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Life on top: cryptoendolithic ascomycetes and microalgae isolated from over 6000 m altitude

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Abstract. Rocks are among the oldest terrestrial niches hosting a multiplicity of life forms, of which diversity has been only partially uncovered. Endolithic metacommunities comprise all major groups of microorganisms, such as chemo-organotrophic, chemo-lithotrophic and phototrophic, represented by bacteria, microalgae and microfungi. Their diversity is often difficult to describe and may remain underestimated. Furthermore, knowledge about the diversity of microorganisms colonizing rocks in peculiar niches is even poorer due to the difficulty to retrieve environmental specimens. Here, we report the phylogenetic and phenotypic characterization of a few endolithic fungi and algae isolated from rock fragments collected at high elevation, i.e., on the top of two mountains over 6000 m altitude, Muztagh Ata (China) and Cerro Mercendario (Argentina). The identity of the strains was confirmed by sequencing the nuclear ITS and LSU, the plastidial *rbcL* loci and by morphological analysis. Three fungal strains belonging to the class *Dothideomycetes* and one algal strain belonging to the genus *Trebouxia* were isolated from Muztagh Ata, while six fungal strains belonging to the order *Chaetothyriales* and four algal strains belonging to the genus *Myrmecia* were isolated from Cerro Mercedario. The detected species diversity is discussed in an evolutionary and ecological context.

Key words: *Chaetothyriales*, *Dothideomycetes*, *Myrmecia*, symbiosis, *Trebouxia*

Introduction

Rocks represent one of the oldest terrestrial niches hosting a multiplicity of life forms, whose diversity has been only partially uncovered to date. The presence of rock-associated microorganisms was documented by Diels (1914) for the first time, but it was only in the 1970s that Friedmann & Galun (1974) highlighted the existence of bacteria and microalgae on rock surfaces and within rocks, giving shape to the modern research field of geobiology. In the evident scenario of climate change, geo(micro)biologists nowadays suggest that rocks are a cradle of habitats where microorganisms find protection from the continuous fluctuation of temperatures, UV radiation, humidity, salinity and deposition of inorganic and organic nutrients (Gorbushina 2007; Onofri et al. 2007a; Selbmann et al. 2015; Coleine et al. 2021; De Los Ríos et al. 2014).

Rock-associated organisms are recognized into the two main categories of epiliths (those colonizing the exposed, external rock surfaces) and endoliths (those colonizing the rock matrices inside; Golubic et al. 1981). The endoliths further differentiate into cryptoendolithic (those hiding inside the rocks, i.e., colonizing natural empty pores and fissures within the rock, but connected indirectly to the rock surface), chasmoendolithic (those which grow in fissures and cracks connected to the rock surface), and hypoendolithic (those which colonize the underside of rocks in contact with the underlying soil (Wierzchos et al. 2011, 2012). Particularly in extreme environments, climate conditions play a key role in determining the type of endolithic growth the microbes can adopt. Epilithic organisms can build extensive and massive covers over rocks and are usually dominated by lichens (e.g., Carter & Viles 2003; Baur et al. 2007; Bjelland et al. 2011; Selbmann et al. 2013) and mosses (e.g., Spitale & Nascimbene 2012; Jackson 2015; Nelson et al. 2020). Lichens are successful colonizers of rocks in extreme environments: such when conditions become harsher, even lichens can enter the first millimeters of rocks, and become endolithic (Friedmann 1982; Nienow et al. 1988; Onofri et al. 2007a), further enriching the community of other microfungi, microalgae and bacteria. This phenomenon is extremely evident and well documented for high mountain peaks and crests, and Antarctica, where other organisms cannot cope with high solar radiation, long snow cover, very low temperature or strong winds (Boustie et al. 2011; Zucconi et al. 2016; Armstrong 2017). In such cases, it seems like there would be a loss of biodiversity, which is partially compensated for by the diversity of endolithic microorganisms. Rock pits and crevices provide them with more

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protected niches. Their body structures adapt to become more and more inconspicuous, optimizing the surface/volume ratio and becoming difficult to detect (Zucconi et al. 2016). The dominance of endoliths is indicative of harsh environmental conditions, which select a relatively low phylogenetic diversity of microorganisms in which many closely related species diversify into their own lineages (Walker & Pace 2007). Walker & Pace (2007) described the endolithic metacommunities formed by bacteria, cyanobacteria, microalgae and microfungi as true ecosystems which can be specifically adapted to different rock types. In fact, endolithic organisms usually colonize orthoquartzite rocks initially, but they are also able to settle on granite (De Los Ríos et al. 2005; Selbmann et al. 2017), halite (Gómez-Silva 2018), gypsum (Wierzchos et al. 2015) and carbonatic rocks (Di Ruggiero et al. 2013; Crits-Christoph et al. 2016). Indeed, the different 'rock architectures', given by the type of the rock and its porosity (Wierzchos et al. 2015), influence the possibility to colonize the rocks only superficially or deeper into the first millimeters. At the same time, the endolithic microorganisms play an important role in the biogeochemical degradation processes of the lithological substrate by the extracellular release of secondary metabolites (acids), which contribute to the transformation of mineral and metals according to the chemical and structural features of the rocks (Gadd 2007).

Endolithic metacommunities are represented by all major groups of microorganisms, such as chemo-organotrophic, chemo-lithotrophic and phototrophic (Gorbushina 2007), including microalgae, bacteria, microfungi – either free-living or building lichens or lichen-like symbioses – and protozoa (Friedmann & Ocampo-Friedmann 1984; Gorbushina & Petersen 2000; Burford et al. 2003; Omelon 2008; Cutler et al. 2015). Algae (e.g., *Chlorella*, *Desmococcus*, *Phycopeltis*, *Printzina*, *Trebouxia*, *Trentepohlia* and *Stichococcus*) and cyanobacteria (e.g., *Calothrix*, *Gloeocapsa*, *Nostoc*, *Stigonema*, *Phormidium*) live on rock surfaces or within the rocks depending on the water availability (Hoffmann 1989; Ortega-Morales et al. 2000; Peraza Zurita et al. 2005; Gaylarde et al. 2006). However, when the solar radiation becomes too high and the environmental conditions are too extreme, these phototrophic organisms find protection in the rock fissures or pores, and localize in a well-delimited layer at about 0.5–5 mm deep inside the rock, where they can still absorb sufficient light necessary to photosynthesize (Matthes et al. 2001; Wierzchos et al. 2006; De Los Ríos et al. 2007; Robinson et al. 2015). Nevertheless, the endolithic photoautotrophs have developed certain adaptations to survive in dark habitats as well, such as the biosynthesis of certain biliproteins and a higher intracellular pigment concentration (Vincent 1988; Samsonoff & MacColl 2001). Being primary producers, the photosynthetic activity of these microorganisms plays a key ecological role within the rocks. They act as a source of nutrients exploited by the heterotrophic endolithic organisms, such as fungi (Gómez-Alarcón et al. 1995; Hirsch et al. 1995; Souza-Egipsy et al. 2004).

Fungi indeed are the organisms best adapted to endolithic growth, especially in habitats where conditions are at the edge of life. These rock-inhabiting fungi (RIF; Ruibal et al. 2009) usually are heavily melanized and present a microcolonial type of growth (Wollenzien et al. 1995). Their peculiar morphology and physiological traits allow them to thrive under prolonged desiccation, high solar/UV irradiation, osmotic stress, extreme temperatures and limited nutrient availability (Sterflinger 1998; Ruibal et al. 2005; Onofri et al. 2007b; Gorbushina et al. 2008). The strong melanization of their cell walls (Dadachova & Casadevall 2008) provides them with a polyextremotolerance towards a multiplicity of abiotic stress factors (Gostinčar et al. 2011, 2012b; Pacelli et al. 2020), while the typical isodiametrical (meristematic) growth allows them to reach the optimal volume/surface ratio to cope with extreme temperatures and desiccation (Wollenzien et al. 1995), but also to thrive in the tiniest spaces of the rocks. RIF belong to two main classes of fungi of *Eurotiomycetes* (mostly *Chaetothyriales*) and *Dothideomycetes* (mostly *Capnodiales*, *Pleosporales*, *Myriangiales* and *Dothideales*; Gueidan et al. 2008, 2011; Ruibal et al. 2009; Muggia et al. 2016, 2020; Coleine et al. 2020; Gostinčar 2020). Previous evolutionary studies inferred a rock-inhabiting ancestor for both classes (Gueidan et al. 2011), hypothesizing the evolution of *Dothideomycetes* RIF in cold and dry conditions, whereas *Eurotiomycetes* RIF would have specialized in hot, semi-arid and also humid environments (Gueidan et al. 2008, 2011).

While endolithic RIF communities have been abundantly studied from warm and cold dry habitats (e.g., deserts and Antarctica; Coleine et al. 2020, 2022; de Menezes et al. 2021; Selbmann et al. 2021), from Mediterranean regions (Ruibal et al. 2005, 2009; Harutyunyan et al. 2008), as biodeteriogens on monuments (Onofri et al. 2014; Isola et al. 2016), and even as plant and animal pathogens (Gueidan et al. 2008; Alves et al. 2019; De Hoog et al. 2019), the investigations on the diversity of microbial communities able to grow in harsh environments at high elevations are still scarce (Egidi et al. 2014; Selbmann et al. 2014). Only a few contributions are reported on the characterization of the bacteria communities associated with soil (Khan et al. 2020) and glaciers on the mountain Muztagh Ata, China (Xiang et al. 2005). In the present work, we report on the phylogenetic and phenotypic characterization of endolithic fungi and algae isolated from rock fragments collected on the top of two mountains over 6000 m altitude, i.e., Muztagh Ata (China) and Cerro Mercendario (Argentina) and we discuss their phylogenetic affiliation.

Materials and methods

Sampling

Small rocks were collected on the top of the two mountains, Cerro Mercedario and Muztagh Ata (Fig. 1A–B), both 6700 m above sea level (a.s.l.). Both mountain summits represent extreme environments at the edge of life, as they remain completely covered by snow for most of the year. However, the very strong winds can blow snow away and let the high solar radiation hit the rock surfaces.

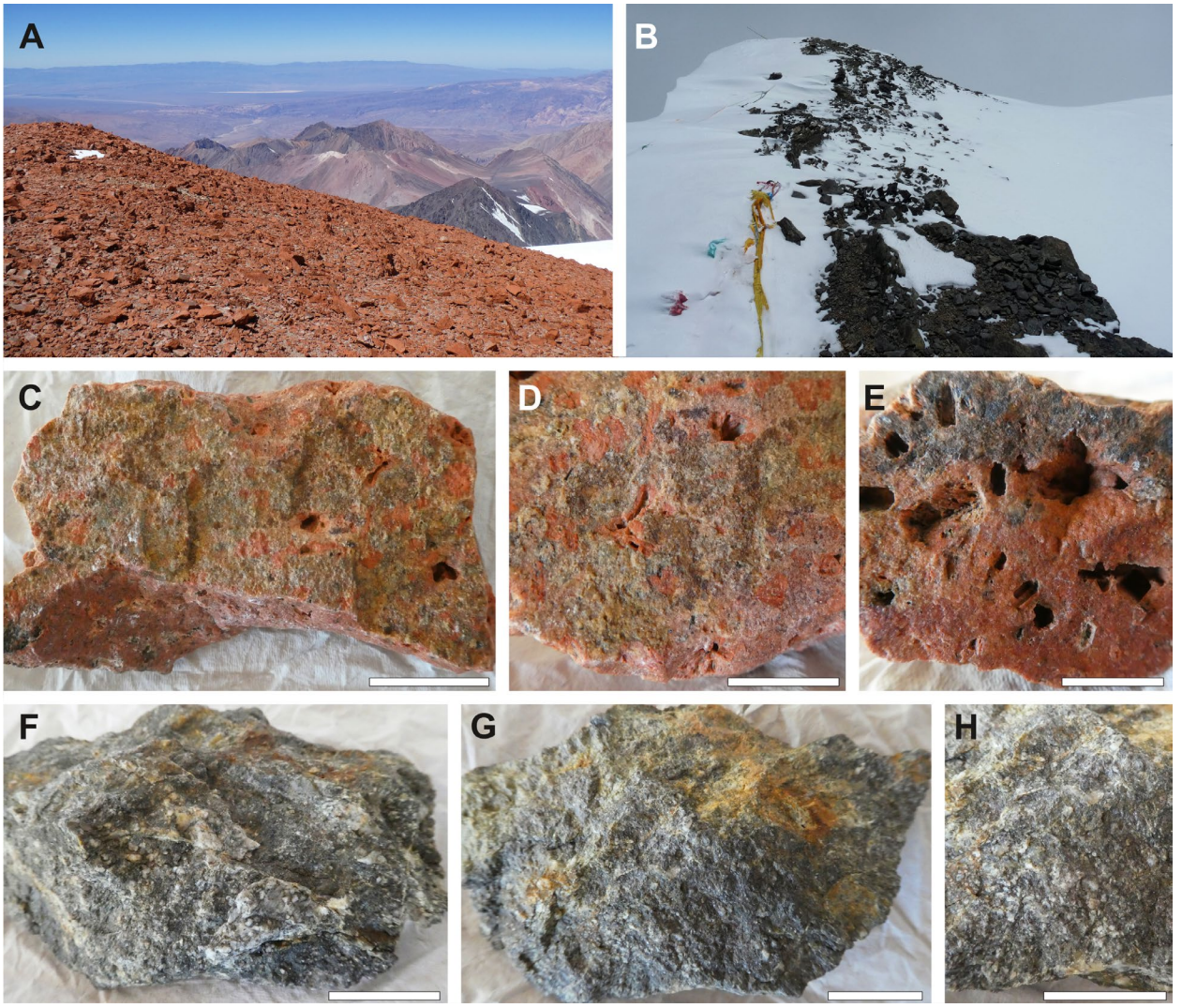

Figure 1. Original environments and rock samples from which the fungal and algal strains were isolated. A – Cerro Mercedario summit 6720 m a.s.l.; B – Muztagh Ata summit 7534 m a.s.l.; C–E – volcanic acidic rock with feldspar and pyroxene/amphibolite from Cerro Mercedario; F–H – low degree metamorphic, pelitic rock with sedimentary mica-schist from Muztagh Ata. Scales: C, D, F–H = 2 cm; E = 1 cm.

This, together with the very low precipitation regimes, makes the environment extremely dry and both summits are completely devoid of vegetation.

Cerro Mercedario is the highest peak of the Cordillera de la Ramada range and the eighth highest mountain of the Cordillera de los Andes (Argentina, province of San Juan, 31°58′45″S/70°06′45″W; Fig. 1A). It is 6720 m a.s.l. high and made of volcanic acidic rocks with feldspar and pyroxene/amphibolite (Fig. 1C–E). Temperature minimum can reach below −40°C, though when no wind blows, at the rock surface it can reach several degrees above 0°C. The nearest meteorological station, about 80 km to the south, is Cristo Redentor, set at 3109 m a.s.l.: here the mean annual temperature measured is about −1.6°C and the total annual precipitation is 360 mm (De Jong et al. 2013; Kinnard et al. 2020).

Muztagh Ata (literally the name means "ice-mountain-father") is the second highest peak of the mountains forming the northern edge of the Tibetan Plateau, although it is also part of the Pamir Mountain Chain (China, Xinjiang region, 38°16′42″N / 75°6′57″E). It measures 7534 m a.s.l. (Fig. 1B) and is made of low degree metamorphic, pelitic rocks with sedimentary mica-schists (Fig. 1F–H). The mountain emerges in one of the driest glacier areas of China and one of the coldest environments in these low- and mid-latitude regions (Shangguan et al. 2006; Zhou et al. 2014). It ranges in elevation from about 3000 to a maximum 7534 m a.s.l, from which several valley glaciers descend. The Taxkorgan is the only meteorological station in this region, situated above 3000 m a.s.l and about 50 km south of Muztagh Ata: in this area the mean annual temperature (measured from 1957 to 2010) is 3.4°C and the the mean annual precipitation is about 70 mm (Yan et al. 2013). Seong et al. (2009) estimated a mean annual precipitation, in the glacier accumulation zone, of 300 mm at 5910 m a.s.l. The summer precipitation is associated with the south Asian monsoon.

Both these regions are classified, according to Köppen climate classification, as tundra climate (group ET; Peel et al. 2007). The winters are long and cold with temperatures below 0°C and precipitation in dry snow form; whereas, the summers are generally mild with a mean temperature of about 10°C.

Culture isolation

Rock pieces were fragmented from three different rocks collected at each mountain summit. The pieces were washed 10 minutes in sterile water, followed by 15 minutes of washing with Tween80 (0.1%) and rinsed four times for 10 minutes with sterile water to remove the detergent. The rock pieces were dried in sterile conditions under the biological hood, wrapped in sterilized aluminum foil, and fragmented by hammering them. The small fragments were taken with a sterile forceps and placed on solid Bold Basal Medium (BBM; Bischoff & Bold 1963) in Petri dishes. Six plates were prepared for each locality, and each plate was inoculated with five rock fragments. The plates were incubated under three different conditions: a) at 17°C with a light/dark cycle of 14/10 h and 20 µmol × photons $m^{-2} \times s^{-1}$; b) 20°C with a light/dark cycle of 14/10 h and 20 µmol × photons $m^{-2} \times s^{-1}$; c) at 3°C in complete darkness in the fridge. The Petri dishes were inspected every month until colonies of fungi or algae appeared. After about 10 months, we observed the first development of a few fungal and algal colonies which started to grow out of the rock fragments. After about 16 months, these colonies grew sufficient biomass to allow sub-cultivation, and were transferred individually on new BBM, malt yeast (MY, for fungi) and *Trebouxia* media (TM, for algae; Ahmadjian 1987). After an additional six months, the subcultures had grown sufficiently to allow molecular analyses and morphological identifications.

Molecular analyses: DNA extraction, PCR amplification and sequencing

Small parts of the cultured fungal and algal colonies were taken with a sterile inoculation loop and transferred into 1.5 ml reaction tubes, containing three sterile tungsten beads for homogenization, frozen and ground using a TissueLyserII (Retsch). The DNA extractions were performed following the CTAB protocol of Cubero et al. (1999), with minor adjustments. The identity of fungal strains was checked with sequences of the nuclear internal transcribed spacers (nucITS) and 5.8S rDNA ribosomal gene, amplified with the primers ITS1F (Bruns & Gardes 1993) and ITS4 (White et al. 1990), and the D1/D2 domain of the 28S nuclear large ribosomal subunit (nucLSU), amplified with the primers LR0R and LR5 (Vilgalys & Hester 1990; http://www.biology.duke.edu/fungi/mycolab/primers.htm). The identity of the algal strains was checked with the sequences of the nucITS, amplified with the primers ITS1T and ITS4T (Kroken & Taylor 2000) and of the ribulose-1,5- biphosphate carboxylase large subunit (*rbcL*), amplified with the primers *rbcL*320 and *rbcL*803 (Nelsen et al. 2011). Polymerase chain reactions (PCR) were prepared for a 25 µl final volume containing 5 µl DNA, 12.5 µl of AccuStart II PCR ToughMix, 0.4 µl for each of the 10 µM primers. PCR amplifications were performed under the following conditions: one initial heating step of 3 minutes at 94°C linked to 35 cycles of 45 seconds at 94°C, 45 seconds at 55°C, 1 minute at 72°C, and one final extension step of 5 minutes at 72°C after which the samples were kept at 4°C. A negative control reaction was always used to check for contamination. All of the amplicons were checked for their quality and size by 1% agarose gel electrophoresis stained with Green Safe Gel (Sigma-Aldrich) and purified using Mag-Bind® Normalizer Kit (Omega bio-tek). Clean amplicons were sent for Sanger sequencing to Macrogen Europe (The Netherlands).

Phylogenetic analyses

The identity of the newly generated fungal nucITS and nucLSU and the algal nucITS and *rbcL* sequences was checked with BLAST similarity search (Altschul et al. 1990) using sequences available in the Genbank (NCBI) database. To delineate the systematic relationships of the isolated taxa, we built a multiple sequence alignment (MSA) for each sequenced locus, and for each of the four major taxonomic groups identified, i.e., *Chaetothyriales* and *Dothideomycetes* for the fungal strains, and *Trebouxia* s.str. and *Trebouxiophyceae* s. lat. – including *Myrmecia* spp. – for the algal strains. The MSAs included both the closest Genbank matches that were recovered for our strains and a broader taxon sampling comprising closely related genera and families selected from previous studies (Supplementary Material Tables S1–S4). In particular, the *Chaetothyriales* dataset was based on Quan et al. (2020) and Muggia et al. (2021), that of *Dothideomycetes* was based on Ametrano et al. (2019a), that of *Trebouxia* s.str. on Muggia et al. (2020) and De Carolis et al., (2022), and that of *Trebouxiophyceae* s. lat. including *Myrmecia* spp. was based on Samolov et al. (2020) and Moya et al. (2018). The MSAs were prepared in Bioedit v.7.2.5 (Hall 1999) and aligned with MAFT v.7 (Katoh & Standley 2013) using the g-ins-I alignment strategy. Ambiguously aligned positions and introns were manually removed from the alignments. Single locus phylogenies were inferred with Maximum Likelihood (ML) and Bayesian Inference (BI) approaches. The analyses were run on CIPRES Science Gateway v.3.3 (Miller et al. 2011). RAxML v.8.2 (Stamatakis 2014) was used for the ML analysis applying GTRGAMMA substitution model and 1000 bootstrap pseudoreplicates. The BI analysis was performed with the program MrBayes v.3.2 (Ronquist et al. 2012) running five million generations with six chains and a random starting tree. Tree sampling was performed every 100 generations with the first 25% of data discarded as burn-in. After checking the phylogenetic concordance of the two loci, they were concatenated (nucITS and nucLSU for fungi, nucITS and *rbcL* for algae) with MEGA (Kumar et al. 2018) and then analysed with both RAxML and MrBayes with the same settings of the single locus analyses. Normalized Robinson-Fould (nRF; Robinson & Foulds 1981) distance between the ML and BI phylogenies was calculated using ETEtoolkit v3.1.2 (Mutawalli et al. 2019). To visualize the mismatches between the ML and BI topologies, we used a R script with the cowplot library (R Development Core Team 2019). The phylogenetic trees were plotted in ITOL (Letunic & Bork 2019).

Morphological analysis

The morphological traits of algal and fungal isolates were analysed using stereo- and compound light microscopes. Plates of the isolated strains were photographed with a Zeiss Axioncam placed on a Stemi 508 Zeiss microscope. A tiny part of the colony was removed using a sterile inoculation loop and mounted in water. Digital light microscope photographs were taken with a Zeiss AXIO Imager A2 coupled to a Thorlabs digital camera. The photos were adjusted for color saturation and sharpness with Adobe Photoshop 7.0 (Adobe System Incorporated, San Jose, CA, USA) and photograph tables were assembled using CorelDRAW X7 (Corel Corporation, Ottawa, Canada).

Results

Culture isolation

A total of nine fungal and five algal strains were isolated and identified (Figs 2–5). In particular, six fungal strains (L3140, L3141, L3144, L3156, L3157 and L3159) belonging to the order of *Chaetothyriales* (Fig. 2), and

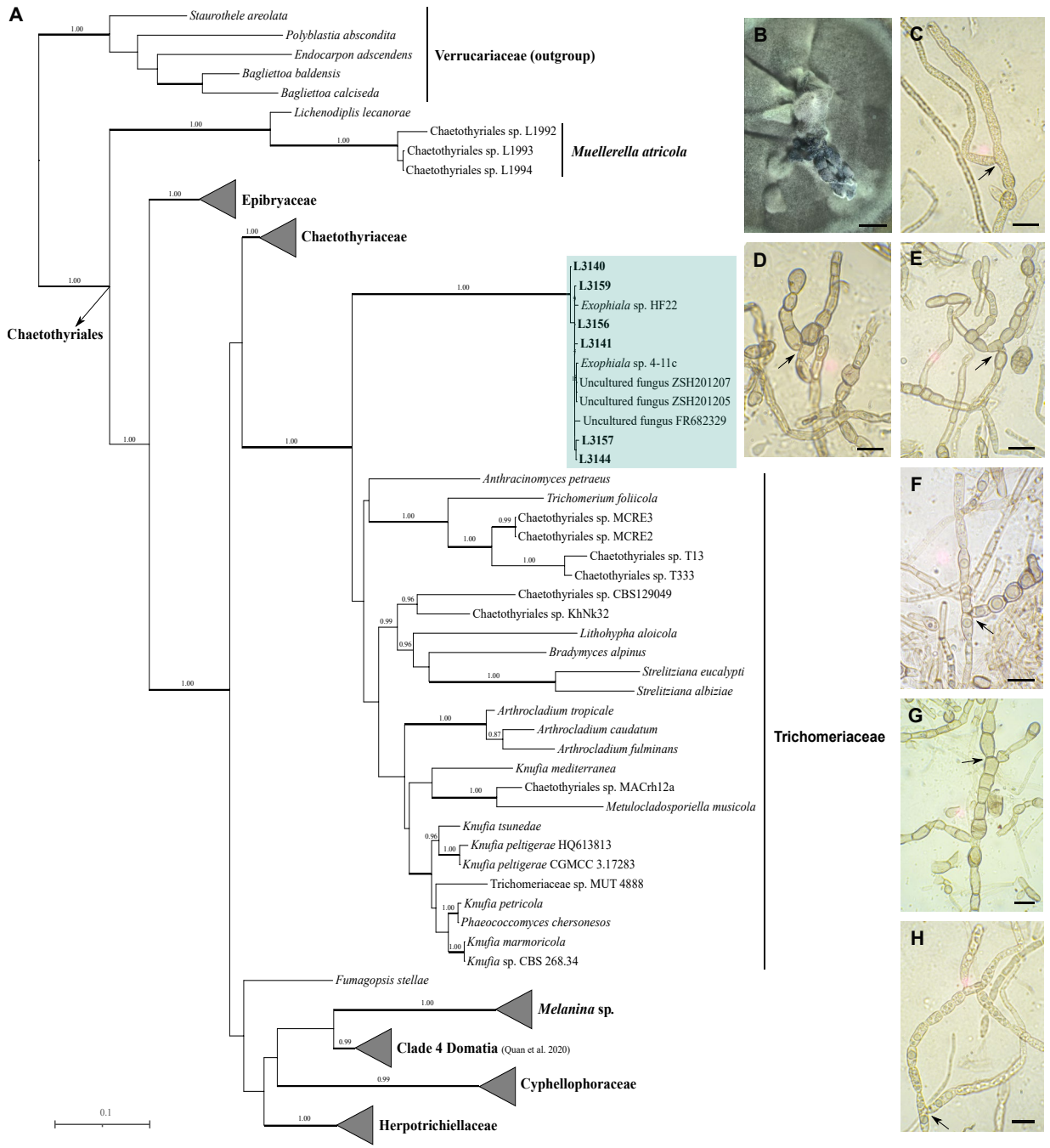

Figure 2. A – Phylogenetic inference of *Chaetothyriales*: Maximum Likelihood analysis based on the concatenated nuclear ITS-LSU dataset; branches in bold denote RAxML bootstrap support ≥75%; Bayesian posterior probabilities ≥0.8 are reported above branches; newly obtained sequences are in bold and the corresponding clade is highlighted in light blue; B–H – Morphology of six-month old representative cultured *Chaetothyriales* strains: B – colony shape on solid malt yeast medium of L3144; C – filamentous and septate hyphae with branches of C – L3140, D – L3159, E – L3156, F – L3141, G – L3157 and H – L3144. Scales: B = 2 mm; C–H = 10 µm.

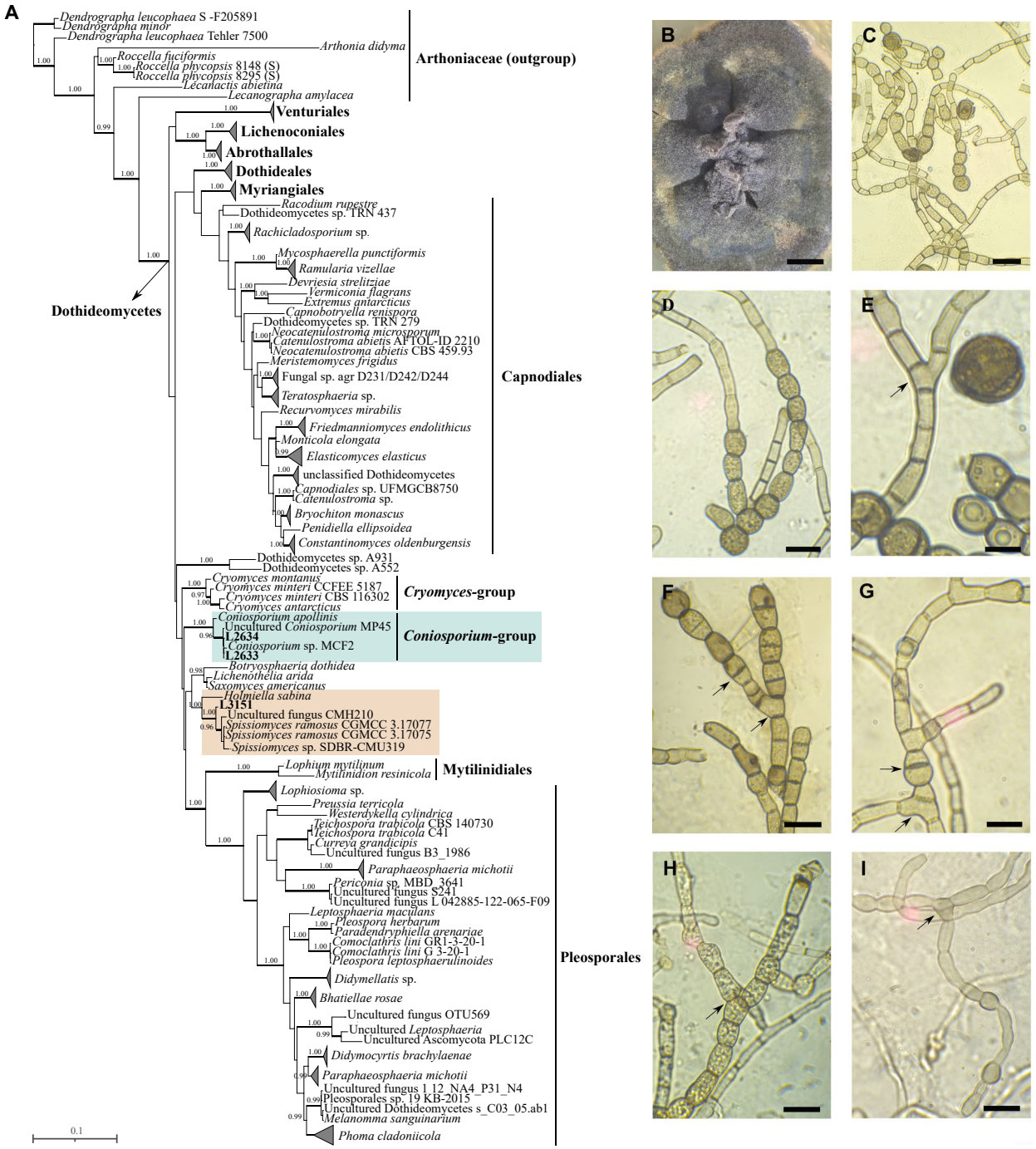

Figure 3. A – Phylogenetic inference of *Dothideomycetes*: Maximum Likelihood analysis based on the concatenated nuclear ITS-LSU dataset; branches in bold denote RAxML bootstrap support ≥75%; Bayesian posterior probabilities ≥0.8 are reported above branches; newly obtained sequences are in bold and the corresponding clades are highlighted in light blue and orange; B–I – Morphology of six-month old representative cultured *Dothideomycetes* strains: B – colony shape on solid malt yeast medium of L3151, filamentous and septate hyphae with branches of C–E – L2633; F, G – L2634 and H, I – L3151. Scales: B = 2 mm; C = 20 µm; D–H = 10 µm.

four algal strains (L3142, L3145, L3147 and L3148) belonging to the genus *Myrmecia* (Fig. 4) were isolated from the rocks collected on Cerro Mercedario, while three fungal strains (L2633, L2634 and L3151) belonging to the class *Dothideomycetes* (Fig. 3) and one algal strain (L3150) belonging to the genus *Trebouxia* (Fig. 5) were isolated from the rocks collected on Muztagh Ata.

These fungal and algal strains were successfully isolated and grew on BBM medium, on MY (for fungi) and on TM (for algae) under different conditions (see Table 1). The strains belonging to *Chaetothyriales* grew at 17°C (L3140, L3141 and L3144) and 3°C (L3156, L3157 and L3159). The strains belonging to *Dothideomycetes* grew at 20°C (L2633 and L2634) and 17°C (L3151). The single *Trebouxia* strain (L3150) and the four *Myrmecia* strains (L3142, L3145, L3147 and L3148) grew at 17°C.

Phylogenetic and morphological analysis

We obtained a total of nine new nucITS and nucLSU fungal sequences, and five new nucITS and *rbcL* algal sequences. We report the phylogenetic analyses performed

for each taxonomic group – *Chaetothyriales*, *Dothideomycetes*, *Myrmecia* and *Trebouxia* – based on the concatenated datasets of fungal nucITS-nucLSU and algal nucITS-*rbcL*, respectively. The Bayesian and the ML phylogenetic inferences were highly concordant, and clades were well supported and topologically congruent with previously published phylogenies (Moya et al. 2018; Ametrano et al. 2019b; Muggia et al. 2020, 2021; Quan et al. 2020; Samolov et al. 2020). The phylogenetic distance between the ML and BI topologies was 0.23 nRF for

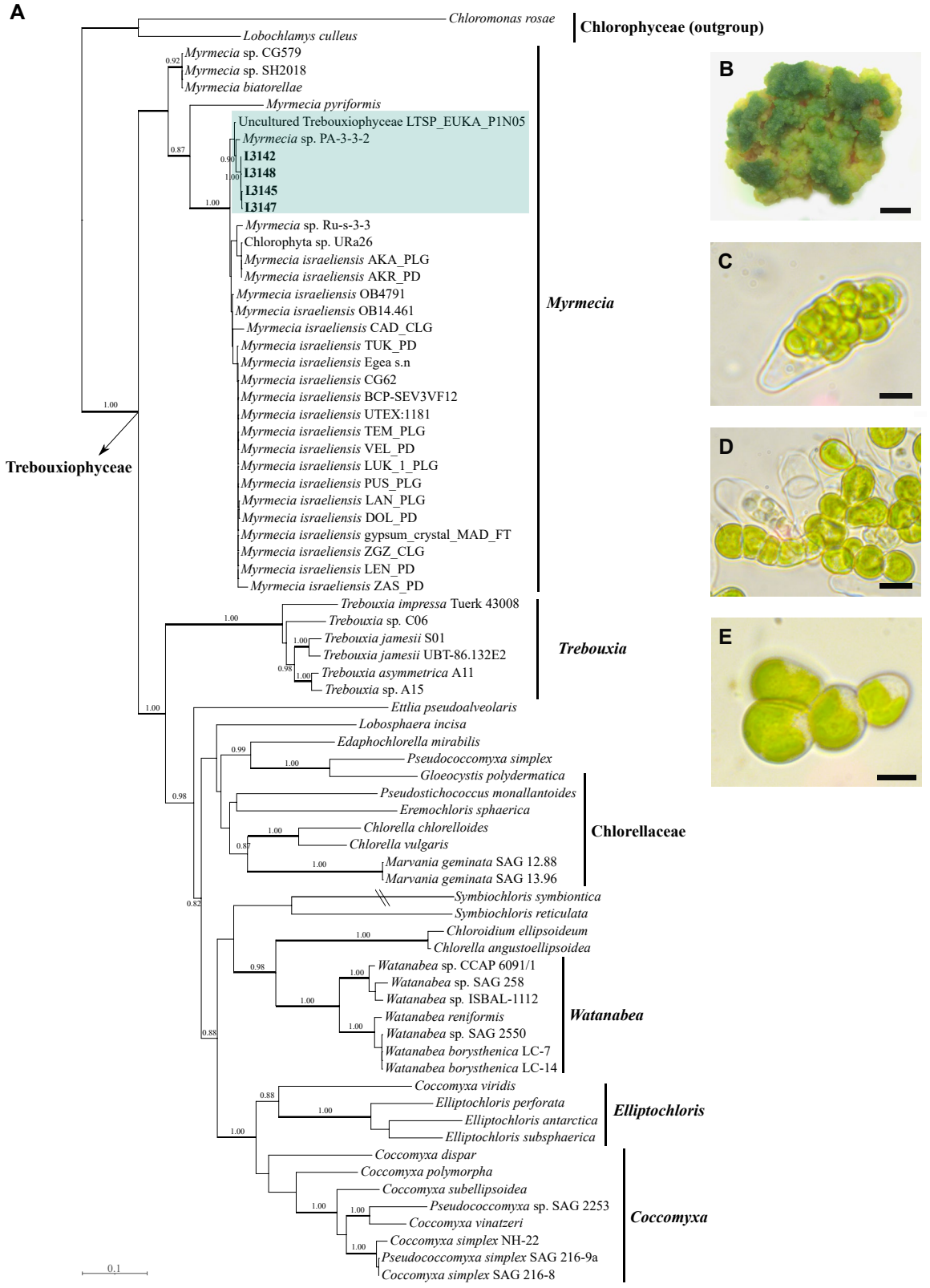

Figure 4. A – Phylogenetic inference of *Myrmecia*: Maximum Likelihood analysis based on the concatenated nuclear ITS-*rbcL* dataset; branches in bold denote RAxML bootstrap support ≥75%; Bayesian posterior probabilities ≥0.8 are reported above branches; newly obtained sequences are in bold and the corresponding clade is highlighted in light blue; B–E – Morphology of six-month old representative cultured *Myrmecia* strains: B – colony shape on solid *Trebouxia* medium of strain L3145; C – autospore; D, E – mature cells. Scales: B = 2 mm; C–E = 10 µm.

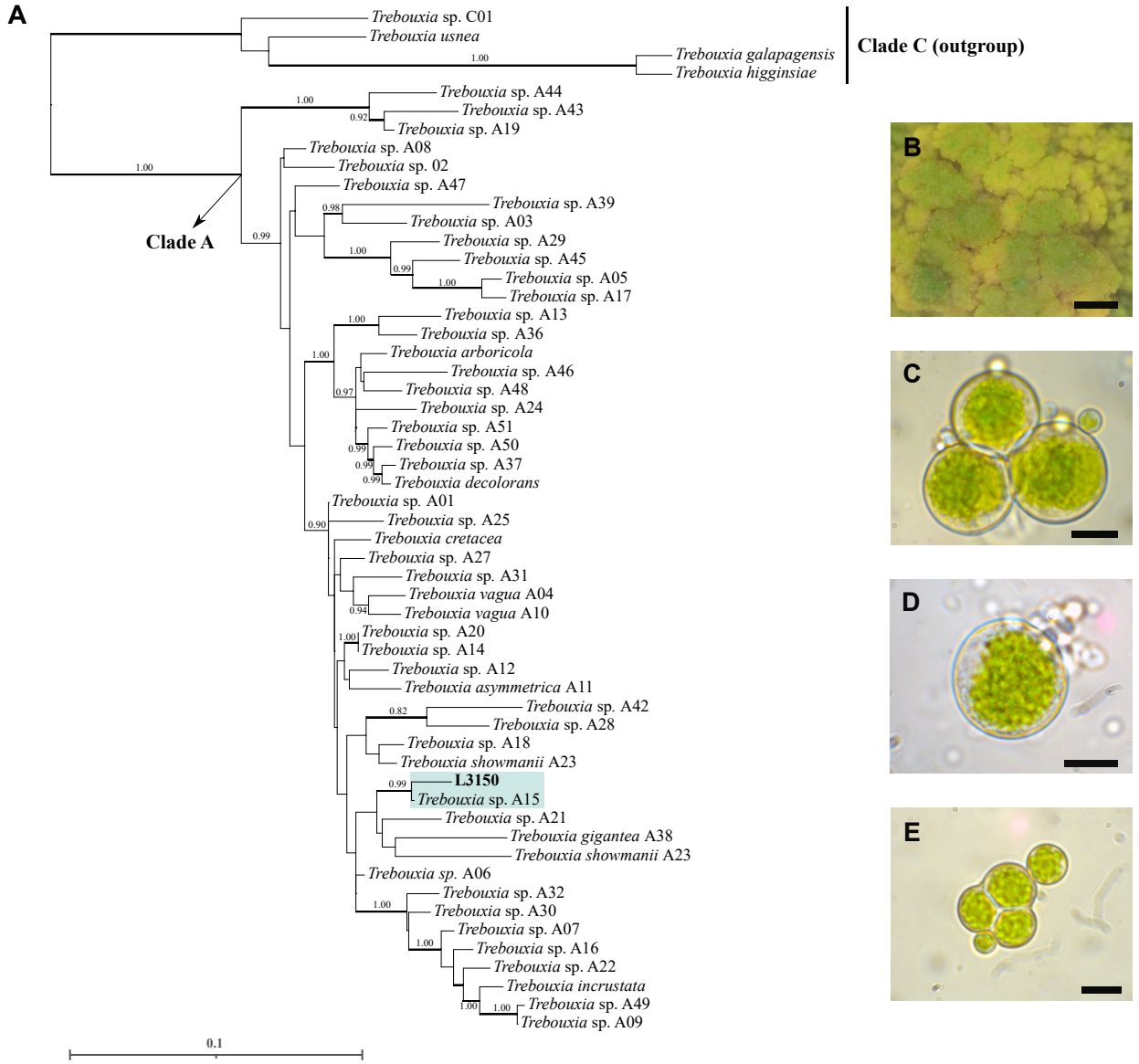

Figure 5. A – Phylogenetic inference of *Trebouxia*: Maximum Likelihood analysis based on the concatenated nuclear ITS-*rbcL* dataset; branches in bold denote RAxML bootstrap support ≥75%; Bayesian posterior probabilities ≥0.8 are reported above branches; the newly obtained sequence is in bold and the corresponding clade is highlighted in light blue; B–E – Morphology of six-month old representative cultured *Trebouxia* strain L3150: B – colony shape on solid *Trebouxia* medium; C–E – mature cells. Scales: B = 1 mm; C–E = 10 µm.

Chaetothyriales, 0.24 nRF for *Dothideomycetes*, 0.30 for *Myrmecia* and 0.17 for *Trebouxia*. At the family level, only one conflicting placement was reported. The family *Chaethothyriaceae* (*Chaetothyriales*) was placed closely related to *Trichomeriaceae* and the lineage formed by our newly sequenced samples in the ML analysis, whereas it was placed closely related to the clade *Melanina* by the Bayesian inference (Supplementary Material Figs S1, S2, S3, S4).

Chaetothyriales (Fig. 2) – The strains L3140, L3141, L3144, L3156, L3157 and L3159 isolated from Cerro Mercedario formed a separate and well supported clade together with two specimens named *Exophiala* sp. HF22 and *Exophiala* sp. 4–11C, and three uncultured fungi (FR682329, ZSH201207 and ZSH201205; Fig. 2A). These strains are characterized by a dense, melanized mycelium, which reached about 2 cm in diameter after growing about six months on MY medium (Fig. 2B). The hyphae are hyaline to heavily melanized, septate, cells are elongated, almost rectangular in shape (12 × 4 µm; Fig. 2C–H).

Dothideomycetes (Fig. 3) – The two strains L2633 and L2634 isolated from Muztagh Ata, formed a well-supported clade with *Coniosporium appollinis, Coniosporium* sp. MCF2 and one uncultured *Coniosporium* MP45, and are here recognized as *Coniosporium* sp.. The strain L3151 instead was placed closely related to a clade formed by an uncultured fungus CMH210, two samples of *Spissiomyces ramosus* (CGMMCC 3.17077 and CGMMCC 3.17075) and a sample of *Spissiomyces* sp. SDBR-CMU319. Thus L3151 is recognized as *Dothideomycetes* sp. The species *Holmiella sabina* is sister to all these taxa (Fig. 3A).

On MY medium, L2633 and L2634 (Fig. 3B) developed a melanized mycelium which reached about 2 cm in diameter after about six months. These strains were

Table 1. Origin data and sequence accession numbers of fungal and algal strains newly isolated in culture: culture ID, origin of the rock samples, temperature (T) of the growth chamber, phylogenetic placement of the strains estimated by the phylogenetic analyses of Figures 2–5, and the new corresponding NCBI accession numbers are reported.

ID culture	Rocks of origin	Temperature growth chamber	Phylogentic placement	ITS	nucLSU	*rbcL*
L2633	Muztagh Ata	20°C	*Coniosporium* sp.	ON620069	ON569432	–
L2634	Muztagh Ata	20°C	*Coniosporium* sp.	ON620070	ON569433	–
L3151	Muztagh Ata	17°C	*Dothideomycetes* sp.	ON620071	ON569434	–
L3150	Muztagh Ata	17°C	*Trebouxia* sp. A15	ON620064	–	ON603529
L3140	Cerro Mercedario	17°C	*Chaetothyriomycetes* sp.	ON620072	ON569435	–
L3141	Cerro Mercedario	17°C	*Chaetothyriomycetes* sp.	ON620073	ON569436	–
L3144	Cerro Mercedario	17°C	*Chaetothyriomycetes* sp.	ON620074	ON569437	–
L3156	Cerro Mercedario	3°C	*Chaetothyriomycetes* sp.	ON620075	ON569438	–
L3157	Cerro Mercedario	3°C	*Chaetothyriomycetes* sp.	ON620076	ON569439	–
L3159	Cerro Mercedario	3°C	*Chaetothyriomycetes* sp.	ON620077	ON569440	–
L3142	Cerro Mercedario	17°C	*Myrmecia* sp.	ON620065	–	ON603530
L3145	Cerro Mercedario	17°C	*Myrmecia* sp.	ON620066	–	ON603531
L3147	Cerro Mercedario	17°C	*Myrmecia* sp.	ON620067	–	ON603532
L3148	Cerro Mercedario	17°C	*Myrmecia* sp.	ON620068	–	ON603533

characterized by two types of hyphal cells: 1) melanized, rectangular (10 × 5 µm) cells along the hyphae (Fig. 3C, E–G) and 2) moniliform, ovoid to round and heavily melanized cells resembling conidia (10 µm) towards the terminal parts of the hyphae (Fig. 3C–F).

L3151 grown on the MY medium built compact, dark brown to black colonies, with a diameter of 2 cm after about six months. The mycelium was composed by slightly melanized hyphae, characterized by rectangular cells (12 × 5 µm) which were sometimes intercalated by thicker cells with a septum dividing them into two halves (Fig. 3F–I).

Myrmecia (Fig. 4) – The strains L3142, L3145, L3147 and L3148 isolated from Cerro Mercedario rocks were identified as *Myrmecia* sp. and formed, together with the sample *Myrmecia* sp. PA-3-3-2 and an uncultured *Trebouxiophyceae* LTSP_EUKA_PIN05, a small, well supported clade sister to that of *Myrmecia israeliensis* (Fig. 4A). The four strains are genetically and morphological identical, being characterized by spherical to subovoid cells of about 13 µm in diameter and a bipartite, slightly cup–shaped parietal chloroplast (Fig. 4C–E). We also observed the presence of many autosporangia (30 × 15 µm) containing up to 13 cells (Fig. 4C–D) and open autosporangia from which autospores were just released (Fig. 4D).

Trebouxia (Fig. 5) – The strain L3150 isolated from Muztagh Ata rocks was found in the *Trebouxia* clade 'A' (Fig. 5A) and it is closely related to the species level lineage *Trebouxia* 'A15' (*sensu* Leavitt et al. 2015; Muggia et al. 2020). The colony develops three-dimensionally in a coralloid type of growth typical of *Trebouxia* colonies (Fig. 5B). The cells are coccoid, of about 18 µm in diameter; the chloroplast is massive and occupies almost the entire volume of the cytoplasm and the nucleus is confined at one side of the cell (Fig. 5C–D). Autospores were not observed in L3150 culture, but several cells seemed to be in the first mitotic division phase.

Discussion

We have documented here for the first time the isolation and the taxonomic characterization of fungi and algae from altitudes over 6000 m a.s.l. The fungal and algal strains started to grow in culture after ten months from the rock fragments that were inoculated on the media and have developed extremely slowly their mycelia and cell colonies, respectively. This hints to the generally extremely slow growth of these microorganisms used to cope with harsh conditions (i.e., endolithic and in selective environments), as well as to their need to adapt to *in vitro* culture. The isolation of microorganisms in axenic culture is crucial to perform the morphological characterization of taxa, especially for species new to science, which otherwise could not be characterized if only environmental DNA (eDNA) was analyzed. In the present study, the cultural approach was preferred, the samples were indeed characterized by an extremely low amount of mixed biomass embedded in the rock, which was often unsuitable for direct extraction, amplification and Sanger sequencing. The culture-dependent approach is flawed by the fact that only a fraction of the microorganisms can grow in culture, leaving the rest of the diversity undetected. This certainly leads to an underestimation of the entire biodiversity of the rock communities (Wijayawardene et al. 2021). Moreover, cultures may be affected by contaminant microorganisms which can be erroneously included in the original community. These contaminants can even overgrow the native species from extreme environments, which often need a longer time to develop in culture. Nevertheless, the low success rate of culture isolation, the low number of isolated strains and their extremely slow growth rate are reassuring about the fact that we detected part of the actual diversity of these extreme rock environments. The algal cultures also showed a certain preference for lower temperatures, as all the strains grew at 17°C. In contrast, fungal cultures did

not show any preference to the temperature, as *Chaetothyriomycetes* sp. grew at 17 and 3°C, *Coniosporium* sp. at 20°C and *Dothideomycetes* sp. L3151 at 17°C. However, the optimal growth temperature should be properly tested with *ad hoc* experiments, potentially considering a larger temperature gradient, which was, though, beyond the scope of this study.

The molecular characterization of all these strains was performed based on the concatenated alignments of fungal nucITS-nucLSU or algal nucITS-*rbcL*, running both ML and Bayesian phylogenetic inferences. The results of the two approaches were mostly congruent, showing only a minor difference in the position of the clade *Chaetothyriaceae*. This is possibly explained by insufficient phylogenetic signal due to the low variability in the nucLSU locus (Yang & Warnow 2011). However, our samples were placed within a monophyletic clade with full support in both ML and Bayesian tree.

The six fungal strains belonging to the order *Chaetothyriales* (isolated from Cerro Mercedario: L3140, L3141, L3144, L3156, L3157 and L3159) segregated into a fully supported clade basal to the family *Trichomeriaceae*, which includes epiphytic (Chomnunti et al. 2012) and rock inhabiting species (Isola et al. 2016). This clade consists of additional black fungi, i.e., two sample named *Exophiala* sp. HF22 and *Exophiala* sp. 4–11C, and three uncultured taxa (FR682329, ZSH201207 and ZSH201205; Fig. 2A). The sequence diversity of both nuclear LSU and ITS markers is very low among our strains and between these and the two '*Exophiala'* samples and the three uncultured taxa. As this new clade is supported by a long branch, it seems to have diverged significantly from the rest of *Tricomeriaceae*. This phylogenetic placement may suggest the clade as a potential new taxon deserving formal description, however this goes beyond the scope of the present study and additional data are needed. Notwithstanding the phylogenetic position of the here isolated strains, the lineage comprises two samples named *Exophiala* which may represent misidentified samples. Indeed *Exophiala* is a genus confirmed to belong to *Herpotrichiellaceae* (Quan et al. 2020; Muggia et al. 2021) and this has been strengthened by our results. The present dataset, indeed, includes 13 species of *Exophiala* (Supplementary Material Table S1) which, among others, represents the collapsed *Herpotrichiellaceae* clade in the phylogenetic analyses (Fig. 2). All these black fungi share rather peculiar and selective environments of origin, as they come either from saline, rock or contaminated substrate, which may support their monophyletic lineage, likely hinting to the recognition of a new taxon. In fact, our strains were isolated from volcanic acidic rock with feldspar and pyroxene/amphibolite, whereas the sample named *Exophiala* sp. HF22 was isolated from a 3100-year-old staircase in the salt mine of Hallstatt (Austria; Piñar et al. 2016), and that named *Exophiala* sp. 4-11c from cadmium contaminated soil (Long & Zhu unpublished work). The uncultured fungus FR682329 was detected from building material (Pitkäranta et al. 2011), whereas, the other two uncultured fungi were sequenced from rainwater samples (Du et al., direct submission to NCBI). It is well known that black melanized fungi have adapted to halophythic and endolithic growth (Kogej et al. 2005; Gunde-Cimerman et al. 2011), but they also have been detected in waters and rainwater (Babič et al. 2017), where melanization protects them from the high UV radiation in the atmosphere, thus making rainwater an extreme environment as well. The identification of this clade comprising potentially polyextremotolerant fungi (Gunde-Cimerman et al. 2011; Gostinčar et al. 2012a) within *Chaetothyriales* further strengthens the renown of this order as one of the fungal lineages in which the greatest diversity of lifestyle and a complex ecological versatility has evolved (De Hoog 2014; Teixeira et al. 2017; Zhang et. al. 2020).

Interestingly, three dothideomycetous strains were instead isolated from the Muztagh Ata rocks. These are identified in two different clades, both formed by rock inhabiting fungi (RIF) and closely related to other RIF clades, here represented by *Cryomyces* spp., *Saxomyces* and *Lichenothelia*. The two strains, L2633 and 2634, are nested with two *Coniosporium* specimens isolated from limestones and one endophytic *Coniosporium* (uncultured *Coniosporium* MP45). *Dothideomycetes* sp. L3151 is basal to a clade formed by two samples of *Spissiomyces ramosus* isolated form rocks (Su et al. 2015) and one from a plant (*Spissiomyces* sp. SDBR-CMU319); the epiphytic fungus *Holmiella sabina* is basal to these samples (Fig. 3A). The phylogenetic position of the RIF *Coniosporium* species was originally discussed by Ruibal et al. (2009), who demonstrated that *Coniosporium apollinis* and *C. uncinatum* belong to *Dothideomycetes*. Later, Selbmann et al. (2005, 2011) described two new species of Antarctic rocks-inhabiting meristematic fungi able to form cryptoendolithic communities, i.e., *Cryomyces antarticus* and *C. minteri*, and showed that they were closely related to the *Coniosporium* clade. Our present results further support these evolutionary relationships, as the strains we identified seem to enrich the diversity of already known lineages. Also, the close phylogenetic placement of the two RIF genera *Lichenothelia* with *Saxomyces* inferred by our analysis was previously presented by Ametrano et al. (2019a, b). Interestingly, these two genera do come from environments similar to that of Muztagh Ata, which is compatible with their close relationships with our strain.

It is of particular interest that the isolated algal strains correspond to *Trebouxia* and *Myrmecia*, as both genera can occur either free living (Bubrick et al. 1984; Yung et al. 2014) or, more notably, as photobionts in lichen symbioses (Rambold et al. 1998; Tschermak-Woess 2019). However, we could neither detect any sign of lichen thalli on the rocks, nor it was possible to spot any algal colony by inspecting the original rock fragments using stereo-microscopy. It is likely that these algae would reside in the tiniest and most hidden rock crevices, being invisible to the naked eye. More specifically, the genus *Trebouxia* is one of the most common and best studied lichen photobionts (see Muggia et al. 2020; Bordenave et al. 2021; De Carolis et al. 2022), for which the genetic and morphological diversities were recently clarified by

pursuing an integrative taxonomic approach. The strain L3150 here isolated is sister to the species-level lineage *Trebouxia* 'A15', included in the clade 'A' (Beck et al. 2002; Leavitt et al. 2015; Muggia et al. 2020; De Carolis et al. 2022) and it is closely related to the species-level lineages recognized as the '*gigantea*-group' (Muggia et al. 2020). So far, this strain could be only genetically identified, but future analyses should address its morphological characterization by investigating the ultrastructure of its chloroplast (pyrenoid included), likely confirming its affiliation to the 'gigantea-group' (Bordenave et al. 2021).

The *Myrmecia* sp. strains (L3142, L3145, L3147 and L3148) form a clade together with a *Myrmecia* sp. and uncultured *Trebouxiophyceae* samples identified from biological soil crusts and forest soil, respectively (Samolov et al. 2020; Hartmann et al. 2009). This clade is unresolved with *M. israeliensis* (Fig. 4A) which is the primary symbiotic microalga in the lichen genera *Psora* spp., *Placidium* spp. and *Clavascidium* spp. (Moya et al. 2018). Lichen species of these three genera grow on soil and often form conspicuous biological soil crusts in either cold or warm, arid and desert habitats (Lewis & Lewis 2005; Flechtner et al. 2013; Fučíková et al. 2014; Samolov et al. 2020). As on Cerro Mercedario, the rocks were laying on soil. It is reasonable that *Myrmecia* cells/colonies isolated from there could have resided in the rock crevices, and thus have grown in culture. As we neither detect any layer of melanized fungi that could form a protective layer above the algae (as often documented for cryptoendolithic microbial communities; Selbmann et al. 2013; Gorbushina et al. 2005), nor any lichen mycobiont was isolated, we assume that these algae were growing free-living in/on the rock (Yung et al. 2014). They would likely receive protection from high solar UV radiation by the rock matrix itself, colonizing the inner pits. Indeed, Wong et al. (2010) identified even free-living *Trebouxia* in hypolithic communities of the Tibetan tundra, supporting the hypothesis of a general phenomenon for extreme cold-arid landscapes.

We hypothesize that the two different fungal-algal communities identified on the two mountains may depend on the type of the rock substrate. Both sampling sites are in fact located much above 6000 m a.s.l and are characterized by a highly similar climate (Peel et al. 2007). However, Muztagh Ata rocks are pelitic rock with sedimentary mica-schist, whereas, Cerro Mercedario are acidic volcanic. These different rock substrates could influence the microbial biodiversity. Otherwise, Walker & Pace (2007) suggested that the site-specific characteristics, such as local climate or water chemistry, could have a stronger influence than rock type, while only few works reported the correlation between the type of rocks and the bacterial communities (De la Torre et al. 2003; Pointing et al. 2009). One of the main drivers of the diversity of endolithic communities is the porosity of the rocks. It has been shown, that the homogeneous distribution of the pores, such as in sandstone, favors the microbial endolithic colonization/growth and biodiversity (Cockell et al. 2003). However, as we identified only a few taxa for each site, we cannot trace statistically supported differences of diversity between the two sites.

The altitude factor may also play an important role in the species selections. Stevens (1989, 1992) introduced the term Rapoport's elevation (RE) gradients to indicate the broader distribution range of organisms living at a higher altitude than of those distributed at lower elevation. This concept is only partially in accordance with what we found in these two high-elevation, far apart, environments. The high selective pressure possibly led to few species able to adapt to these extreme, but rather homogeneous, ecological niches. We indeed detected a very low diversity of algae and fungi. However, we have no evidence that the same algal or fungal species are present in both sites.

Fungal diversity of mountain regions is only partially known, as most of the works focused on specific fungal groups, such as arbuscular mycorrhizal (AM) fungi, macromycetes, ectomycorrhizal (EcM; Liu et al. 2011; Velázquez et al. 2016). Also, microbial composition of soil at low-mid elevations is usually dominated by *Basidiomycota* in comparison to *Ascomycota* and *Zygomycota* (Praeg et al. 2020; see James et al. 2020 for the new reappraisal of the Phylum *Zygomycota*). At high elevations, instead, the abundance of *Ascomycota*, *Chytridiomycota*, and *Glomeromycota* tend to increase and the dominant classes have been shown to be *Agaricomycetes*, *Sordariomycetes*, *Dothideomycetes*, *Leotiomycetes* and *Zygomycota* (Ogwu et al. 2019), supporting our findings on the fungal diversity in rocks collected at high elevation. The culturable fungi we detected were indeed all ascomycetes.

Other factors could influence the rock colonization by microorganisms in these harsh habitats, such as the dispersion of fungal spores by wind currents, which can carry them up to these high altitudes (Yamamoto et al. 2012). For example, *Cladosporium* and other *Dothideomycetes* were found in airborne samples collected at high altitude in Japan (Tanaka et al. 2019).

To our knowledge this is the first study which reports on fungi and algae isolated from rocks at altitudes above 6000 m a.s.l. While several researches have focused on the diversity of bacteria communities on soil and rocks (Wei et al. 2016; Kumar et al. 2019; Tang et al. 2020), there is still much to discover on the eukaryotic diversity in high alpine and nival zones. Furthermore, understanding the diversity of organisms able to colonize high altitude environments would help to understand how diversity could change in the near future under a global warming scenario, where species would move higher up towards these borderline ecosystems (Frenot et al. 2005; Farrell et al. 2011; Olech & Chwedorzewska 2011; Selbmann et al. 2013), potentially causing the loss of the stenoecious species adapted to extreme environments such as mountain tops.

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Supplementary electronic materials

Figure S1. Tanglegram of ML and BI phylogenies (*Chaetothyriales*). Download file

Figure S2. Tanglegram of ML and BI phylogenies (*Dothideomycetes*). Download file

Figure S3. Tanglegram of ML and BI phylogenies (*Myrmecia*). Download file

Figure S4. Tanglegram of ML and BI phylogenies (*Trebouxia*). Download file

Table S1. List of taxa included in the phylogenetic analysis of *Chaetothyriales* and their NCBI accessions. Download file

Table S2. List of taxa included in the phylogenetic analysis of *Dothideomycetes* and their NCBI accessions. Download file

Table S3. List of taxa included in the phylogenetic analysis of *Myrmecia* and their NCBI accessions. Download file

Table S4. List of taxa, reported as ID of the species level lineage according to Muggia et al. (2020), included in the phylogenetic analysis of *Trebouxia* and their NCBI accessions. Download file

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