

## Phylogeny of rock-inhabiting fungi related to *Dothideomycetes*

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**Abstract:** The class *Dothideomycetes* (along with *Eurotiomycetes*) includes numerous rock-inhabiting fungi (RIF), a group of ascomycetes that tolerates surprisingly well harsh conditions prevailing on rock surfaces. Despite their convergent morphology and physiology, RIF are phylogenetically highly diverse in *Dothideomycetes*. However, the positions of main groups of RIF in this class remain unclear due to the lack of a strong phylogenetic framework. Moreover, connections between rock-dwelling habit and other lifestyles found in *Dothideomycetes* such as plant pathogens, saprobes and lichen-forming fungi are still unexplored. Based on multigene phylogenetic analyses, we report that RIF belong to *Capnodiales* (particularly to the family *Teratosphaeriaceae* s.l.), *Dothideales*, *Pleosporales*, and *Myriangiales*, as well as some uncharacterised groups with affinities to *Dothideomycetes*. Moreover, one lineage consisting exclusively of RIF proved to be closely related to *Arthoniomycetes*, the sister class of *Dothideomycetes*. The broad phylogenetic amplitude of RIF in *Dothideomycetes* suggests that total species richness in this class remains underestimated. Composition of some RIF-rich lineages suggests that rock surfaces are reservoirs for plant-associated fungi or saprobes, although other data also agree with rocks as a primary substrate for ancient fungal lineages. According to the current sampling, long distance dispersal seems to be common for RIF. *Dothideomycetes* lineages comprising lichens also include RIF, suggesting a possible link between rock-dwelling habit and lichenisation.

**Key words:** *Arthoniomycetes*, *Capnodiales*, *Dothideomycetes*, evolution, extremotolerance, multigene phylogeny, rock-inhabiting fungi.

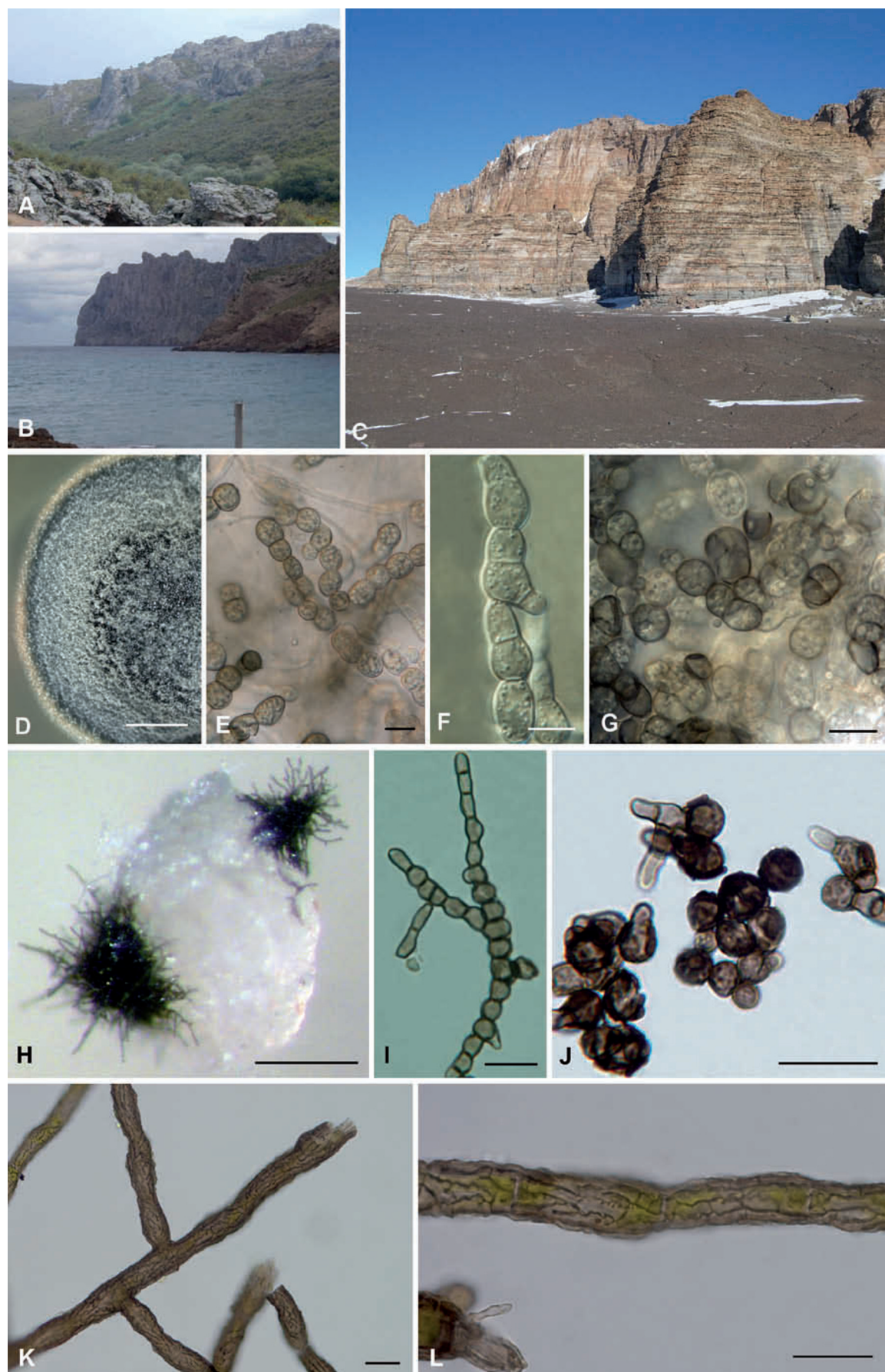
## INTRODUCTION

The *Dothideomycetes* constitute the largest class of ascomycetes with approximately 19 000 species, which are currently classified in 11 orders and 90 families (Kirk *et al.* 2008). This class is ecologically diverse, with many pathogens or saprobes on plants, some coprophilous species, and a few lichen-forming fungi (Schoch *et al.* 2009b; this volume). Early studies have shown that a large part of the non-lichenised, slow-growing melanised fungi isolated from rock surfaces (here referred to as rock-inhabiting fungi) also belong to this class (Sterflinger *et al.* 1997, 1999). Subsequent sampling efforts revealed a higher diversity of species than expected for these rock-inhabiting fungi (Ruibal 2004, Ruibal *et al.* 2005, 2008, Selbmann *et al.* 2005, 2008).

Rock-inhabiting fungi (RIF) are peculiar organisms that apparently lack sexual reproductive structures and form compact, melanised colonies on bare rock surfaces (Fig. 1). Although very common, RIF have often been overlooked due to their small size, their slow growth and the lack of diagnostic features. First discovered in hot and cold deserts (Krumbein & Jens 1981, Friedmann 1982, Staley *et al.* 1982), RIF are now known to be ubiquitous on hard surfaces, in extreme as well as in temperate climates (Urzi *et al.* 1995, Sterflinger & Prillinger 2001, Gorbushina 2007, Gorbushina & Broughton 2009). RIF are well adapted to nutrient-poor and dry habitats where they are particularly successful colonisers due to restricted competition with other microbes (Gorbushina 2007) and their extremotolerance.

Extremotolerance comprises some specific universally present adaptations that enable these fungi to tolerate surprisingly wide ranges of temperatures, irradiation and osmotic stresses (Palmer *et al.* 1990, Sterflinger 1998, Gorbushina *et al.* 2003, Ruibal 2004, Onofri *et al.* 2007, Gorbushina *et al.* 2008). Melanisation protects cells against UV radiations (Dadachova & Casadevall 2008), whereas the typical isodiametrical (meristematic) growth form ensures an optimal volume : surface ratio and, therefore, allows them to survive extreme temperatures and desiccation (Wollenzien *et al.* 1995). These oligotrophic organisms are able to rely only on sparse, airborne nutrients available on rock surfaces. Their growth on these substrates is limited, and, for some of them, the production of internal asexual spores further allows to save energy. All adaptations contribute to the amazing survival capabilities of RIF in hostile habitats. The environmental tolerance of these fungi, and, in some cases, their capacity to penetrate minerals, make them an attractive subject for studies in microbial ecophysiology and applied research, such as biodeterioration of monuments and exobiology (Gorbushina *et al.* 1993, Diakumaku *et al.* 1995, Wollenzien *et al.* 1997, Gorbushina *et al.* 2002, Gorbushina 2003, Onofri *et al.* 2008).

Sterflinger *et al.* (1997) provided the first molecular evidence of RIF phylogenetic affiliations, and they are known to belong to two groups of ascomycetes, namely *Dothideomycetes* and *Eurotiomycetes* (de Hoog *et al.* 1999, Sterflinger *et al.* 1999, Ruibal 2004, Ruibal *et al.* 2005, 2008, Sert *et al.* 2007a). In *Eurotiomycetes*, multigene phylogenetic analyses have shown that



**Fig 1.** Rock-inhabiting fungi related to *Dothideomycetes*. A–C: sampling localities (photos C. Ruibal and L. Selbmann). A. Metamorphic black slate from Atazar, Central Mountain System, Spain. B. Limestone from Cala Sant Vicenç, Serra de Tramuntana, Mallorca, Spain. C. Sandstone from Alatna Valley, McMurdo Dry Valleys, Antarctica. D–G: *Coniosporium apollinis*, a rock-inhabiting species from the Mediterranean region (CBS 100213, photos C. Gueidan). D. Colony on MEA. E. Melanised torulose hyphae. F. Hypha disarticulating into bi- to multi-cellular clumps; G. Meristematic growth. H–J: Antarctic rock-inhabiting fungi (photos L. Selbmann). H. RIF growing on a crystal of sandstone. I. Melanised hypha of *Friedmanniomyces endolithicus*. J. Meristematic growth of *Cryomyces antarcticus*. K–L: *Cystocoleus ebeneus*, a lichenised species assigned to *Capnodiales* (photos L. Muggia). K. Microfilamentous thallus. L. Melanised hyphae of the mycobiont forming a furrow around the filamentous algae. Scale bars: D = 2 mm, E–G and I–J = 10 µm, H = 0.5 mm, K–L = 20 µm.



RIF cluster in early diverging lineages of *Chaetothyriales*, whereas two species seem to be more closely related to the lichenised order *Verrucariales*, the sister group of *Chaetothyriales* (Gueidan *et al.* 2008). Gueidan *et al.* (2008) also demonstrated that the most recent common ancestor of both lichenised *Verrucariales* and pathogen-rich *Chaetothyriales* was probably a rock-inhabiting fungus. It was hypothesised that adaptations to life in extreme conditions might have been a prerequisite for the evolution of human pathogenicity (de Hoog 1993, Haase *et al.* 1999, Gueidan *et al.* 2008) and lichenisation in this class (Gueidan *et al.* 2008). In contrast, despite the high diversity of RIF within *Dothideomycetes*, only very few human pathogens are known from this class of *Ascomycota* (de Hoog *et al.* 2000). Alternatively, associations with plants and in particular plant pathogenicity are very common (Schoch *et al.* 2006, Arzanlou *et al.* 2007, Crous *et al.* 2007a–c, 2009; this volume). Additionally, lichenised species also appeared to be nested within *Dothideomycetes* (Lutzoni *et al.* 2004, James *et al.* 2006, Del Prado *et al.* 2006, Muggia *et al.* 2008, Nelsen *et al.* 2009). Presently no strong phylogenetic hypothesis is available to assess the placement of RIF within *Dothideomycetes*. Moreover, no studies have investigated phylogenetic relationships among RIF, lichen-forming fungi and plant-associated fungi within *Dothideomycetes*. Our main goal was to infer phylogenetic relationships of RIF within *Dothideomyceta*, a lineage including *Dothideomycetes* and *Arthoniomycetes*, to explore more specifically their diversity, origins and evolution.

## MATERIAL AND METHODS

### Taxon and gene sampling

Representative taxa of most of the main orders and families of *Dothideomyceta* (*Dothideomycetes* and *Arthoniomycetes*) were sampled. Two separate sets of data matrices were assembled. The first set (three-gene analysis; Table 1 - see online Supplementary Information) is composed of 182 taxa (including 102 rock-inhabiting strains) for which DNA sequences of three ribosomal genes have been obtained: the large and small subunits of the nuclear ribosomal RNA gene (nucLSU and nucSSU, respectively) and the small subunit of the mitochondrial ribosomal RNA gene (mtSSU). Because this first set of data matrices included only ribosomal genes, low phylogenetic confidence was expected for deep relationships within *Dothideomyceta*. To overcome this problem, a second set of data matrices was assembled (five-gene analysis; Table 1 in Supplementary Information) consisting of DNA

sequences of five loci from 113 taxa (including 40 rock-inhabiting strains): the largest and second largest subunits of the RNA polymerase II (*RPB1* and *RPB2*, respectively), nucLSU, nucSSU and mtSSU. The outgroup for the three-gene analysis included *Hyphozyma lignicola*, *Symbiotaphrina buchneri* and *S. kochii*, whereas only the latter two species were selected as outgroup for the five-gene analysis. These species were chosen because they constituted a sister group to *Dothideomyceta* in a previous study (Schoch *et al.* 2009a).

### DNA isolation and sequencing

Different laboratories contributed data using various protocols, but most DNA sequence information was produced as follows: genomic DNA was isolated from cultures grown on MEA. Fungal biomass was transferred to a tube with 500 µL of TES buffer and ground with a micro-pestle for 1–2 min, with or without silica-mix (2/3 silica-gel, 1/3 Celite® 545). A volume of 140 µL of 5 M NaCl was then added, followed by 65 µL of 10 % (w/v) CTAB (cetyltrimethylammoniumbromide). After an incubation of 30 min at 65 °C, 700 µL of (24:1) chloroform/isoamylalcohol was added, the tubes were mixed carefully by hand, stored on icy water for 30 min, and centrifuged for 10 min at 4 °C (10 000 × g). The supernatant was recovered and the genomic DNA precipitated using isopropanol. After washing the pellets with 70 % ethanol, they were dried in a vacuum centrifuge and re-suspended in 60 µL of TE buffer (protocol modified from Möller *et al.* 1992).

Six regions covering five genes were amplified: nucLSU, nucSSU, mtSSU, *RPB1* region A–D, *RPB2* region 5–7, and *RPB2* region 7–11 (see Table 2 for primers used). Genomic DNA (1 µL of a 1/10 or 1/100 dilution) was added to a PCR mix comprising 2.5 µL of PCR buffer (buffer IV with 15 mM MgCl<sub>2</sub>, Abgene, Epsom, U.K.), 2.5 µL of dNTPs (2 mM), 2.5 µL of BSA (10 mg/mL), 2.0 µL of primers (10 µM), 0.15 µL *Taq* polymerase (5 U/µL, Denville, Metuchen NJ, U.S.A.), and water for a total volume of 25 µL. Amplification cycles for nucLSU, nucSSU and *RPB1* (same conditions applied for *RPB2*) are described in Gueidan *et al.* (2007), and in Zoller *et al.* (1999) for mtSSU. The PCR products were purified using Microcon PCR cleaning kits (Millipore, Billerica MA, U.S.A.). Sequencing was carried out using Big Dye Terminator Cycle sequencing Kits (ABI PRISM version 3.1, Perkin-Elmer, Applied Biosystems) on ABI 3730xl DNA Analyzers (Applied Biosystems, Foster City CA, U.S.A.) from the Duke Center for Evolutionary Genomics (Durham NC, U.S.A.) and the Hubrecht Institute (Utrecht, Netherlands).

**Table 2.** List of primers for the five genes used in this study (*RPB2* was amplified in two regions).

| Gene regions            | PCR primers   | Additional primers used for sequencing   |
|-------------------------|---|--|
| nucLSU                  | LR0R <sup>a</sup> , LR7 <sup>a</sup>                            | LR3, LR3R, LR5, LR5R, LR6, LR6R <sup>a</sup>   |
| nucSSU                  | nssu131 <sup>c</sup> , NS24 <sup>d</sup>                        | nssu1088, nssu1088R, nssu897R, nssu634 <sup>c</sup> , SR11R <sup>e</sup> , NS23, NS22 <sup>d</sup> , SR7R, SR7, SR10R <sup>f</sup> |
| mtSSU                   | mtSSU1, mtSSU3R <sup>g</sup>                                    | mtSSU2, mtSSU2R <sup>g</sup>   |
| <i>RPB1</i> region A–D  | <i>RPB1</i> -AF <sup>h</sup> , <i>RPB1</i> -6R1asc <sup>i</sup> | –  |
| <i>RPB2</i> region 5–7  | <i>RPB2</i> -5F, <i>RPB2</i> -7cR <sup>j</sup>                  | –  |
| <i>RPB2</i> region 7–11 | <i>RPB2</i> -7cF, <i>RPB2</i> -11aR <sup>j</sup>                | –  |

<sup>a</sup>Rehner & Samuels (1994), <sup>b</sup>Vilgalys & Hester (1990), <sup>c</sup>Kauff & Lutzoni (2002), <sup>d</sup>Gargas & Taylor (1992), <sup>e</sup>Spatafora *et al.* (1995), <sup>f</sup>Vilgalys (unpubl.; [www.biology.duke.edu/fungi/mycolab/primers.htm](http://www.biology.duke.edu/fungi/mycolab/primers.htm)), <sup>g</sup>Zoller *et al.* (1999), <sup>h</sup>Hall (unpubl.; <http://faculty.washington.edu/benhall/>), <sup>i</sup>Hofstetter *et al.* (2007), <sup>j</sup>Liu *et al.* (1999).

## Alignments and phylogenetic analyses

Sequences were assembled and edited using Sequencher (Gene Codes Corporation, Ann Arbor MI, U.S.A.). Manual alignments were performed using MacClade v. 4.08 (Maddison & Maddison 2003). Ambiguous regions (*sensu* Lutzoni *et al.* 2000) and introns were delimited manually and excluded from the alignments. Congruence was tested using a 70 % reciprocal bootstrap criterion (Mason-Gamer & Kellogg 1996, Reeb *et al.* 2004). For the three-gene dataset, the test was performed using Compat (Kauff & Lutzoni 2002) on all possible gene pairs (mtSSU vs. nucSSU, mtSSU vs. nuLSU, and nuLSU vs. nucSSU) and based on bootstrap consensus trees. Bootstrap trees were obtained using Neighbor-Joining bootstrap analyses with Maximum Likelihood distances in PAUP v. 4.0b10 (Swofford 2003). Models of molecular evolution were estimated using the Akaike Information Criterion implemented in Modeltest v. 3.7 (Posada & Crandall 1998). For the five-gene dataset, congruence was also tested using a 70 % reciprocal bootstrap criterion, but the comparison was done manually based on trees obtained with 500 bootstrap replicates using RAxML VI-HPc (Stamatakis *et al.* 2005, 2008) on the Cipres Web Portal ([www.phylo.org/sub\\_sections/portal/](http://www.phylo.org/sub_sections/portal/)). Taxa or sequences responsible for incongruence were removed from the dataset, and the markers were combined. Final phylogenetic analyses of the three-gene and five-gene datasets were performed using RAxML on the Cipres Web Portal. The ML search followed a GTRMIX model of molecular evolution applied to the following nine partitions: *RPB1* first, second and third codon positions, *RPB2* first, second and third codon positions, nuLSU, nucSSU and mtSSU. Support values were obtained with bootstrap analyses of 1 000 pseudoreplicates using RAxML.

## RESULTS

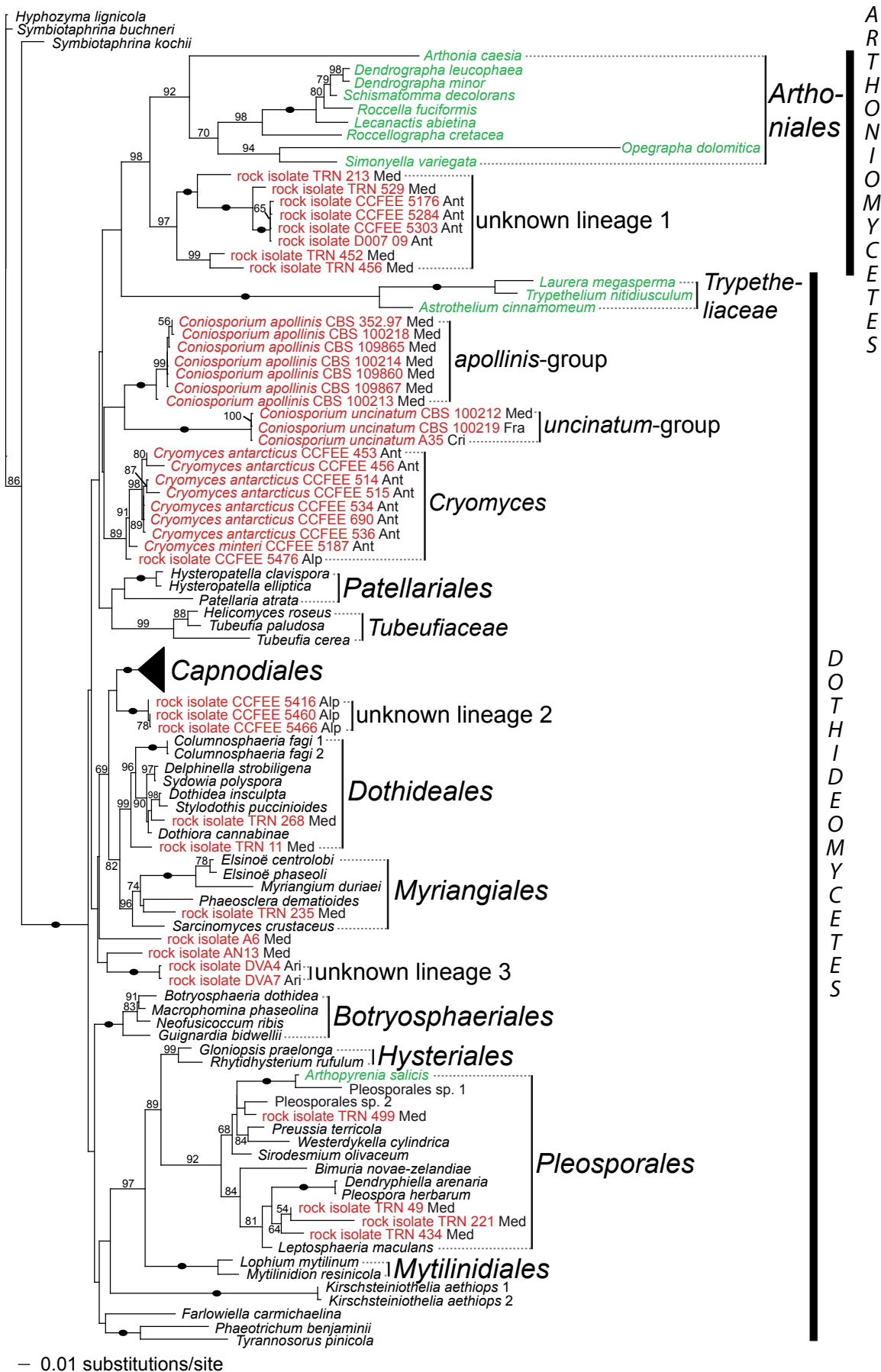
### DNA sequence alignments

Not all markers were recovered or available for all taxa. For the three-gene dataset, 20 nuLSU, 11 nucSSU and 54 mtSSU sequences were missing. Among the 182 taxa, 119 had sequences for three genes, 61 for two genes, and 12 for one gene (Table 1 in Supplementary Information). After exclusion of ambiguous regions and introns, the combined dataset included 3 274 characters (1 106 for nuLSU, 1 616 for nucSSU and 552 for mtSSU). Among these, 2 063 were constant while 931 were parsimony-informative. For the five-gene dataset, missing data comprised 5 nuLSU, 8 nucSSU, 30 mtSSU, 48 *RPB1* and 30 *RPB2* sequences. Among the 113 taxa, 32 had sequences for five genes, 46 for four genes, 30 for 3 genes, and 5 for 2 genes (Table 1 in Supplementary Information). After exclusion of ambiguous regions and introns, the combined dataset included 6 045 characters (1 133 for nuLSU, 1 607 for nucSSU, 593 for mtSSU, 1 011 for *RPB1* and 1 701 for *RPB2*). Among these, 2 912 were constant while 2 693 were parsimony-informative.

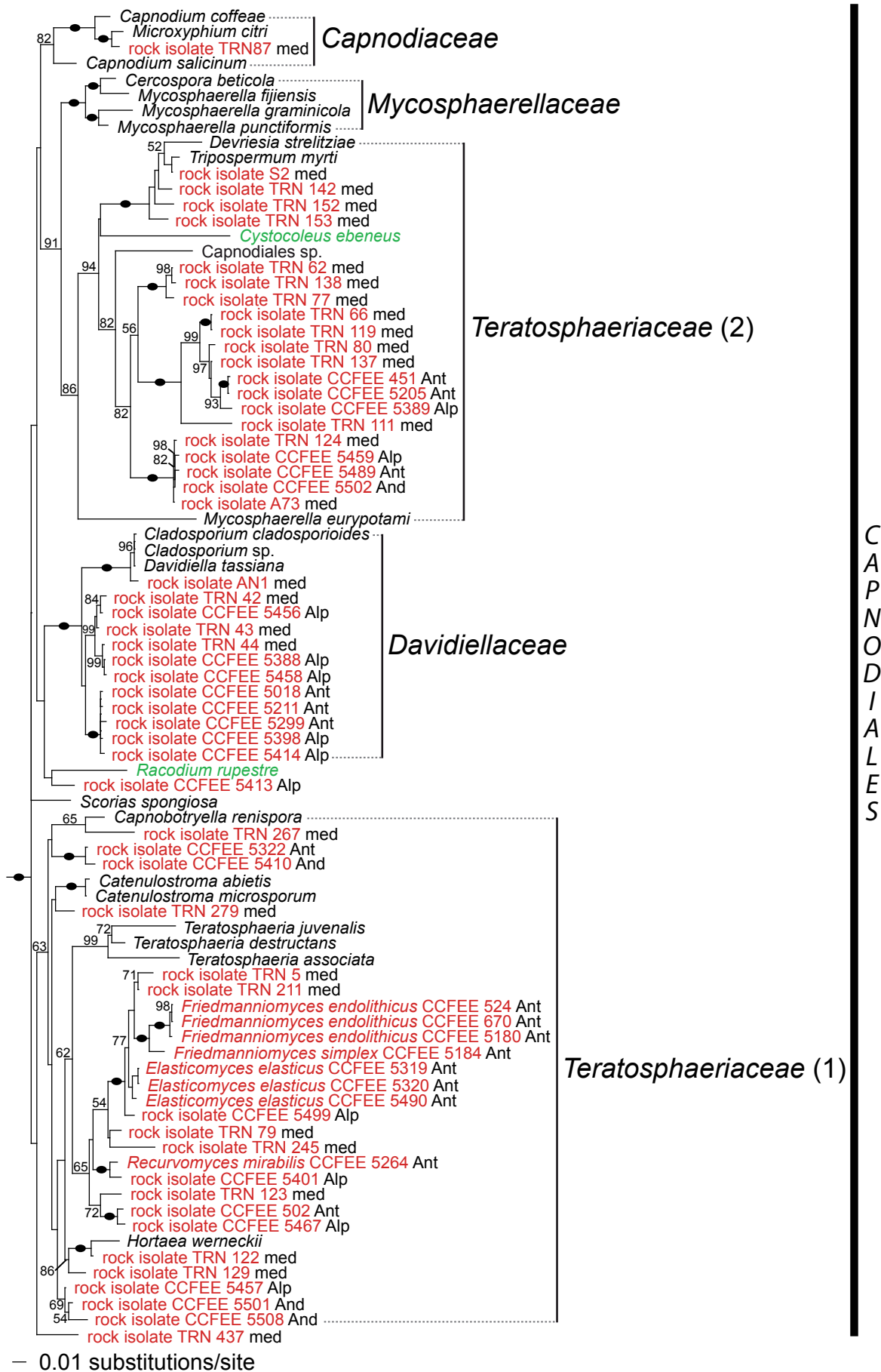
## Phylogenetic inference

For the three-gene analysis (Figs 2–3), results show that, within the two classes *Dothideomycetes* and *Arthoniomycetes*, rock-inhabiting fungi belong to 13 groups, either well-known orders or families, or lineages that have not previously been characterised. Among the rock-inhabiting fungi clustering with well-known groups of *Dothideomycetes*, two strains are found in the order *Dothideales*, four in the order *Pleosporales*, one in *Myriangiales*, 12 forming a monophyletic group sister to the remaining members of *Davidiellaceae*, and one in the family *Capnodiaceae*. The family *Teratosphaeriaceae* is not monophyletic in this analysis (also see Crous *et al.* 2009; this volume). In a first group including the generic type *Teratosphaeria fibrillosa* (*Teratosphaeriaceae* 1, Fig. 3), many rock-inhabiting strains are present, including taxa from the three genera *Friedmanniomyces*, *Elasticomyces* and *Recurvomyces*. The second group (*Teratosphaeriaceae* 2, Fig. 3), including the three leaf-colonising species *Devriesia strelitziae*, *Mycosphaerella eurypotami* and *Tripospermum myrti*, an unknown species of *Capnodiales*, the lichen species *Cystocoleus ebeneus* as well as 20 undescribed rock inhabiting strains, is supported as sister to the family *Mycosphaerellaceae* (91 % bootstrap). The two rock-inhabiting species *Coniosporium uncinatum* and *C. apollinis* are well supported (100 % bootstrap), but their sister relationship is not. Neither these two species of *Coniosporium* nor the Antarctic genus *Cryomyces* can be assigned to any known family or order sampled here. Amongst the unknown lineages, one does not seem to be part of *Dothideomycetes* (lineage 1, Fig. 2), and appears as sister to *Arthoniomycetes* (98 % bootstrap). Due to the lack of support for many deep internodes, it is not possible to determine if lineages 2 and 3 can be accommodated by the expansion of known groups of *Dothideomycetes*, or if the recognition of new taxonomical entities are needed. Finally, the rock isolates A6, AN13, TRN 437 and CCFFEE 5413 do not significantly cluster with any other taxa.

With the five-gene analysis (Fig. 4), the inferred deep branching pattern within *Dothideomycetes* is still poorly supported, but additional well-supported nodes are recovered (e.g., *Capnodiaceae* as sister to the lineage including *Mycosphaerellaceae* and *Teratosphaeriaceae*, and the monophyly of *Teratosphaeriaceae* 1). As in the three-gene analysis, the sister relationship between lineage 1 and *Arthoniomycetes* obtains high support (100 % bootstrap), even though the two rock-inhabiting strains included do not seem to form a monophyletic group. The placement of the lichen family *Trypetheliaceae* as sister to *Arthoniomycetes* (70 % bootstrap) might be an artifact, as this relationship was not recovered in any other studies (Del Prado *et al.* 2006, Spatafora *et al.* 2006, Nelsen *et al.* 2009). Within *Dothideomycetes*, the orders *Dothideales* and *Myriangiales* form a sister group (100 % bootstrap), and are sister to the well-supported *Capnodiales* (100 % bootstrap), which includes most of the rock-inhabiting strains. Within *Capnodiales*, the second group of *Teratosphaeriaceae* (*Teratosphaeriaceae* 2; Fig. 4) is still supported as sister to *Mycosphaerellaceae* (89 % bootstrap). Other lineages comprising exclusively RIF (*Cryomyces*, *Coniosporium uncinatum*, and *C. apollinis*) do not significantly cluster with any known group of *Dothideomycetes*.

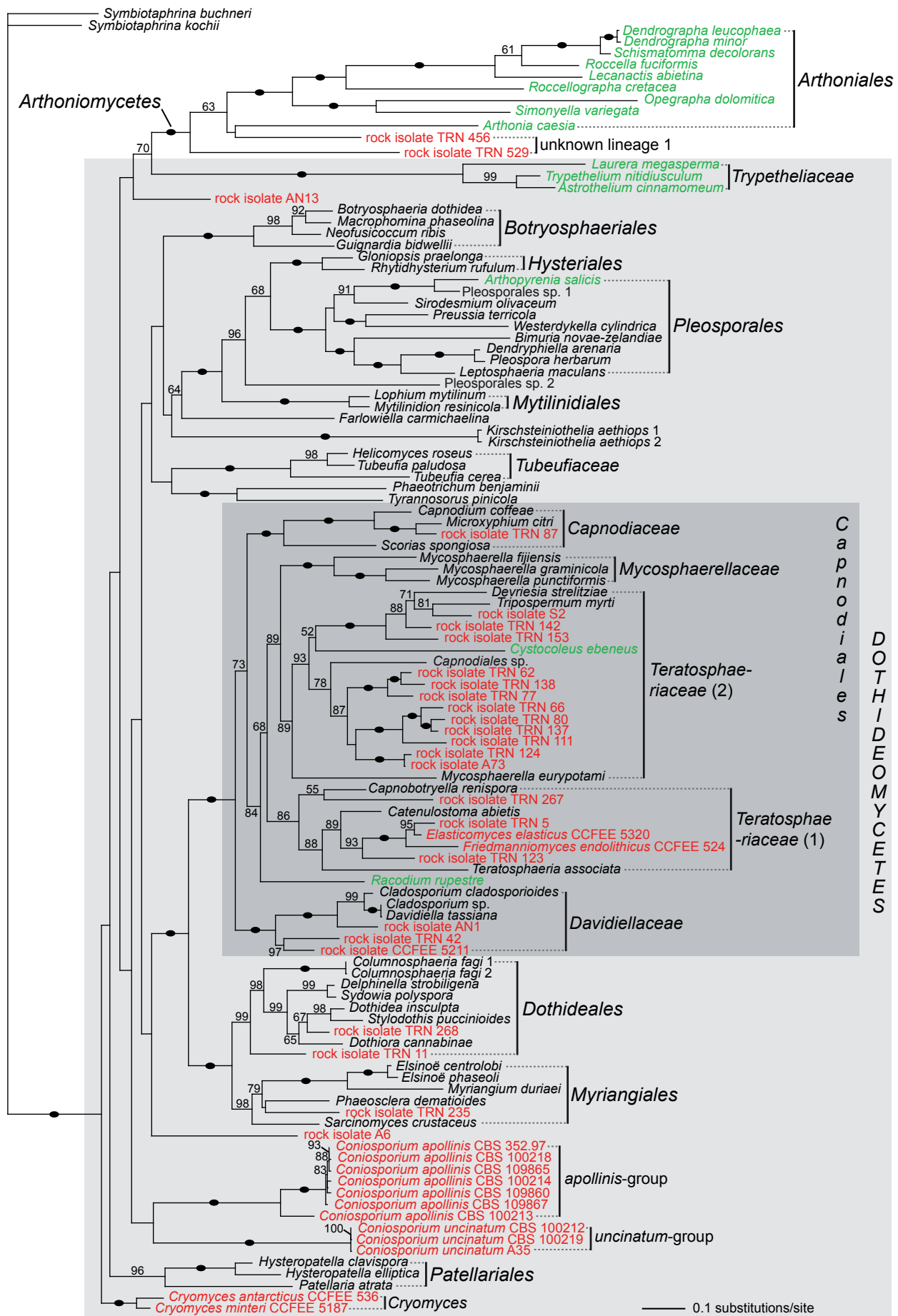


**Fig. 2.** Phylogenetic placement of 102 rock-inhabiting strains within *Dothideomycetes* (*Dothideomycetes* and *Arthoniomycetes*). The tree is based on a Maximum Likelihood analysis of the combined nucLSU, nucSSU and mtSSU (three-gene analysis). A black oval on a branch indicates a bootstrap support value of 100 %. Other bootstrap values  $\geq 50$  % are shown below or above branches. RIF are highlighted in red and lichens in green. Geographical origins are also labeled for RIF (Alp = Alps, And = Andes, Ant = Antarctica, Ari = Arizona desert, Cri = Crimea, Fra = France, Med = Mediterranean region, including Greece, Israel, Italy, Slovenia, Spain and Turkey). Phylogenetic relationships within *Capnodiales* are detailed in Fig. 3.



**Fig. 3.** Phylogenetic placement of RIF within the order *Capnodiales*. The tree is based on a Maximum Likelihood analysis of the combined nuclSU, nucSSU and mtSSU (three-gene analysis). A black oval on a branch indicates a bootstrap support value of 100 %. Other bootstrap values  $\geq 50$  % are shown below or above branches. RIF are highlighted in red and lichens in green. Geographical origins are also labeled for RIF (Alp = Alps, And = Andes, Ant = Antarctica, Ari = Arizona desert, Cri = Crimea, Fra = France, Med = Mediterranean region, including Greece, Israel, Italy, Slovenia, Spain and Turkey).





**Fig. 4.** Phylogenetic relationships of rock-inhabiting lineages with known groups of Dothideomyceta based on a Maximum Likelihood analysis of the combined nuclSU, nucSSU, mtSSU, *RPB1* and *RPB2* (five-gene analysis). A black dot on a branch indicates a bootstrap support value of 100 %. Other bootstrap values  $\geq 50$  % are shown below or above the branches. RIF are highlighted in red and lichens in green.

## DISCUSSION

### Species diversity in *Dothideomycetes*

The *Dothideomycetes* are very diverse in term of species, some of which are well known for their pathogenicity on crops (e.g., *Mycosphaerella fijiensis*, the agent of the leaf spot disease of banana, or *Leptosphaeria maculans*, the agent of the blackleg disease of cabbage). Whilst many species are associated with plants (either as pathogens or as epiphytes), saprobic, coprophilous, lichen-forming and rock-inhabiting fungi are also present in this class. The importance of RIF in term of species richness is still under-investigated. A thorough sampling of dothideomycetous RIF from few localities in Mallorca and Central Spain formed the basis of the analyses described here (Ruibal 2004, Ruibal *et al.* 2005, 2008). RIF from Antarctica, the Alps and the Andes (Selbmann *et al.* 2005, 2008), as well as the Arizona and Negev deserts (Staley *et al.* 1982, A.A. Gorbushina, unpubl. data) extended the geographical range of the sampled taxa. Finally, isolates from monuments in the Mediterranean area supplemented the sampling (Gorbushina *et al.* 1996, Sterflinger *et al.* 1997, Volkmann & Gorbushina 2006). In comparison to known RIF habitats (Gorbushina 2007), our sampling was very restricted and does not permit a realistic overview of fungal diversity on rock surfaces. Nevertheless, an impressive number of rock-inhabiting species is already evident. Our data show that rock-inhabiting fungi are not only present in well-known orders, such as *Capnodiales* or *Pleosporales*, but also in novel lineages (e.g., lineage 1, Fig. 2). Moreover, very few species with overlapping distribution were recovered from neighbouring geographical localities in Mallorca and Central Spain (Ruibal *et al.* 2005, 2008). Therefore, we can hypothesise that species richness within *Dothideomycetes* remains woefully underestimated, and that many more species will need to be described within this class in the future, especially for fungi colonising rocky substrates.

### Classification of rock fungi related to *Dothideomycetes*

Although very diverse within *Dothideomycetes*, RIF have not been included in recent phylogenetic studies of this class (Lumbsch *et al.* 2001, Schoch *et al.* 2006). Only very few of these rock-inhabiting species have been taxonomically described (Sterflinger *et al.* 1997, Bills *et al.* 2005, Sert *et al.* 2007b), and the molecular marker available for most of these species (ITS) does not allow their inclusion in large-scale phylogenetic analyses. The few attempts to produce phylogenies involving RIF have shown that they belong to two diverse classes of *Ascomycota*, namely *Eurotiomycetes* (particularly the order *Chaetothyriales*) and *Dothideomycetes* (preponderantly the orders *Capnodiales*, *Dothideales* and *Pleosporales*) (Sterflinger *et al.* 1999, Ruibal 2004, Ruibal *et al.* 2005, 2008).

Our results confirm the placement of RIF in the same orders of *Dothideomycetes*, although some lineages are shown to belong to additional groups. Based on our results, many RIF should be classified within *Dothideales*, *Pleosporales* and *Capnodiales*, the latter order holding the largest number in rock-colonising species. The genera *Elasticomyces* and *Recurvomyces*, as well as the Antarctic genus *Friedmanniomyces*, were previously attributed to *Capnodiales* based on nucSSU data (Selbmann *et al.* 2008). Our multigene analyses confirm this placement, and show that these three genera belong to *Teratosphaeriaceae* s. str., the family currently showing the highest diversity in RIF (Fig. 3). We

also showed that one RIF (TRN 235) previously thought to be related to the *Dothideales* (Ruibal *et al.* 2008) actually belongs to *Myriangiales*, along with *Sarcinomyces crustaceus*, a species similarly melanised and meristematic, but isolated from plant material (Sigler *et al.* 1981).

Several well-supported groups of RIF could not be attributed to any known families and orders according to our data. As a consequence, *Cryomyces* should still be considered as *Dothideomycetes incertae sedis*, as no close relationship was recovered for this enigmatic Antarctic genus (Selbmann *et al.* 2005). The positions of RIF-rich genera *Coniosporium* and *Sarcinomyces* are also problematic. Previous studies placed them either in *Dothideales* or *Chaetothyriales* based on ITS or nucSSU data (de Leo *et al.* 1999, Sterflinger *et al.* 1999, Sert *et al.* 2007a). Yet, the limited taxon and gene sampling on which these analyses were based was probably insufficient to demonstrate clear phylogenetic relationships. Our results show that *Coniosporium apollinis* (including the type strain CBS 352.97), *C. uncinatum* (including the type strain CBS 100219) and *Sarcinomyces crustaceus* belong to *Dothideomycetes* (Fig. 4). However, a previous multigene analysis showed that two other species, *Coniosporium perforans* and *Sarcinomyces petricola*, belong to *Chaetothyriales* (Gueidan *et al.* 2008). These anamorphic genera are therefore not monophyletic, and additional research is required to clarify their status.

Among lineages lacking known reference taxa, two groups seem to belong to *Dothideomycetes* (unknown group 2, a lineage comprising RIF from the Alps, and unknown group 3, a lineage including strains isolated in Arizona; Fig. 2). Another unknown group (lineage 1) clusters outside *Dothideomycetes*, sister to the *Arthoniales* (Figs 2, 4). A previous study had noted the problematic placement of this latter group (Ruibal *et al.* 2008). Many lineages including RIF still need to be named. In the past, several melanised meristematic species and genera have been described such as *Lichenothelia* (Hawksworth 1981; see also Henssen 1987), which could potentially correspond to some of these RIF lineages. However, little is known about these formerly named taxa, and no molecular data or cultures are available for many of them. Naming RIF will therefore require an extensive study of both rock-inhabiting species and formerly described melanised meristematic species, whether they grow on rock or not.

### Rock surfaces: “terroirs” for ancient lineages or reservoirs for plant-associated fungi?

Despite the prevailing extreme conditions, rock surfaces host a large variety of specialised fungi. Fungal colonisation of subaerial rocks can be explained by two non-exclusive hypotheses. Firstly, atmosphere-exposed rock substrates could constitute “terroirs” for ancient fungal lineages. Rock surfaces were among the first terrestrial substrates available for living organisms on earth (Gorbushina & Broughton 2009). It is therefore likely that, early on, some species became adapted to colonise rock surfaces. RIF are persistent to different types of physical stress, but are poor competitors and surrender to more combative organisms (Gorbushina *et al.* 2008). Increasing competition with other rock-inhabiting organisms living under more permissive conditions may have restricted some of these ancient, morphologically reduced, slow-growing, fungal relicts to extreme habitats. The presence of lineages comprising exclusively RIF that diverged early in the evolution of *Dothideomycetes* (e.g., *Cryomyces* and lineage 1, Fig. 2) supports this hypothesis of rock surfaces as substrates for ancient fungal lineages.



Secondly, rock surfaces could form reservoirs for plant-associated or saprobic fungi. Through spore or propagule dispersal, some species of various unrelated groups of plant pathogens, epiphytes or saprobes can reach rock substrates. Their ability to survive in these environments will depend on some key features, namely oligotrophy, melanisation and pleiomorphism (or diversity of growth forms, amongst which meristematic growth). Under extreme conditions prevailing on rock surfaces, fungi possessing these key features can survive due to their slow, meristematic, clumpy growth and thick-walled, heavily melanised cells. These key features seem to have evolved several times in *Dothideomycetes*, allowing different lineages to colonise rock substrates. In *Dothideales*, phyllosphere fungi such as *Aureobasidium pullulans* and relatives, which have a filamentous or yeast-like growth under moist conditions, but convert to a meristematic form when colonising inert substrates, have also been isolated from rock surfaces (Ruibal *et al.* 2008). The family *Teratosphaeriaceae* s. l. is another example of a group in which some leaf-colonising species can also grow meristematically and form dark, thick-walled cells. According to our results, this family (as traditionally delimited; i.e., including *Teratosphaeriaceae* 1 and 2) is also extremely diverse in RIF (Fig. 3). Rocks supporting growth of subaerial biofilms (Gorbushina & Broughton, 2009) may be viewed as a reservoir for all types of melanised meristematic fungi, from where other habitats can be re-colonised. Survival of new comers is probably additionally facilitated by the existing microbial community on rocks (Gorbushina & Broughton 2009) in a fashion known for immigrant bacteria on leaf surfaces (Monier & Lindow 2005).

Alternatively, rock-colonising lichens may supply buffered environments and refugia for RIF or organisms otherwise occupying other niches (Selbmann *et al.* 2010). Recent studies have shown that lichens harbour an amazing diversity of ascomycetous endophyte-like (endolichenic) fungi (Arnold *et al.* 2009), and phylogenetic relatedness was found between some endolichenic fungi isolated from saxicolous lichens and RIF (Harutyunyan *et al.* 2008). If in most cases, species from rock surfaces can still go back to their primary habitats, in some cases, these fungi keep specialising and get trapped in these extreme habitats. This may be the case for groups with no close relationships with plant-associated fungi, such as the genus *Friedmanniomyces* (Fig. 3).

## Geographical distribution of rock-inhabiting fungi

The large majority of rock-inhabiting strains isolated thus far originated from rocks in the Mediterranean region or Antarctica (Sterflinger *et al.* 1999, Ruibal 2004, Ruibal *et al.* 2005, 2008 Selbmann *et al.* 2005, 2008). In Antarctica, RIF tend to grow within rocks, together with the cryptoendolithic lichen communities, finding shelter from extreme conditions prevailing on rock surfaces. In the Mediterranean area, RIF tend to grow on the rock surface or in cracks, causing damages to the substrate (e.g., biopitting of marble). Despite differences in temperature, they share similar morphological and physiological adaptations, such as melanisation, meristematic growth and oligotrophism.

Similarly to previous studies (Selbmann *et al.* 2005, Ruibal *et al.* 2008), our results show that Antarctic RIF often share an evolutionary history with RIF from semi-arid areas. In our study, RIF sampled in geographically disjoint localities (Antarctica versus Mediterranean region) cluster together in *Davidiellaceae*, the two groups of *Teratosphaeriaceae*, and unknown lineage 1 (Figs 2–3). In some cases, Antarctic and Mediterranean strains are even phylogenetically very closely related, showing a recent

common evolutionary history (e.g., in *Teratosphaeriaceae* 2, the Mediterranean rock isolates TRN 124 and A73 with the Antarctic strain CCFFEE 5489). Likewise, some strains of *Recurvomyces mirabilis* and *Elasticomyces elasticus* have been recorded in the Antarctic as well as in high peaks of the Alps and Andes (Selbmann *et al.* 2008). Therefore, it seems that an efficient mechanism of dispersal, most probably wind-mediated (Gorbushina *et al.* 2007, Gorbushina & Broughton 2009), have led to a colonisation spanning different continents.

## Rock-dwelling habit and evolution of lichenisation

Most of the diversity in lichen-forming fungi is found in *Lecanoromycetes*, a large and diverse class of ascomycetes including approximately 14 000 species (Miadlikowska *et al.* 2006, Kirk *et al.* 2008). Yet, the classes *Lichinomycetes* (with the single order *Lichinales*), *Eurotiomycetes* (with the orders *Pyrenulales* and *Verrucariales*), *Arthoniomycetes* (with the single order *Arthoniales*), and *Dothideomycetes* also include lichens. Although *Lichinales*, *Pyrenulales*, *Verrucariales* and *Arthoniales* are monophyletic lineages containing mostly lichenised species, lichens in *Dothideomycetes* seem to encompass a broader phylogenetic spectrum: the *Trypetheliaceae*, a family of mostly tropical bark-colonising lichens, forms a monophyletic group within *Dothideomycetes* (Del Prado *et al.* 2006, Nelsen *et al.* 2009, Schoch *et al.* 2009a). *Arthopyrenia salicis*, a corticolous, temperate lichen species nests within the order *Pleosporales* (Del Prado *et al.* 2006, Nelsen *et al.* 2009). Two melanised micro-filamentous lichens, *Cystocoleus ebeneus* and *Racodium rupestre*, were assigned to the order *Capnodiales* (Muggia *et al.* 2008, Nelsen *et al.* 2009). Finally, the two lichen families *Strigulaceae* (mostly leaf-colonising tropical species) and *Monoblastiaceae* (temperate and tropical species) are now shown to belong to *Dothideomycetes* (Nelsen *et al.* 2009; this volume).

Whether these lichen lineages, that are unrelated to *Lecanoromycetes*, originated from independent gains of lichenisation is not clear (Lutzoni *et al.* 2001, James *et al.* 2006, Gueidan *et al.* 2008, Arnold *et al.* 2009, Schoch *et al.* 2009a, b). Within *Eurotiomycetes*, phylogenetic data suggest that the lineage including *Pyrenulales* and *Verrucariales* possibly results from an independent gain of lichenisation (Gueidan *et al.* 2008, Schoch *et al.* 2009a). Phylogenetic data suggest that lichens in *Verrucariales* may have evolved from rock-inhabiting fungi (Gueidan *et al.* 2008), a result in agreement with experimental data demonstrating that some RIF and one melanised lichen-colonising fungus could form associations with lichen-associated algae (Gorbushina *et al.* 2005, Brunauer *et al.* 2007). This rock-inhabiting ancestor may have evolved associations with epilithic microalgae in order to get a more constant supply in nutrients. If the evolution of fungal-algal associations occurred in *Eurotiomycetes*, it most likely also occurred in different fungal groups. It is therefore interesting to see if in *Dothideomycetes*, where rock fungi are so diverse, similar transitions in lifestyles can be suggested.

Although many lichenised species in *Dothideomycetes* are either corticolous or only secondarily or occasionally saxicolous, *Cystocoleus ebeneus* and *Racodium rupestre* are true rock inhabitants. Amongst lichens in *Dothideomycetes*, these two species are the most likely to have evolved from a rock-inhabiting ancestor. They share substrate preference and some morphological features, such as their melanised hyphae, with RIF. Strikingly, in our result, *Cystocoleus ebeneus* is nested within a lineage comprising almost exclusively RIF (*Teratosphaeriaceae* 2, Fig. 3).

*Racodium rupestre* is also related to a RIF, but this relationship is not supported (Fig. 3). This result agrees with a rock-inhabiting ancestor for these two lichenised species, but further data will however be necessary to test this hypothesis. Also of interest is the close phylogenetic relationship between the lichen order *Arthoniales* and the lineage 1 of RIF (Figs 2, 4). Although mostly corticolous or foliicolous, *Arthoniales* also comprises saxicolous species (Ertz *et al.* 2009). Further data is needed to explore the relationships between saxicolous species of *Arthoniales* and RIF. In conclusion, these preliminary results suggest that there might be a link between rock-dwelling habit and lichenisation. However, additional taxon and gene sampling are needed to confirm the phylogenetic placements of some of the lichenised taxa and to clarify their relationships to RIF. Only then the hypothesis of RIF as ancestors of lichenised lineages can be adequately tested.

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