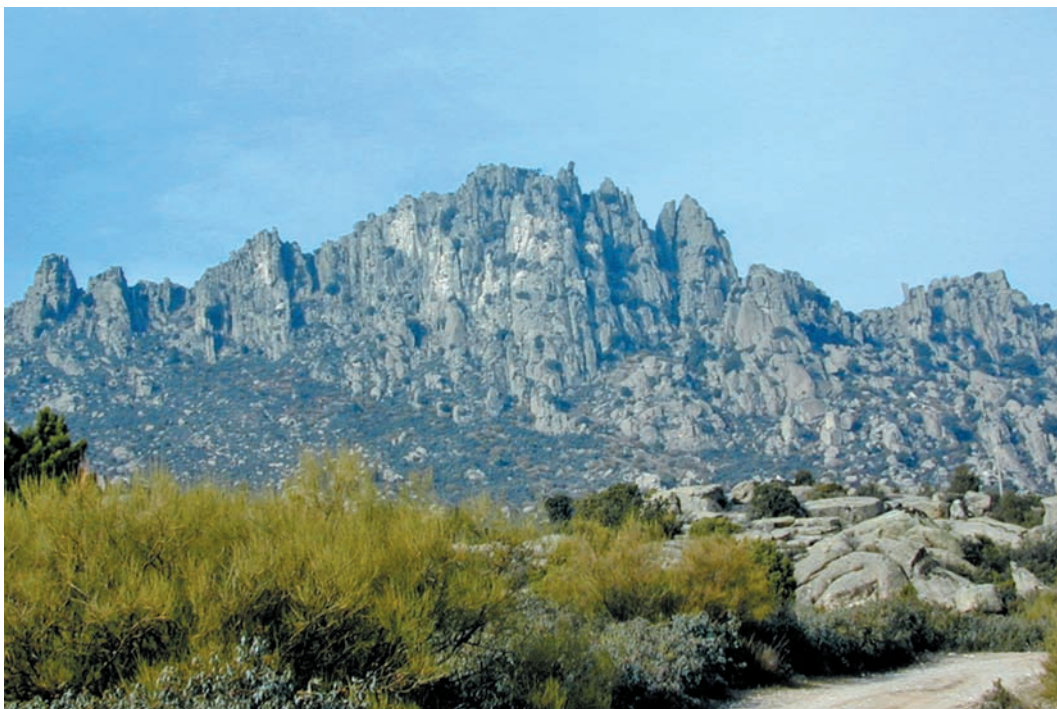


Fig. 1 General view of La Cabrera study site.

The vegetation was sparse, consisting of shrubs (dominated by *Cistus* species) and pasture. The Patones site (40°53'N, 3°27'W) was located c. 5 km to the east of the village of the same name along the road M-102, at c. 800 m asl, on a ridge crest that delimits the valley of the Jarama River. The area was at the southern border of the Central Mountain System and was formed of cretaceous limestone and dolomites (Pérez González & Portero 1990). The site was sampled in May 2003. The vegetation was chaparral-like and dominated by *Quercus ilex*, *Retama sphaerocarpa*, and *Juniperus oxycedrus*. The Puebla de la Sierra site (41°4'N, 3°29'W), sampled in October 2003, was located c. 2 km on the trail towards La Peña de Cabra at km 9 of the road M-130, between the villages of Prádena del Rincón and Puebla de la Sierra at an elevation of c. 1700 m asl. The zone was formed mainly of quartzite, with mixed small quantities of sandstone and conglomerates, from the Ordovician period (García & Aparicio 1987). The site was the only one that was tree-covered, mainly with planted Scots pine (*Pinus sylvestris*) and Spanish fir (*Abies pinsapo*).

Isolation of fungi from rock surfaces

Sample selection was determined by locating a clean and easily collectible sample. Rock samples were taken from clean, vertical, lichen-free surfaces. At each site, 2–5 rock faces separated by 20–200 m and in different orientations were sampled. A cold chisel was inserted into an existing crack, and the pieces of superficial rock were chipped off the surface with a hammer. Rock fragments were packed in sterile, clean paper bags and transported to the laboratory at ambient temperature. Samples were processed for isolation within 1 wk.

The rock surfaces were rinsed with 96 % ethanol and allowed to dry to reduce the influence of dust and airborne spores. Samples for isolation plates were prepared by chipping or scraping surfaces with a sterile knife and chisel blade to remove small superficial fragments from 10–20 cm² area from the outer layer of the field-collected rock. Penetration into the rock was c. 0.5–3 mm, depending on the rock texture. The fragments were further pulverised in a sterile mortar. About 2–3 cm³ of pulverised rock were added to a sterile 50 mL centrifuge tube and washed once with a weak sterile detergent solution (aqueous Tween 20, Sigma -Aldrich, Inc., St. Louis, MO, USA), followed by five washes, three with distilled water and two more with sterile water. Washed samples were resuspended in 40 mL of sterile water and aliquots were spread homogeneously onto isolation media plates. For each rock sample, aliquots of 500, 300, 200, 100, and 50 µL, were spread onto three plates

of each of dichloran-rose bengal agar base (DRBC) (Oxoid Ltd., Basingstoke, Hampshire, GB); and dichloran-glycerol agar base (DG18) (Oxoid Ltd.). Media were supplemented with streptomycin sulfate and terramycin (50 µg/mL) to suppress bacterial growth.

Plates were incubated for 2–3 wk with 12 h fluorescent light at 22 °C. Within a few days of the first appearance of colonies on the plates, all colonies that had dark hyphae were isolated, while faster-growing hyaline mycelia were eliminated by dissection. Continual removal of rapidly extending unpigmented colonies permitted more time and space for development of slower growing pigmented colonies. Plates were examined for up to 1 mo until no more new colonies appeared.

Strain characterisation

The darkly pigmented isolates obtained from isolation plates were grown in duplicate on potato-dextrose agar (PDA, Difco Laboratories) plates at 22 °C for 2 wk. Grouping the isolates into gross morphological types by colony colour, texture, margin type, and radial extension was attempted. However, the crude grouping was inadequate to discriminate numbers of different species. To recognise numbers of unique genotypes and which isolates were genotypically identical, we used a microsatellite-primed PCR (MP-PCR) fingerprinting technique (Longato & Bonfante 1997). Band patterns of PCR products in agarose gels were used to group isolates. The groupings based on MP-PCR were further corroborated by the macroscopic morphology of isolates in PDA plates.

Once the isolates were grouped into genotypes, at least one representative member of each group was processed and analysed. Our underlying assumption was that this isolate was representative of a single species and that the rest of the isolates of the group were the same species, although it was recognised that genetically distinct individuals of the same population could belong to the same species. At least one, and sometimes more than one, isolate of each group were preserved when morphological deviants were observed, or when multiple strains were sequenced. Isolates (Table 1) were frozen as vegetative mycelia in 10 % glycerol at -80 °C at Merck Sharp & Dohme de España, S.A.

The representative isolates were cultured in triplicate in five different media to study their macro- and microscopic characters and to attempt identification. The set of media included: potato-carrot agar (PCA) (Gams et al. 2007), malt extract agar (MEA, Difco Laboratories), Czapek-Dox agar (CZA, Difco Laboratories), oatmeal agar (OA, Difco Laboratories), and

Table 1 Numbers of melanised fungal isolates and genotypes isolated, sequenced and preserved from four stone formations in the Central Mountain System.

Sampling sites	Isolates	Rock type	Genotypes	Isolates ITS sequenced	Preserved representative isolates
La Cabrera	69	Granite	33	39	63
Atazar	85	Slate	57	57	81
Patones	47	Limestone	34	35	41
Puebla de la Sierra	65	Quartzite	39	40	57
Totals	266		163	171	242

Table 2 Melanised fungal isolates from rock of the Central Mountain System. Origin, primers used in the amplification of the ITS region¹ and GenBank accession numbers of the sequences obtained.

Strain(s)	Site (sample no.)	N° of isolates	ITS region primers	ITS region accession ²	Identification
TRN1	La Cabrera (1)	1	ITS1F/ITS1-3	AY843039	Sterile, ' <i>Sarcinomyces petricola</i> ' clade, Chaetothyriales
TRN3, TRN31	La Cabrera (1)	2	ITS5/ITS4A	AY843040, AY616206	<i>Homonema carpetanum</i> , Dothideales (core), Dothideales
TRN4	La Cabrera (3)	1	ITS5/ITS4A	AY843041	<i>Phaeooccomyces catenatus</i> , <i>Sacinomyces petricola</i> clade, Chaetothyriales
TRN5	La Cabrera (2)	1	ITS5/ITS4A	AY843042	Sterile, Mycosphaerellaceae
TRN6	La Cabrera (1)	3	ITS1F/ITS1-3	AY843043	Torula-like, <i>Sacinomyces petricola</i> clade, Chaetothyriales
TRN7	La Cabrera (1)	1	ITS1F/ITS1-3	AY843044	Torula-like, <i>Sacinomyces petricola</i> clade, Chaetothyriales
TRN9	La Cabrera (1)	1	ITS1F/ITS1-3	AY843045	' <i>Stigmira</i> ' sp., Dothideales (core), Dothideales
TRN13v	La Cabrera (1)	1	ITS5/ITS4A	AY843048	Sterile, Mycosphaerellaceae
TRN14	La Cabrera (4)	1	ITS1F/ITS4	AY843049	<i>Exophiala</i> sp., <i>Capronia</i> clade, Chaetothyriales
TRN24, TRN40	La Cabrera (1)	2	ITS5/ITS4A	AY616202, AY616201	<i>Homonema carpetanum</i> , Dothideales (core), Dothideales
TRN25	La Cabrera (1)	5	ITS5/ITS4A	AY616205	<i>Homonema carpetanum</i> , Dothideales (core), Dothideales
TRN30	La Cabrera (2)	1	ITS5/ITS4A	AY843051	<i>Exophiala</i> sp., <i>Capronia</i> clade, Chaetothyriales
TRN35	La Cabrera (2)	1	ITS5/ITS4A	AY843052	Sterile, ' <i>Sarcinomyces petricola</i> ' clade, Chaetothyriales
TRN37	La Cabrera (1)	3	ITS5/ITS4A	AY843054	<i>Aureobasidium pullulans</i> , Dothideales (core), Dothideales
TRN41	La Cabrera (1)	1	ITS5/ITS4A	AY843055	Sterile, ' <i>Cryomyces</i> ' group
TRN42	La Cabrera (1)	1	ITS1F/ITS1-3	AY843056	Fusicladium-like, <i>Mycosphaerella</i> clade, Mycosphaerellaceae
TRN43	La Cabrera (1)	1	ITS5/ITS4A	AY843057	Fusicladium-like, <i>Mycosphaerella</i> clade, Mycosphaerellaceae
TRN44	La Cabrera (1)	1	ITS5/ITS4A	AY843058	Sterile, <i>Mycosphaerella</i> clade, Mycosphaerellaceae
TRN45	La Cabrera (3)	1	ITS1F/ITS1-3	AY843059	<i>Lecythophora</i> sp., ' <i>Cryomyces</i> ' group
TRN47	La Cabrera (1)	2	ITS5/ITS4A	AY843060	Pleosporales
TRN49	La Cabrera (1)	1	ITS5/ITS4A	AY843061	Pleosporales
TRN50, TRN15	La Cabrera (1)	18	ITS5/ITS4A, ITS1F/ITS1-3	AY843062, AY843050	<i>Homonema</i> sp., Dothideales (core), Dothideales
TRN157	La Cabrera (1)	1	ITS5/ITS4A	AY843064	Fusicladium-like, <i>Teratosphaeriaceae</i> clade, Capnodiales
TRN158	La Cabrera (1)	1	ITS5/ITS4A	AY843065	<i>Cladophialophora</i> sp., unknown zone 3, Chaetothyriales
TRN159	La Cabrera (1)	1	ITS5/ITS4A	AY843066	Sterile, Mycosphaerellaceae
TRN162	La Cabrera (1)	1	ITS5/ITS4A	AY843068	<i>Exophiala</i> sp.?
TRN165, TRN11, TRN13n	La Cabrera (1)	3	ITS5/ITS4A, ITS1F/NL1R (TRN13n)	AY843069, AY843046, AY843047	Sterile, Dothideales (core), Dothideales
TRN170, TRN172	La Cabrera (3)	2	ITS5/ITS4A	AY843070, AY843071	<i>Sclerococcum</i> sp., ' <i>Cryomyces</i> ' group
TRN173	La Cabrera (1)	1	ITS5/ITS4A	AY843072	Sterile, Mycosphaerellaceae
TRN174	La Cabrera (1)	3	ITS5/ITS4A	AY843073	Unknown zone 1, <i>Capronia</i> clade, Chaetothyriales
TRN206	Atazar (1)	3	ITS1F/NL1R	AY843074	<i>Phaeotheca</i> sp., <i>Phaeotheca</i> clade, Mycosphaerellaceae
TRN209	Atazar (3)	1	ITS1F/NL1R	AY843075	<i>Phaeotheca</i> sp., <i>Phaeotheca</i> clade, Mycosphaerellaceae
TRN210	Atazar (1)	1	ITS5/ITS4A	AY843076	<i>Cladophialophora</i> sp., unknown zone 3, Chaetothyriales
TRN211	Atazar (1)	1	ITS5/NL1R	AY843077	Phoma-like, Mycosphaerellaceae
TRN212	Atazar (2)	1	ITS1F/NL1R	AY843078	<i>Phaeotheca</i> sp., <i>Phaeotheca</i> clade, Mycosphaerellaceae
TRN213	Atazar (1)	1	ITS1F/NL1R	AY843079	<i>Phaeooccomyces</i> sp.
TRN214	Atazar (1)	1	18S-1/ITS4A	AY843080	<i>Cladophialophora</i> sp.?, Chaetothyriales
TRN215	Atazar (1)	1	ITS5/ITS4A	AY843081	Sterile, Pleosporales
TRN216	Atazar (2)	3	ITS5/NL1R	AY843082	Sterile, Pleosporales
TRN219	Atazar (1)	1	ITS5/NL1R	AY843083	Sterile, Pleosporales
TRN220	Atazar (1)	1	ITS5/NL1R	AY843084	Sterile, ' <i>Cryomyces</i> ' clade
TRN221	Atazar (3)	1	ITS5/NL1R	AY843085	Sterile, Pleosporales
TRN222	Atazar (1)	2	ITS5/NL1R	AY843086	Sterile, Pleosporales
TRN225	Atazar (2)	3	ITS5/NL1R	AY843087	Sterile, Pleosporales
TRN227	Atazar (1)	2	ITS5/ITS4A	AY843088	Unknown zone 1, <i>Capronia</i> clade, Chaetothyriales
TRN230	Atazar (2)	2	ITS5/ITS4A	AY843089	Sterile, ' <i>Sarcinomyces petricola</i> ' clade, Chaetothyriales
TRN231	Atazar (1)	1	ITS5/NL1R	AY843090	Sterile, Mycosphaerellaceae
TRN232	Atazar (1)	2	ITS5/NL1R	AY843091	Sterile, <i>Teratosphaeriaceae</i> clade, Capnodiales
TRN235	Atazar (1)	1	18S-3/NL1R	AY843092	<i>Phaeosclera</i> sp., Dothideales (<i>Phaeosclera</i> clade), Dothideales

TRN236	Atazar (2)	1	18S-3/ITS4A	AY843093	<i>Phaeosclera</i> sp., Dothideales (<i>Phaeosclera</i> clade), Dothideales
TRN237	Atazar (3)	3	ITS5/ITS4A	AY843094	Unknown zone 1, <i>Capronia</i> clade, Chaetothyriales
TRN240	Atazar (2)	1	ITS5/ITS4A	AY843095	Unknown zone 1, <i>Capronia</i> clade, Chaetothyriales
TRN242	Atazar (1)	1	ITS5/ITS4A	AY843097	Sterile, Chaetothyriales
TRN244	Atazar (2)	2	ITS5/NL1R	AY843098	Sterile, Mycosphaerellaceae
TRN245	Atazar (1)	1	ITS5/NL1R	AY843099	Sterile, Mycosphaerellaceae
TRN246	Atazar (2)	1	ITS5/NL1R	AY843100	Unknown zone 1, <i>Capronia</i> clade, Chaetothyriales
TRN247	Atazar (1)	1	ITS5/NL1R	AY843101	Unknown zone 1, <i>Capronia</i> clade, Chaetothyriales
TRN248	Atazar (1)	3	ITS5/NL1R	AY843102	Unknown zone 1, <i>Capronia</i> clade, Chaetothyriales
TRN252	Atazar (3)	2	ITS5/NL1R	AY843103	Unknown zone 1, <i>Capronia</i> clade, Chaetothyriales
TRN254	Atazar (3)	2	18S-3/ITS4A	AY843104	<i>Phaeosclera</i> sp., Dothideales (<i>Phaeosclera</i> clade), Dothideales
TRN256	Atazar (1)	2	18S-3/ITS4A	AY843105	<i>Phaeosclera</i> sp., Dothideales (<i>Phaeosclera</i> clade), Dothideales
TRN258	Atazar (3)	2	ITS1F/NL1R	AY843106	Sterile, Dothideales (core), Dothideales
TRN260	Atazar (1)	1	ITS1F/NL1R	AY843107	Unknown zone 1, <i>Capronia</i> clade, Chaetothyriales
TRN261	Atazar (1)	1	18S-1/ITS4A	AY843108	<i>Phaeosclera</i> sp., Dothideales (<i>Phaeosclera</i> clade), Dothideales
TRN262	Atazar (3)	1	ITS1F/NL1R	AY843109	Sterile, Mycosphaerellaceae
TRN263	Atazar (3)	1	ITS1F/NL1R	AY843110	<i>Phaeotheca</i> sp., <i>Phaeotheca</i> clade, Mycosphaerellaceae
TRN264	Atazar (1)	1	18S-3/NL1R	AY843111	<i>Exophiala</i> sp., Chaetothyriales
TRN267	Atazar (3)	2	ITS1F/NL1R	AY843112	<i>Acrodontium crateriforme</i> , Mycosphaerellaceae
TRN268	Atazar (3)	1	ITS1F/NL1R	AY843113	<i>Hormonema</i> sp., Dothideales (core), Dothideales
TRN269	Atazar (3)	1	ITS1F/NL1R	AY843114	<i>Aureobasidium pullulans</i> , Dothideales (core), Dothideales
TRN270	Atazar (1)	2	ITS1F/NL1R	AY843115	<i>Phaeoocomyces</i> sp., 'black yeasts' group
TRN272	Atazar (3)	3	ITS1F/NL1R	AY843116	<i>Endocoonidioma</i> sp., Dothideales (core), Dothideales
TRN275	Atazar (1)	3	ITS1F/NL1R	AY843117	<i>Endocoonidioma</i> sp., Dothideales (core), Dothideales
TRN278	Atazar (2)	1	ITS1F/NL1R	AY616199	<i>Hormonema carpetanum</i> , Dothideales (core), Dothideales
TRN279	Atazar (3)	1	ITS1F/NL1R	AY843118	<i>Catenulostroma</i> sp.?, Teratosphaeriaceae clade, Capnodiales
TRN280	Atazar (3)	1	ITS1F/NL1R	AY843119	<i>Catenulostroma</i> sp.?, Teratosphaeriaceae clade, Capnodiales
TRN281	Atazar (1)	1	18S-3/NL1R	AY843120	Sterile, 'Cryomyces' group
TRN282	Atazar (1)	1	18S-3/NL1R	AY843121	Sterile, Mycosphaerellaceae
TRN283	Atazar (1)	1	ITS1F/NL1R	AY843122	Sterile, Mycosphaerellaceae
TRN284	Atazar (1)	1	ITS1F/NL1R	AY843123	Sterile, Mycosphaerellaceae
TRN286	Atazar (3)	1	ITS1F/NL1R	AY843124	Sterile, Mycosphaerellaceae
TRN287	Atazar (3)	1	ITS5/ITS4A	AY843125	Sterile, Mycosphaerellaceae
TRN288	Atazar (1)	1	ITS1F/NL1R	AY843126	Sterile, Teratosphaeriaceae clade, Capnodiales
TRN289	Atazar (1)	1	ITS5/ITS4A	AY843127	<i>Catenulostroma</i> sp.?, Teratosphaeriaceae clade, Capnodiales
TRN290	Atazar (1)	1	18S-1/NL1R	AY843128	<i>Catenulostroma</i> sp.?, Teratosphaeriaceae clade, Capnodiales
TRN431	Patones (4)	1	18S-3/ITS4A	AY843129	Sterile, Mycosphaerellaceae
TRN432	Patones (1)	1	18S-3/ITS4A	AY843130	<i>Coniosporium apollinis</i> , 'Coniosporium apollinis' clade
TRN433	Patones (4)	1	18S-3/ITS4A	AY843131	Sterile, Mycosphaerellaceae
TRN434	Patones (3)	1	18S-3/ITS4A	AY843132	Pleosporales
TRN436	Patones (2)	1	18S-3/ITS4A	AY843134	<i>Wardomyces</i> sp.?, Chaetothyriales
TRN437	Patones (4)	1	18S-3/ITS4A	AY843135	Sterile, Mycosphaerellaceae
TRN438	Patones (4)	1	18S-3/ITS4A	AY843136	Sterile, Mycosphaerellaceae
TRN439	Patones (1)	1	18S-3/ITS4A	AY843137	<i>Cladosporium</i> sp., Mycosphaerellaceae
TRN440	Patones (4)	1	18S-3/ITS4A	AY843138	Sterile, Mycosphaerellaceae
TRN441	Patones (1)	1	18S-1/ITS4A	AY843139	Sterile, <i>Trimmatostroma</i> clade, Mycosphaerellaceae
TRN442	Patones (4)	1	18S-1/ITS4A	AY843140	Sterile, Mycosphaerellaceae
TRN443	Patones (4)	1	18S-3/ITS4A	AY843141	Sterile, Mycosphaerellaceae
TRN444	Patones (3)	1	18S-3/ITS4A	AY843142	Sterile, Dothideales
TRN445	Patones (4)	1	18S-3/ITS4A	AY843143	<i>Catenulostroma</i> sp.?, Teratosphaeriaceae clade, Capnodiales
TRN446	Patones (1)	1	18S-3/ITS4A	AY843144	<i>Catenulostroma</i> sp.?, Teratosphaeriaceae clade, Capnodiales
TRN447	Patones (3)	1	18S-3/ITS4A	AY843145	<i>Phaeosclera</i> sp., Dothideales (<i>Phaeosclera</i> clade), Dothideales
TRN448	Patones (1)	1	18S-1/ITS4A	AY843146	<i>Phaeosclera</i> sp., Dothideales (<i>Phaeosclera</i> clade), Dothideales
TRN449	Patones (2)	1	18S-3/ITS4A	AY843147	<i>Phaeoocomyces</i> sp., 'black yeasts' group
TRN450	Patones (2)	1	18S-3/ITS4A	AY843148	<i>Cylindroxiphium</i> sp., Capnodiales s.str., Mycosphaerellaceae

Table 2 (cont.)

Strain(s)	Site (sample no.)	N° of isolates	ITS region primers	ITS region accession ²	Identification
TRN451	Patones (2)	1	18S-3/ITS4A	AY843149	<i>Phaeoocomyces catenatus</i> , <i>Saccharomyces petricola</i> clade, Chaetothyriales
TRN452	Patones (1)	1	18S-3/ITS4A	AY843150	<i>Phaeoocomyces</i> sp., 'black yeasts' group
TRN453	Patones (1)	1	18S-3/ITS4A	AY843151	<i>Phaeotheca</i> sp., <i>Phaeotheca</i> clade, Mycosphaerellaceae
TRN454	Patones (3)	1	18S-3/ITS4A	AY843152	<i>Phaeotheca</i> sp., <i>Phaeotheca</i> clade, Mycosphaerellaceae
TRN455	Patones (1)	1	18S-3/ITS4A	AY843153	<i>Phaeoocomyces</i> sp., 'black yeasts' group
TRN456	Patones (4)	1	18S-3/ITS4A	AY843154	<i>Phaeoocomyces nigricans</i> , 'black yeasts' group
TRN458	Patones (2)	1	18S-3/ITS4A	AY843156	<i>Phaeoocomyces</i> sp., 'black yeasts' group
TRN461	Patones (4)	4	18S-3/ITS4A	AY843158	Sterile, Mycosphaerellaceae
TRN465	Patones (1)	1	18S-3/ITS4A	AY843160	<i>Coniosporium</i> sp., ' <i>Coniosporium apollinis</i> ' clade
TRN467	Patones (4)	2	18S-3/ITS4A	AY843161	Sterile, Mycosphaerellaceae
TRN468	Patones (4)	1	18S-3/ITS4A	AY843162	Sterile, Mycosphaerellaceae
TRN472	Puebla de la Sierra	3	18S-3/ITS4A	AY843164	Sterile, Unknown zone 2, Chaetothyriales
TRN473	Puebla de la Sierra	3	18S-3/ITS4A	AY843165	Sterile, Unknown zone 2, Chaetothyriales
TRN475	Puebla de la Sierra	3	18S-3/ITS4A	AY843166	Sterile, Unknown zone 2, Chaetothyriales
TRN481	Puebla de la Sierra	1	18S-3/ITS4A	AY843167	Sterile, Unknown zone 2, Chaetothyriales
TRN482	Puebla de la Sierra	2	18S-3/ITS4A	AY843168	Sterile, Unknown zone 2, Chaetothyriales
TRN484	Puebla de la Sierra	1	18S-1/ITS1-3	AY843169	Sterile, Venturiaceae
TRN485	Puebla de la Sierra	1	18S-1/ITS1-3	AY843170	Unknown zone 2, Chaetothyriales
TRN486	Puebla de la Sierra	1	18S-3/NL1R	AY843171	Sterile, <i>Capronia</i> clade, Chaetothyriales
TRN487	Puebla de la Sierra	1	ITS5/ITS4A	AY843172	Sterile, <i>Capronia</i> clade, Chaetothyriales
TRN488	Puebla de la Sierra	1	ITS5/ITS4A	AY843173	<i>Cladophialophora</i> sp., Chaetothyriales
TRN489	Puebla de la Sierra	1	ITS5/ITS4A	AY843174	<i>Cladophialophora</i> sp., Chaetothyriales
TRN491	Puebla de la Sierra	1	ITS5/ITS4A	AY843175	Sterile, Mycosphaerellaceae
TRN492	Puebla de la Sierra	1	ITS5/ITS4A	AY843176	<i>Phaeoocomyces</i> sp., 'black yeasts' group
TRN493	Puebla de la Sierra	3	18S-3/ITS4A	AY843177	<i>Exophiala</i> sp., <i>Capronia</i> clade, Chaetothyriales
TRN495	Puebla de la Sierra	1	18S-3/ITS4A	AY843178	<i>Capronia</i> clade, Chaetothyriales
TRN497	Puebla de la Sierra	1	18S-3/ITS4A	AY843179	<i>Exophiala</i> sp., <i>Capronia</i> clade, Chaetothyriales
TRN498	Puebla de la Sierra	1	18S-3/ITS4A	AY843180	<i>Exophiala</i> sp., <i>Capronia</i> clade, Chaetothyriales
TRN499	Puebla de la Sierra	2	18S-3/ITS4A	AY843181	Sterile, Pleosporales
TRN500	Puebla de la Sierra	1	18S-3/ITS4A	AY843182	Sterile, <i>Phaeotheca</i> clade, Mycosphaerellaceae
TRN502, TRN504	Puebla de la Sierra	4	18S-3/ITS4A	AY843183, AY843184	<i>Cladophialophora</i> sp., unknown zone 3, Chaetothyriales
TRN506	Puebla de la Sierra	1	18S-3/ITS4A	AY843185	<i>Cladophialophora</i> sp., unknown zone 3, Chaetothyriales
TRN507	Puebla de la Sierra	1	18S-3/ITS4A	AY843186	<i>Cladophialophora</i> sp., unknown zone 3, Chaetothyriales
TRN508	Puebla de la Sierra	3	ITS5/ITS4A	AY843187	<i>Cladophialophora</i> sp., unknown zone 3, Chaetothyriales
TRN509	Puebla de la Sierra	3	ITS5/ITS4A	AY843188	<i>Cladophialophora</i> sp., unknown zone 3, Chaetothyriales
TRN510	Puebla de la Sierra	1	ITS5/ITS4A	AY843189	<i>Cladophialophora</i> sp., unknown zone 3, Chaetothyriales
TRN515	Puebla de la Sierra	5	18S-3/ITS4A	AY843190	<i>Cladophialophora</i> sp., unknown zone 3, Chaetothyriales
TRN520	Puebla de la Sierra	1	18S-3/ITS4A	AY843191	<i>Phaeoocomyces</i> -like, 'black yeasts' group
TRN521	Puebla de la Sierra	1	18S-3/ITS4A	AY843192	<i>Phaeosclera</i> sp., Dothideales (<i>Phaeosclera</i> clade), Dothideales
TRN522	Puebla de la Sierra	1	18S-3/ITS4A	AY843193	<i>Cladophialophora</i> sp., unknown zone 3, Chaetothyriales
TRN523	Puebla de la Sierra	1	18S-3/ITS4A	AY843194	<i>Phaeosclera</i> sp., Dothideales (<i>Phaeosclera</i> clade), Dothideales
TRN524	Puebla de la Sierra	1	18S-1/ITS1-3	AY843195	<i>Phaeosclera</i> sp., Dothideales (<i>Phaeosclera</i> clade), Dothideales
TRN525	Puebla de la Sierra	1	ITS1f/NL1R	AY843196	<i>Phaeosclera</i> sp., Dothideales (<i>Phaeosclera</i> clade), Dothideales
TRN526	Puebla de la Sierra	1	18S-3/ITS4A	AY843197	Sterile, Dothideales (core), Dothideales
TRN527	Puebla de la Sierra	1	18S-3/ITS4A	AY843198	Sterile, unknown zone 2, Chaetothyriales
TRN528	Puebla de la Sierra	3	18S-3/ITS4A	AY843199	<i>Cladophialophora</i> sp., unknown zone 3, Chaetothyriales
TRN529	Puebla de la Sierra	1	18S-3/ITS4A	AY843200	<i>phaeoocomyces</i> -like, 'black yeasts' group
TRN531	Puebla de la Sierra	1	18S-3/ITS4A	AY843201	Sterile, <i>Capronia</i> clade, Chaetothyriales
TRN533	Puebla de la Sierra	1	ITS5/ITS4A	AY843202	Sterile, Dothideales (core), Dothideales

¹ Sequences length from 455 to 568 bp.² ITS region accession numbers are in the same order as strain numbers, unless indicated otherwise.

water agar supplemented with 0.2 % yeast extract (AI, Difco Laboratories). Colony diameter, texture, pigmentation, margin appearance, exudates, and colours in the descriptions were recorded after 2 wk at 22 °C.

Microscopic features were evaluated by 1) observing hyphae and other structures mounted in 5 % KOH; 2) growing fungal colonies on cover glasses immersed in cornmeal agar (Difco Laboratories) and malt agar (0.5 % malt extract, 1.5 % agar) and supported on 2 % water agar. Three-week-old colonies on cover glasses were fixed in 95 % ethanol, mounted in cotton blue-lactophenol, and photographed.

DNA extraction, PCR amplification, and DNA sequencing

DNA extraction was performed following previously described methods (Peláez et al. 1996). The MP-PCR was performed using (GTG)⁵ primer (Longato & Bonfante 1997). Reactions (25 µL) were carried out in the following conditions: 0.2 mM of each dNTPs (geneAmp dNTPs Perkin Elmer, Norwalk, CT, USA), primer at a final concentration of 0.4 µM, 5 µL of fungal DNA solution (1/100 dilution of the extraction solution with a concentration between 0.1 and 0.01 µg/mL) and 0.2 U of *Taq* polymerase (QBiogene, Inc., Carlsbad, CA, USA) in a reaction buffer. The steps of the PCR reactions were: 40 cycles of 30 s at 95 °C, 30 s at 53 °C and 2 min at 72 °C and the process was completed with a final step of 10 min at 72 °C for the polymerisation of incomplete fragments. Reactions were performed following manufacturer's recommendations. The amplification products were checked for appropriate size by electrophoresis in 1.2 % agarose gels (Hispanlab, S.A., Alcobendas, Madrid, Spain) in a Pharmacia LKB-GPS 200 electrophoresis apparatus at 120 V (Amersham Biosciences Ltd., Buckinghamshire, UK).

To analyse and compare the band patterns from MP-PCR, fragments were separated electrophoretically in prefabricated polyacrylamide (12.5 %) gels (GeneCel Excel 12.5/24 Kit, Amersham Biosciences Ltd.) in a GenePhor unit (Amersham Biosciences Ltd.) at 600 V and visualised by silver staining (PLUS ONE DNA Silver Staining Kit, 17-6000-36, Amersham Biosciences Ltd.) following the manufacturer's procedures. Gel band patterns were scanned and used to produce phenetic groupings with Gel Compar 4.1 software package (Applied Biomaths). The combined data of all fingerprints for each isolate were compared in a pairwise manner using Jaccard's index (Sneath & Sokal 1973) to generate a matrix of similarities. A dendrogram showing the relative similarity among the different isolates was calculated by UPGMA (Sneath & Sokal 1973). Isolates with 97 % or greater homology were considered to be the same genotype.

The ITS1-5.8S-ITS2 fragment of the isolates was amplified with the following primers: ITS1F (Gardes & Bruns 1993), ITS4A (Larena et al. 1999), ITS5 and ITS2 (White et al. 1990), NL1R (reverse of NL1) (O'Donnell 1993), 18S-1 (Platas et al. 2004), ITS1-3: 5'-AGTTCAGCGGGTAGTCC-3' (designed in the laboratory) and 18S-3: 5'-GATGCCCTTAGATGTTCTGGGG-3' (designed in-house). The specific primers applied for amplification of each isolate are listed in Table 2. Some additional sequencing of the rDNA region containing the initial

sequence of the 18S rDNA gene of some of the isolates was attempted to correlate isolates with previous studies (Ruibal et al. 2005). The amplifications were performed using primers NS1 and NS2 (White et al. 1990).

PCR amplifications were performed with *Taq* DNA polymerase (QBiogene, Inc.) following the procedures recommended by the manufacturer (5 min at 93 °C followed by 40 cycles of 30 s at 93 °C, 30 s at 53 °C and 2 min at 72 °C). The amplification products (0.1 µg/mL) were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit with an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) following the manufacturer's recommendations. Each strand of the amplification products was sequenced with the same primers used for the initial amplification.

Sequence analyses

Partial sequences obtained in sequencing reactions were assembled using GCG software (GCG Wisconsin Package, v. 10.3-Unix, Accelrys Inc., San Diego, CA) generating a consensus sequence for each fungal strain. Consensus sequences were aligned by CLUSTAL X (Thompson et al. 1997) and the program-generated multiple alignments were visually adjusted with GeneDoc v. 2.5 software (Nicholas & Nicholas 1997). Maximum-parsimony and neighbour-joining analyses were performed with PAUP v. 4 using the default settings (Swofford 1998). Data were resampled with 1 000 bootstrap replicates (Felsenstein 1985) by using the heuristic search option of PAUP. The percentage of bootstrap replicates that yielded greater than 50 % for each group was used as a measure of statistical confidence. Sequence matching with public or proprietary databases was performed with BLAST2N and FastA applications (GCG Wisconsin Package).

RESULTS

Number of isolates and efficiency of the isolation method

A total of 266 strains were isolated from the four locations of the Central Mountain System (Table 1). Sixty nine isolates were obtained from La Cabrera, representing 33 genotypes; from Atazar, 85 isolates were recovered, representing 57 genotypes; from Patones, 47 isolates were obtained that accounted for 34 genotypes; and finally, 65 isolates were recovered from Puebla de la Sierra, representing 39 genotypes. From the total set, only five genotypes were found to be common to two or more sites: 1) TRN156, TRN492, and TRN270; 2) TRN165 and TRN258; 3) TRN280/TRN289, and TRN173; 4) TRN4 and TRN451; and 5) TRN1, TRN230, and TRN35. Seventy five percent (122) of the genotypes established by MP-PCR were isolated only once; the rest varied in abundance (Fig. 2). Only one genotype (TRN50 group) was significantly abundant; it was isolated 18 times from granite of La Cabrera. These results were consistent with our previous studies of limestone in Mallorca, where infrequent and unique genotypes predominated (Ruibal et al. 2005). The even distribution of predominantly single and infrequent genotypes indicated that the isolation method was adequate to recover large number of genetically different strains and

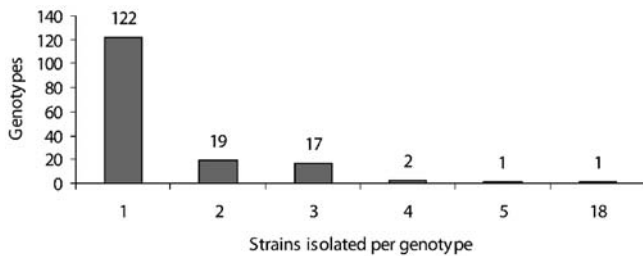


Fig. 2 Isolation frequencies of 162 different fungal genotypes isolated from stone. Genotypes were established based on a grouping by microsatellite-primed PCR products.

suggested an undersampling of high species diversity (Gotelli & Colwell 2001). Because of the high number of single genotypes and scant overlap among the four samples, no obvious pattern with respect to site or rock type was evident. The absence of cosmopolitan, airborne fungi, e.g., *Penicillium* and *Alternaria* species, among the set of isolates indicated that the whole isolation procedure, including both rock surface sterilisation and dissection of fast, hyaline mycelia, was effective.

Morphology, grouping of taxonomic units and identification

The set of isolates generally exhibited a limited spectrum of morphological features. Colonies were strongly pigmented, ranging across a gradient of different tones and intensities from grey, dark-olive, olive-brown to black. Dark pigmentation was the main criterion for colony selection from isolation plates and therefore its predominance limited reliable discrimination among isolates.

Another macromorphological trend among our isolates was their slow radial growth. The mean diameter of a set of isolates selected based on an ITS-sequence similarity grouping ($n = 70$) on the five characterisation media was 14.1 mm, with the largest average radial growth in OTM medium (17.1 mm) and the smallest in AI medium (11 mm). The mycelia of a significant proportion of isolates (10 %) exhibited scarce or nearly imperceptible radial growth from the inoculation point. Also a significant percentage of the plates (10 %) exhibited a pattern of forming tiny punctuate colonies, probably arising from fragmented cell masses, or by forming colonies from cells accumulated in dense, irregular masses via meristematic growth.

Among the array of colony textures observed, filamentous textures with poorly developed aerial mycelia were the most common, although compact and dense colonies with a dry, granulose-crustose texture were evident in a high percentage of the isolates (mainly in CZA medium). Black yeast-like colonies in the classical sense (de Hoog & Hermanides-Nijhof 1977) were infrequent among our set of isolates (see below). Mycelial exudates or soluble pigments in the agar were largely absent among the isolates.

The observation and identification of melanised rock fungi in culture was problematic due to their strong pigmentation and their often hard or granular texture which often made prepara-

tion of isolates for microscopy difficult. The microscopic morphology of the majority of the isolates revealed sterile mycelia with poorly differentiated, few or no specialised structures and tissues. Asexual sporulating states were infrequent and sexual states absent. Common microscopic features observed among isolates included hyphae with densely melanised, firm or thick-walled cells, vegetative hyphae of nearly rectangular cells that develop into torulose/moniliform hyphal pattern, cells that occasionally develop transverse and oblique septa, and that sometimes develop muriform or multicellular structures.

A hundred and fifty three isolates were selected (Table 2) based in their position in the ITS tree and examined microscopically; 64 of them (41.8 %) did not exhibit any conidial or sexual state on the media set and were considered sterile mycelia. The rest exhibited diverse modes of conidiogenesis and, in a few cases, isolates developed pycnidial conidiomata, or apparently multicellular structures that may have been primordial or aborted conidiomata. Unequivocal identification of isolates with conidial states was only possible in the cases of TRN3, TRN31, TRN25, TRN278, TRN40, TRN24 (*Hormonema carpetanum*), TRN37, TRN 269 (*Aureobasidium pullulans*), TRN432 (*Coniosporium apollinis*), TRN456 (*Phaeococcomyces nigricans*), TRN497, TRN498 (*Exophiala spinifera*), TRN4, TRN451 (*Phaeococcomyces catenatus*), and TRN267 (*Acrodontium crateriforme*). In other cases, we classified the isolates into anamorph form genera, such as *Endoconidioma*, 'Stigmia' (see Crous et al. 2006a), *Phaeosclera*, *Coniosporium*, *Phaeococcomyces*, *Sclerococcum*, *Exophiala*, *Cladophialophora*, *Wardomyces*, *Catenulostroma*, *Trimmatostroma*, *Fusicladium*, *Phaeotheca* and *Cylindroxiphium* (Table 2). Lastly, other isolates were assigned to morphological types coinciding with classical, polyphyletic genera, e.g., *Cladosporium* (Table 2). In most cases, however, unequivocal identifications were not possible; therefore more extensive studies will be necessary to determine whether the fungi could be new species or if they were vegetative states of known ascomycetous fungi.

Strain grouping based on position in the ITS tree attempted to reveal correlations between morphological and molecular traits (Table 2). When morphological trends were superimposed onto a sequenced-based framework some molecular groups showed strong morphological cohesion. Clades of fungi corresponding to *Catenulostroma*, *Cladophialophora*, *Exophiala*, *Hormonema*, *Phaeococcomyces*, *Phaeosclera*, and *Phaeotheca*, were apparent (Fig. 3–5).

Recognition of genotypes by microsatellite-primed PCR

Cluster analysis of MP-PCR products of the 266 initial isolates identified 163 different genotypes (analysis not shown). The ITS region from at least one example of each genotype was sequenced. In several cases, the ITS region from more than one representative of each genotype was sequenced to verify the assumption that a group of isolates from a common MP-PCR pattern represented a single species (Table 1). Therefore, ITS sequences were obtained from a total of 171 isolates (Table 2). Phenetically similar isolates from different sites were also sequenced to determine if they shared common genotypes leading to the conclusion that only five genotypes occurred at multiple collection sites.

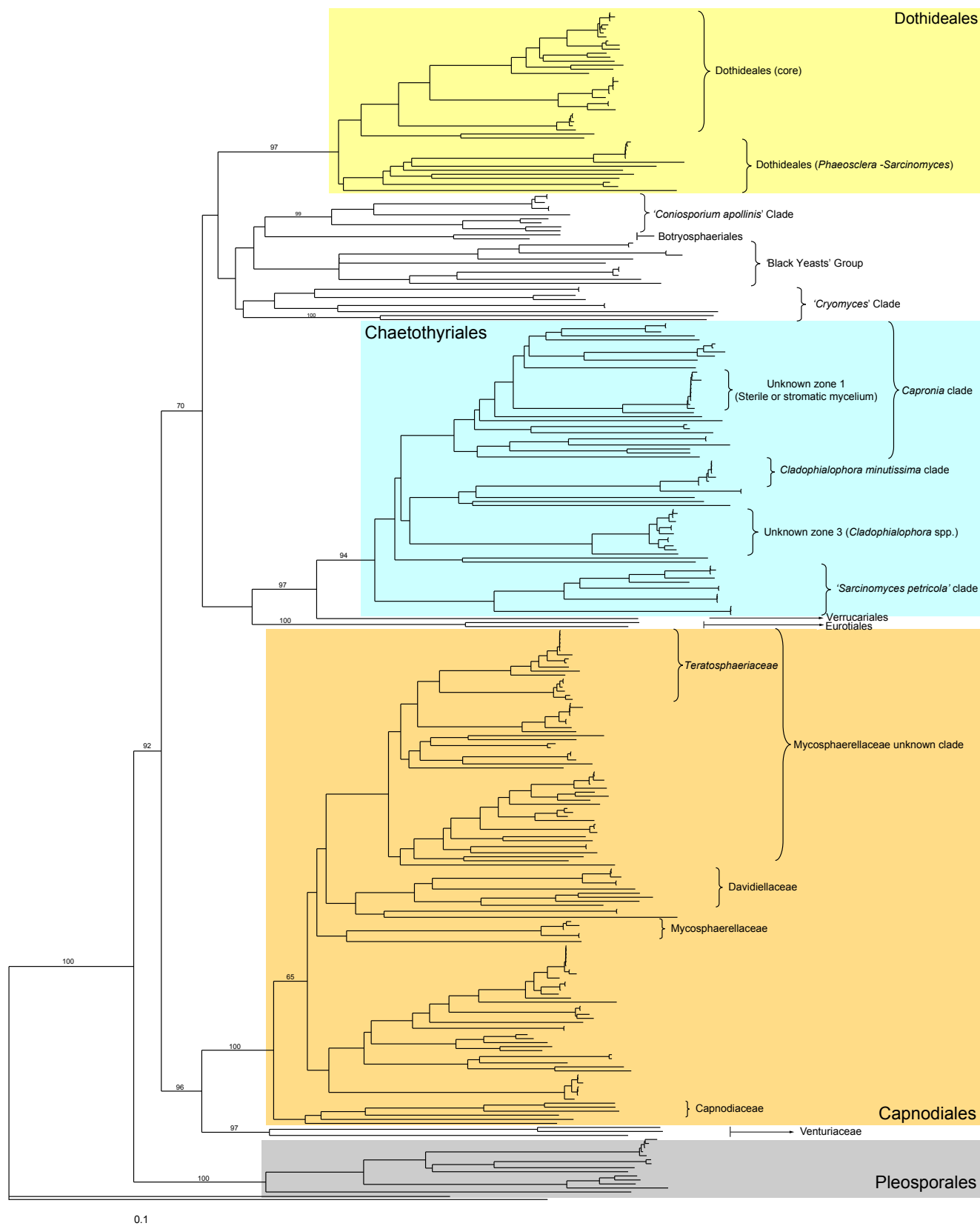


Fig. 3 Phylogram of the neighbour-joining tree obtained from melanised rock surface isolates and selected reference strains sequences of the ITS1-5.8S-ITS2 ribosomal region. Statistical support (1 000 bootstrap replicates) values of indicated at branch points of the main branches.

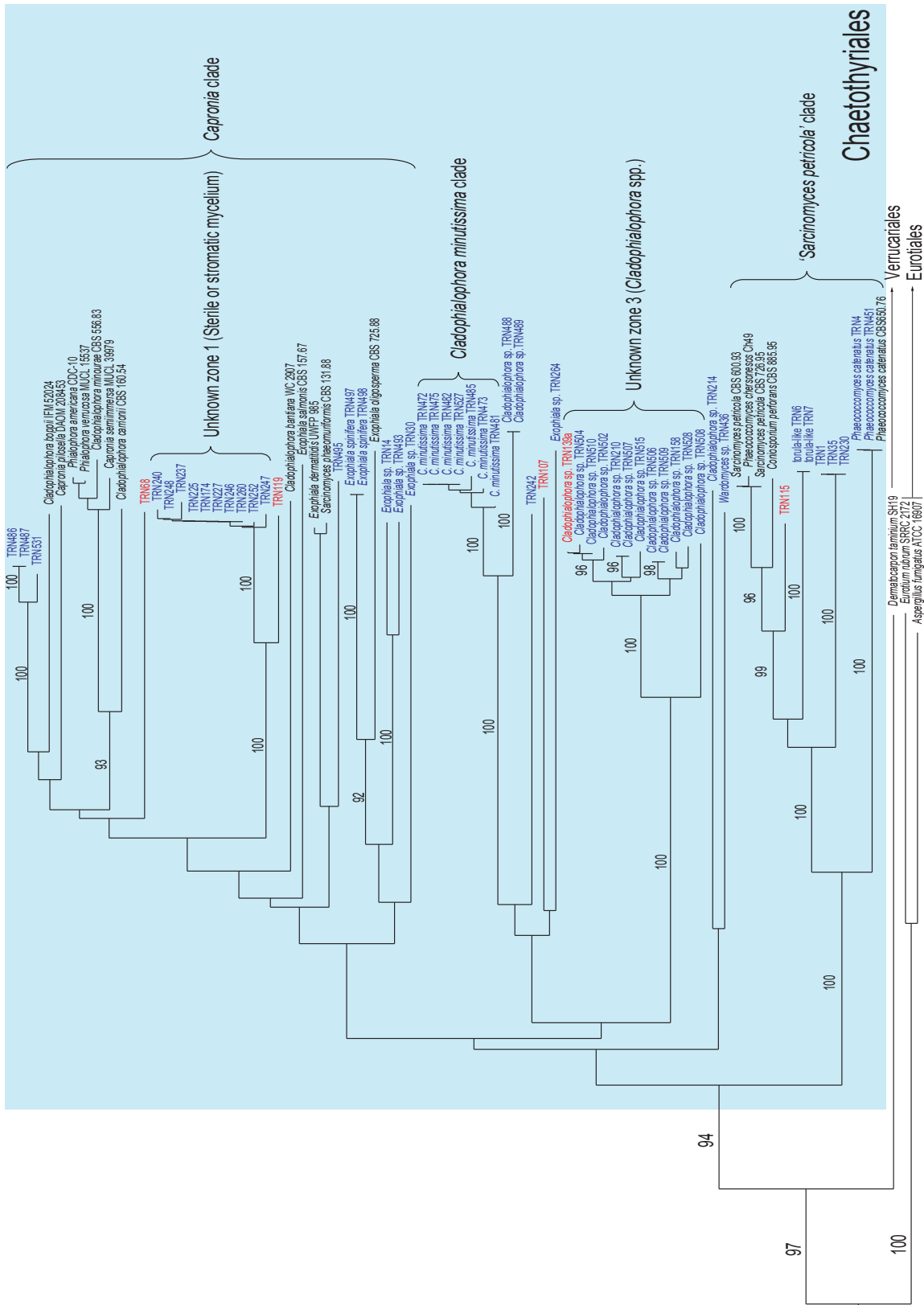


Fig. 5 Partial phylogram of the neighbour-joining tree obtained from melanised rock surface isolates and reference strains sequences of the ITS1-5.8S-ITS2 ribosomal region (Fig. 3). Branches corresponding to the Chaetothyriales, Eurotiales, and Verrucariales clades.

Amplification and sequencing of the ITS region of rDNA

To estimate the relative phylogenetic relationships among the isolates and the reference strains, the ITS sequences of the 159 isolates were aligned with reference sequences from GenBank that were selected based on sequence similarity, or because they were potentially related taxa. A sequence alignment was used to generate a tree by the neighbour-joining method (Fig. 3). A basidiomycete was chosen as outgroup (*Rhizoctonia* sp. Eab-S4) because FASTA analyses indicated the isolates spanned a broad spectrum of the Pezizomycotina. We did not intend to recreate the taxonomic frame of the phylum, but sought to quickly estimate the relative phylogenetic position and if possible arrive at an identification. Results were similar to more specific analyses of other authors in similar groups, and several main clades received strong bootstrap support, so the consistency of the tree seemed to be remarkably high considering that we had attempted to compare sequences of such different fungi. Isolate TRN140 yielded the most disparate ITS sequence and was not clearly associated with any other group of strains (Fig. 3).

ITS-tree, Dothideomycetes

The tree was divided in four main branches (Fig. 3). The first of these branches grouped together (with no relevant support), the order Dothideales and other clades of uncertain taxonomic position. The first branch in the Dothideales (Fig. 4) was the only which received strong bootstrap support (97 %). It was divided into two main sub-branches; the first comprised the reference strains of the Dothideales s.str. and a group of isolates identified as *Hormonema carpetanum* (Bills et al. 2004b), and other *hormonema*-like isolates. The second branch was referable to the reference strains *Sarcinomyces crustaceus* (CBS 156.89) and *Phaeosclera dematioides* (CBS 157.81), both ascomycetous anamorphs of uncertain affinity. All the isolates included in this branch were identified as *Phaeosclera* sp. Although these fungi are of unknown affinities, the branch was monophyletic with that of the Dothideales s.str., so a close relationship could be inferred among the branches.

ITS-tree, unidentified branches associated with the Dothideomycetes

In a major branch parallel to the Dothideales clade appeared a series of sub-branches with no relevant statistical support (Fig. 4). The lack of statistical confidence and the heterogeneous nature of the isolates casted doubts on the phylogenetic significance of this clade. The first among the branches was designated as the '*Coniosporium apollinis*' clade (Fig. 4), and included two reference sequences of *C. apollinis*, a rock-inhabiting fungus from Greece (Sterflinger et al. 1997). This branch, strongly supported by a bootstrap value of 99 %, also contained our isolates; TRN432, identified as *C. apollinis* based on its sequence and its morphology, and TRN465, TRN74, designated as *Coniosporium* sp. The rest of the isolates (TRN71, TRN56, TRN57, and TRN73) may be congeneric with *C. apollinis*. Also two species of the Botryosphaeriaceae, *Lasiodiplodia theobromae* (Alves et al. 2008) and *B. dothidea* (Crous et al. 2006b), appeared associated with the '*Coniosporium apollinis*' clade.

The next unsupported clade was designated as the 'black yeasts' group (Fig. 4) because all strains formed colonies consisting largely of melanised unicellular aggregates. Yeast-like forms were infrequently found among other clades of the tree. However, in this branch, the nearly exclusive yeast-like growth was distinctive and diagnostic. The only reference sequence that grouped within this clade was *Phaeococcomyces nigricans* (CBS 652.76). Due to their distinctive morphological characteristics and their position in the tree, the other isolates in this group were identified as *Phaeococcomyces* spp. The last of the unsupported clades related to the Dothideales was designated as the '*Cryomyces*' clade, because the isolates grouped with reference sequences of *Cryomyces antarcticus* and *C. minteri* (Selbmann et al. 2005) (Fig. 4).

ITS-tree, Eurotiomycetes

Although the second main clade corresponded with the orders Chaetothyriales, Verrucariales, and Eurotiales (Fig. 3), it lacked significant support. These three orders have been proposed to belong to the class Eurotiomycetes (Lutzoni et al. 2004, Miadlikowska & Lutzoni 2004, Reeb et al. 2004). The Eurotiales branch (100 % bootstrap value) was formed only by the reference sequences and included no rock isolates (Fig. 5). Chaetothyriales and Verrucariales appeared grouped with a high bootstrap value (97 %). The order Chaetothyriales was amply represented among our isolates and received a bootstrap value of 94 % (Fig. 5). The clade was divided into a number of sub-branches. The only one with a strong support (bootstrap value of 100 %), contained several previously characterised melanised fungi found in rocks such as *Sarcinomyces petricola* and *Coniosporium perforans*, and sequences of the anamorph species *Phaeococcomyces catenatus*. The remaining branches of the Chaetothyriales (*Capronia* clade, *Cladophialophora minutissima* clade) did not receive relevant support and seemed to correspond to the Chaetothyriales family Herpotrichiellaceae, which includes the genus *Capronia* and some of its recognised or suspected anamorphs, e.g., *Cladophialophora*, *Exophiala*, and *Phialophora*. Also within this region appeared groups of closely related sequences (unknown zones 1 and 2) with no correspondence with any reference sequence (Fig. 5). The last branch of the Eurotiomycetes clade was formed solely by the reference sequence of the lichenised fungus *Dermatocarpon taminium* SH19 (Fig. 5), a member of the order Verrucariales (Heiðmarsson 2003).

ITS-tree, Capnodiales

The third main branch grouped with relatively high bootstrapping (bootstrap value 96 %) both the order Capnodiales (bootstrap support 100 %) and the family Venturiaceae of the order Pleosporales (97 % bootstrap value), (Fig. 3). The Capnodiales clade comprised a number of sub-branches grouped with a bootstrap value of 65 %, and a Capnodiaceae clade. Among the other sub-branches were a Davidiellaceae clade (Crous et al. 2007a, b) and a Mycosphaerellaceae clade, both with high bootstrap values (Schoch et al. 2006) (Fig. 6). A clade (bootstrap value of 89 %) named as 'Mycosphaerellaceae unknown clade', included a number of miscellaneous anamorphic ascomycetes, including the Teratosphaeriaceae (72 % bootstrap value) (Crous

et al. 2007a). Finally, a clade (65 % bootstrap value) containing a number of isolates and *Devriesia americana* (STE-U 5121 = CBS 117726) (Crous et al. 2007c) as unique reference sequence. Finally, the Venturiaceae was represented by isolate TRN484, grouped with the reference strains *Venturia cerasi* and *Fusicladium effusum* (Fig. 6). For the most part, isolates in the Capnodiales formed only vegetative mycelia in culture. The major exceptions were fungi with endoconidia that we tentatively called *Phaeosclera* (Sigler et al. 1981), cladosporium-like, and catenulostroma-like fungi.

ITS-tree, Pleosporales

The last of these branches was identified with the order Pleosporales (bootstrap value of 100 %) and included reference sequences belonging to the family Phaeosphaeriaceae (Pleosporales) (Fig. 3). All the isolates in the clade only formed vegetative mycelia and could not be directly related with any known reference sequences, although the high bootstrap values of all the branches show the high affinity of the isolates with the Phaeosphaeriaceae (Fig. 6).

DISCUSSION

Biogeography and diversity

The abundance and variety of isolates encountered in a previous study of fungi in Mallorcan limestone (Ruibal et al. 2005) was surprisingly high, therefore we wanted to confirm whether this pattern of taxonomic diversity was repeatable in the continental environment of central Spain. In Mallorca, limestone from two sites yielded 117 melanised isolates that were determined to represent 39 different genotypes. Anticipating that levels of diversity could be comparable, we were cautious not to sample widely or extensively until the levels of abundance of fungi in continental rock samples could be evaluated. We started with four different rock types (granite, slate, limestone, and quartzite) at four different sites. The only criteria used to select different kinds of rock samples were that rocks would be relatively clean and free from influence of soil and lichens and could be easily lifted off the rock faces with a chisel. No attempt was made to enrich the samples by choosing stained or biologically deteriorated surfaces or surfaces that were obviously colonised by black fungal structures.

Rock surfaces harboured an easily accessible reservoir of melanised ascomycetes. Even with arbitrary selection of rock substrata, a set of isolates was obtained that exhibited a combination of cryptic morphology and phylogenetic affinities agreeing with, or even surpassing, previous studies on rock-inhabiting fungi in the Mediterranean and other locations. The assemblage of isolates from central Spain resembled that recovered from limestone in the Balearic Islands (Ruibal et al. 2005) and included some genera, species or species assemblages detected by other investigators (Staley et al. 1982, de Hoog et al. 1997, Sterflinger et al. 1997, Wollenzien et al. 1997, de Leo et al. 1999, 2003, Onofri et al. 1999, Bogomolova & Minter 2003). The phylogenetic bias towards the Dothideales, Capnodiales, and Chaetothyriales was especially evident (Fig. 3–5). The high diversity observed in two different regions in

Spain confirms that our isolation technique was an appropriate method for the isolation of melanised fungi on rock surfaces and that the patterns of diversity are probably common for rock surfaces in a semi-arid environment. The patterns of diversity and species assemblages of the rock surfaces contrasted sharply and had little in common with the species assemblages that would be expected from an adjacent community of soil fungi (Bills et al. 2004a).

Genotypic diversity

Because many of the isolates were macroscopically similar, even on sets of media that normally would exaggerate differences in other fungi, we could not confidently use traits such as mycelial growth rate and pigmentation or texture to group them into operational taxonomic units (Arnold et al. 2000, Bills et al. 2004a). We based our comparison and grouping strategy on MP-PCR complemented by the macromorphological appearance of the isolates. The method allowed us to more efficiently analyse fewer isolates without losing diversity information. We were able to check the usefulness of this method by verifying that sequenced isolates with the same MP-PCR pattern grouped together in the ITS tree (Table 2, Fig. 3). However, we found isolates with virtually identical ITS sequences but with different MP-PCR patterns, implying that different individuals of the same species were grouped together. On the other hand, when different strains with identical MP-PCR patterns were sequenced, they consistently had identical ITS sequences. Therefore, the strategy avoided loss of diversity when choosing isolates for sequencing. The assumption that one MP-PCR pattern was equivalent to one species was questionable in many cases. *Hormonema carpetanum* strains with discrete MP-PCR patterns also varied by a few base pairs in their ITS2 sequences, yet upon detailed morphological and fine-scale phylogenetic analyses strains from rocks were considered to be conspecific with plant-associated isolates from the same region (Bills et al. 2004b). Terminal branches consisting of essentially one ITS type (Fig. 2–5) likely represented multiple isolates of single species, such as in the case of TRN261, TRN236, TRN525, TRN524, and TRN235 (Fig. 4), unknown zones 1 and 2 (Fig. 5), or TRN280, TRN446, TRN445, TRN290, TRN289 (Fig. 6).

Morphological data

The convergent morphological features of these fungi are thought to be related to adaptations to environmental conditions. The most obvious was the melanin pigment production, necessary for protection against desiccation and ultraviolet radiation, and possibly the binding of certain metallic ions, which, depending on their nature, may stimulate the growth and survival of melanised fungi or protect them from metal toxicity (Gorbushina 2003). It has been shown that certain metabolites, such as mycosporines, apart from their ultraviolet-protective properties, possess germination self-inhibitory functions, useful for the survival, persistence and longevity of these organisms. The slow and granular meristematic growth is thought to be related with energy conservation, necessary for survival on substrates with scarce nutrient input (Gorbushina 2003, Gorbushina et al. 2003).

Microscopically, sterile mycelia predominated among the isolates (Table 2). Lack of specialised reproductive structures could be an artefact caused by the cultural conditions; however, unspecialised reproduction would be consistent with a hypothesis of specialised organisms living in an oligotrophic, extreme environment. The lack of reproductive structures could also be considered an adaptation for energy conservation (Gorbushina 2003, Gorbushina et al. 2003). In a regime of scarce nutrients and water that exist on rock surfaces, the high energetic costs required for the growth of complex reproductive structures would select for fungi with less elaborate reproduction systems, such as simple modes of conidiogenesis, or melanised, desiccation-resistant, hyphal structures that fragment and act as propagules.

Among the isolates that formed conidial states, varied types of conidiogenesis were observed (Table 2), which was reflected in the diverse taxonomic origins of the isolates. In the rest of the isolates, conidial states were not observed, although in some, hyphal disarticulation could free cells or cells aggregates that could act as propagules. This disarticulation of melanised, torulose hyphae, whose cells could occasionally develop transverse septa, suggested a thallic micronematous conidiogenesis or simple reproduction by propagative fragmentation. Other characters observed in the sterile mycelia, such as torulose and/or moniliform hyphae, chlamydospore-like structures, hyphopodia-like branching, and muriform or meristematic development, even though relatively common, were of limited diagnostic value. Correspondence among morphological types and the groups established by the phylogenetic analysis of the ITS sequences of the isolates was evident only in some cases. Although meristematic strains with black yeast-like growth patterns have been documented in culture (de Hoog et al. 1999, Sterflinger et al. 1999), yeast-like growth forms were infrequent among the isolates.

Diagnostic value of the molecular data: ITS rDNA phylogram

Most isolates could be loosely grouped in two main classes of ascomycetes: Dothideomycetes and Eurotiomycetes, and among four orders: Pleosporales, Dothideales, Capnodiales, and Chaetothyriales. Our analyses were not able to recover monophyly in the Dothideomycetes, as in Schoch et al. (2006), but our data sampling and analyses were inappropriate for this type of resolution.

Dothideomycetes

The order Dothideales (Fig. 4) was amply represented. A diverse set of isolates within this cluster were identified as *Phaeosclera* spp. based primarily on their hard, granular to cerebriform colonies originating from meristematic cell division (Sigler et al. 1981), and forming cell masses disarticulating into single cells or cell aggregates.

Ambiguous clades adjacent to the Dothideales

The taxonomic affiliations of the fungi belonging to the group of ITS clades adjacent to the Dothideales clade (the '*Coniosporium apollinis*' clade, Botryosphaeriales, the 'black yeast' group, and the '*Cryomyces*' clade) were difficult to discern. In the first,

'*Coniosporium apollinis*' clade (Fig. 4), the majority of the reference sequences belonged to strains of the genus *Coniosporium*. This is a heterogeneous form-genus applied to some melanised meristematic fungi that cluster close to the Chaetothyriales (de Leo et al. 1999). The *Coniosporium* species referenced in this tree were all isolated from stone (*C. apollinis* in the '*Coniosporium apollinis*' clade, *C. uncinatum* in the '*Cryomyces*' clade, and *C. petricola* in the Chaetothyriales clade). The polyphyly of *Coniosporium* species in the ITS tree is an example of an artificial genus based on convergent morphologies possibly related to similar adaptations to extreme environments. Two reference strains belonging to the Botryosphaeriaceae corresponded to the recently described Botryosphaeriales (Crous et al. 2006b, Schoch et al. 2006).

The second clade of uncertain affinities, labelled 'black yeast group' (Fig. 4), included isolates with entirely yeast-like growth, and as the only reference strain *Phaeococcomyces nigricans* CBS 652.76. The genus *Phaeococcomyces* is employed for melanised fungi that lack filamentous growth and has been considered a synonym of certain species of *Exophiala* (Haase et al. 1995, de Hoog et al. 2000a). The clade perhaps could represent an unrecognised new genus related to the Chaetothyriales.

The last unsupported clade, '*Cryomyces*' clade (Fig. 4), included reference sequences of *Cryomyces antarcticus* and *C. minteri* (Selbmann et al. 2005), both meristematic, melanised endolithic fungi isolated from Antarctic. The result pointed to a possible cosmopolitan distribution of melanised, meristematic, rock-inhabiting fungi, enabled by their exceptional tolerance to extreme environmental conditions, and emphasised the great unexplored diversity of this ecological group. Reference sequence *Coniosporium uncinatum* CBS 100219 appeared in this group, along with isolate TRN174, identified as the form-genus *Sclerococcum* based on its sporodochia-like colonies, consisting of masses of melanised cells organised in chains and arising from a meristematic tissue (Hawksworth 1979).

Chaetothyriales

This was the best represented order among our isolates. The clade was subdivided in three subclades (Fig. 5). In the first one, identified as the '*S. petricola*' clade, series of isolates were grouped together with reference sequences of uncertain affiliation, e.g., *Coniosporium perforans*, *Phaeococcomyces chersonesos*, and *Sarcinomyces petricola*. All are species of melanised fungi isolated from rock surfaces (Sterflinger et al. 1997, Wollenzien et al. 1997, Bogomolova & Minter 2003) and were assigned to form-genera solely based on their morphologies. Placement of these heterogeneous groups in a particular order was ambiguous based only on ITS sequences analysis. For example, *Sarcinomyces* is a controversial form-genus almost entirely defined by being meristematic growth of cells to form asexual propagules, which may be an inadequate definition for organisms with high morphological plasticity. Note as an example the polyphyly represented in the ITS analysis by *Sarcinomyces petricola*, *Sarcinomyces phaeomuriformis* (in the *Capronia* clade), and *Sarcinomyces crustaceus* (in the *Phaeosclera-Sarcinomyces* branch of the Dothideales clade). These species have been associated with *Phaeococcomyces*

catenatus in a separate clade adjacent to the Herpotrichiellaceae (Chaetothyriales) (Sterflinger et al. 1999). The strong statistical support of these associations suggested that these fungi belong to the Chaetothyriales.

The second subclade in the Chaetothyriales, identified as the *Capronia* clade, included reference sequences of the Herpotrichiellaceae. The subclade also included a group of very similar vegetative isolates with a high statistical support and no related reference strains. This distinctive clade structure (marked as 'unknown zone 1', Fig. 5) might represent individuals of a new species, or members of a species complex.

The third subclade of the Chaetothyriales group comprised two similarly structured groups of isolates. A group apparently conspecific with *Cladophialophora minutissima* (Davey & Currah 2007), a recently described new species from boreal bryophytes, and the unknown zone 3 (Fig. 5).

Capnodiales

The ITS Capnodiales clade (Fig. 6) was composed of five main subclades, three of them, Davidiellaceae, Mycosphaerellaceae, and Capnodiaceae, comprising the Capnodiales as defined by Schoch et al. (2006). The two remaining subclades shared some of the characteristics of other groups of uncertain affiliation that were previously mentioned. The 'Mycosphaerellaceae unknown clade' contained reference strains considered synonyms of *Mycosphaerella* (*Teratosphaeria microspora*) or its anamorphs, some isolated as leaf spot pathogens on Proteaceae (Taylor et al. 2003). These strains, identified initially as *Trimmatostroma* and *Stenella*, have been later renamed as *Catenulostroma* (Crous et al. 2007a). In particular, the *Teratosphaeriaceae* clade (Fig. 6) showed some morphological resemblance to *Trimmatostroma* species characteristic of decayed wood surfaces (Ellis 1971, 1976). *Trimmatostroma*, with the generic type species *T. salicis*, was recently treated as a genus of Leotiales, while the capnodiacean species were reclassified in *Catenulostroma* (Crous et al. 2007a). Teleomorph connections of *Catenulostroma* are in *Teratosphaeria*.

The last subclade in the Capnodiales included several vegetative mycelial strains, mostly isolated from Mallorca. The nearest reference sequence to these series of isolates was *Devriesia americana* (STE-U 5121) (Crous et al. 2000, 2007b). Recently, the phylogeny of the Capnodiales has been extensively treated by Schoch et al. (2006). The resulting phylogeny differentiates two subclasses in the Dothideomycetes, Pleosporomycetidae (Pleosporales) and Dothideomycetidae (Dothideales, Myriangiales, and Capnodiales), Capnodiaceae, Mycosphaerellaceae, Davidiellaceae, and Piedraiaceae are introduced or placed within the Capnodiales. Crous et al. (2007a) have also included *Teratosphaeriaceae* in the Capnodiales. In our analysis, the Capnodiales was poorly delineated, especially regarding rock isolates as in previous studies (Ruibal et al. 2005).

Pleosporales

Isolates and reference sequences belonging to the order Pleosporales, were separated into two groups in the analysis. First, the Pleosporales clade comprised the majority of the isolates and reference sequences belonging to the family Phaeosphaeriaceae. The second was comprised of the Venturiaceae

clade, with only one isolate from rock, TRN484 (Fig. 6). The polyphyletic character of the Pleosporales clade, supported by high bootstrappings, is in agreement with the results of other authors (Braun et al. 2003). *Devriesia americana* and other isolates sequences morphologically identified as fusicladium-like anamorph appeared divergent to the reference sequences of the Venturiaceae (*Venturia cerasi*, *Fusicladium effusum*). These contradictory analyses are an example of the inability of ribosomal rDNA sequences to resolve many relationships among the euascomycetes (Berbee et al. 2000), but also account for the unclear identity and classification of some of the 18S and ITS sequences deposited in GenBank.

Conclusions

The analysis, and that of Ruibal et al. (2005), revealed that a remarkable diversity of ascomycetous fungi can be found in a relatively small surface area of natural rock. Our results confirm those conclusions over a larger geographical scale. Because most isolates were rare, the question as to whether they were restricted to a particular location or rock type was not evident. Our analysis did not reveal any genotypic specificity for the types of rock or sites sampled, as evidenced by few number MP-PCR patterns common to at least two isolation sites and the apparent random distribution of isolates from different sites throughout the ITS tree. A more extensive and intensive microscale analysis emphasizing aspect, shade, waterflow, and other rock patterns possibly could have detected preferential colonisation patterns. On the other hand, rock surfaces may simply provide mechanical support, where organisms disperse at random and can compete in an ecological space with a relatively low population density, and where competitiveness for resources is much lower than in other niches. The mineral composition of rocks may only have minimal effects on fungal nutritional metabolism. Nutrient sources are likely to be exogenous and very transient. Perhaps water from rain, fog, and surface run off carry dissolved or suspended organic and inorganic nutrients to the vicinity of fungal cells. Such an oligotrophic pattern along with the extreme conditions would prevent colonisation by high nutrient-dependent, cosmopolitan organisms, while favouring the settlement and specialisation of a wide range of organisms that share a common set of morphological and physiological features. The characteristics exhibited by these organisms would not necessarily be exclusive to rock surfaces, they might also allow them to colonise other living or inert surfaces in the same ecosystems, such as bark and leaf epiphytes, plant pathogens (*Mycosphaerella*-related strains) or even animal pathogens (human-pathogenic strains in the Chaetothyriales). The physiological and morphological specialisation of these fungi would give them an advantage in such environments that might be also considered 'extreme'. If this hypothesis was true, rock surfaces, with their very oligotrophic environment, could be considered the origin and reservoir of these pathogenic organisms.

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