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**The Antarctic lichens and endolithically colonized granites as
evolutionary niches for microbial diversification**

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ABSTRACT

In the rare ice-free areas of Antarctica, naked rocks play a prominent role for terrestrial life, which, along the coasts, are represented mostly by lichens. Here, lichens develop their thalli in very inconspicuous forms hampering a straightforward species recognition but representing an important group of organisms with high percentage of endemism. Differently, toward the interior of the continent, where the conditions are harsher, microbial life, including lichens and microbes associated, withdraw inside rocks to find a more protected niche, forming endolithic communities. The traditional dual nature of lichens, i.e. symbiotic associations between biotrophic fungi (mycobiont) and phototrophic microorganisms (photobiont), has been recently challenged. Lichen thalli are open structures housing other non-cyanobacterial prokaryotes, algae and additional microfungi. Metabarcoding sequencing, culture experiments, microscopy studies, and phylogenetics have uncovered in particular the diversity of the lichen-associated fungal and bacterial communities, under different ecological settings. However, only two researches hint at Antarctic lichens as pivotal niches for the diversification of microfungi in extreme environments and no study has ever compared lichens with endolithic communities present in the same areas. This thesis aimed at capturing the diversity of fungi and bacteria residing within lichen thalli of selected eight species: the Antarctic endemic *Acarospora flavocordia*, *Buellia frigida*, *Lecanora fuscobrunnea*, *Lecanora physciella*, and *Lecidea cancriformis*, and the cosmopolitan *Pleopsidium chlorophanum*, *Rhizoplaca melanophthalma*, and *Rusavskia elegans*. Furthermore, we conducted a comparative analysis between epilithic communities, consisting of six lichen species, and the endolithic counterparts inhabiting colonized granite samples collected in the same area. At last, we isolated fungal and algal strains from specific lichen thalli using a culture-dependent method, aiming to describe new species and expand the culture collections of the Italian National Antarctic Museum, University of Tuscia, Italy. The results obtained indicated that the mycobiont plays a pivotal role in shaping microbial assemblages associated with each analyzed thallus. The characterization of microbial diversity between the two ecological niches revealed that endolithic communities exhibit higher and specific biodiversity compared to lichens. Moreover, the high presence of still undescribed microbial taxa associated with lichen thalli highlights diversification and evolution that take place in extreme environments.

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CHAPTER 1. INTRODUCTION

1.1 Antarctica

Antarctica is the last discovered and the most inhospitable continent. It is also the driest, coldest, and highest, with an average elevation of 2,500 metres. With its 13.66 million km², it is almost double the size of Australia, and only 2% of the continent is free from ice. The perimeter of Antarctica is subcircular, with diameters around 4000 km from coast to coast. The shape is made less regular by the northward projection of the Antarctic Peninsula and the two large indentations formed by the Weddell and Ross Seas. From a geological perspective, Antarctica is composed of two subcontinents: East Antarctica and West Antarctica (Fig. 1.1). The former is largely covered by the polar ice cap and is bordered towards the sea by the long semi-circular coastline. The Transantarctic Mountains (TAM), a vast mountain range stretching approximately 3,000 km and rising to over 4,500 metres in height, bound it to the west. The latter, situated south of the Pacific and Atlantic Oceans, includes the basins of the Ross Sea and the Weddell Sea, as well as the intermediate lands of the Antarctic Peninsula, the Ellsworth Mountains, and Marie Byrd Land. Antarctica is blanketed by an immense glacier, the ice cap, which is the largest plateau on Earth. Its average thickness is 2,200 m, but in several places, it exceeds 4,000 m, reaching a peak of 4,700 m at the continent's centre. The rocky surface underneath remains concealed beneath the deep ice, forming a buried topography that encompasses tall mountains and extensive depressed basins. Near the coasts, the ice cap slowly flows towards the margins under its own weight, and its elevation rapidly decreases by 2,000-3,000 m over relatively short distances of 200-300 km (Manzoni 2006).

Antarctica encompasses three distinct climatic regions: 1) the sub-Antarctic region, characterized by vascular plants at lower altitudes and *Bryophyta* and lichens at higher altitudes; 2) the maritime Antarctic region, where lichens such as *Acarospora*, *Buellia*, *Caloplaca*, *Lecanora*, *Lecidea*, and *Rhizoplaca* predominate. Only two vascular plant species, *Colobanthus quitensis* (*Caryophyllaceae*) and *Deschampsia antarctica* (*Poaceae*), are present. 3) Continental Antarctica, where microorganisms, more than anywhere else on Earth, dominate the biology due to extreme environmental conditions; in this region, mosses and lichens are confined to a narrow altitudinal range along coastal regions (Convey et al., 2008). Mosses and lichens, along with cyanobacteria, stand out as the primary phototrophic organisms in Antarctica (Vincent et al., 2000). The absence of terrestrial vertebrates means that heterotrophic organisms are limited to invertebrates, alongside microorganisms such as protozoa, fungi, bacteria, and archaea.



Figure 1.1 Map of the Antarctic continent (Abrahamsen, 2012)

1.1.1 Victoria Land

Victoria Land is a region of Continental Antarctica, located in the lower part of the continent, named by James Clark Ross (who discovered it in January 1841), in honour of British Queen Victoria. It covers an area of approximately 140,000 km²; it is bordered to the east with the Ross Sea to the east and Wilkes Land to the west. This region is normally 'divided' into two areas (Northern Victoria Land and Southern Victoria Land). The Northern Victoria Land area stretches from 72° 19' S 170° 16' E to 75° 59' S 159° 30' E while the area of South Victoria Land lies between 76° 55' S 160° 55' E and 78° 12' 166° 25' and includes the characteristic McMurdo Dry Valleys (Figure 1.2).

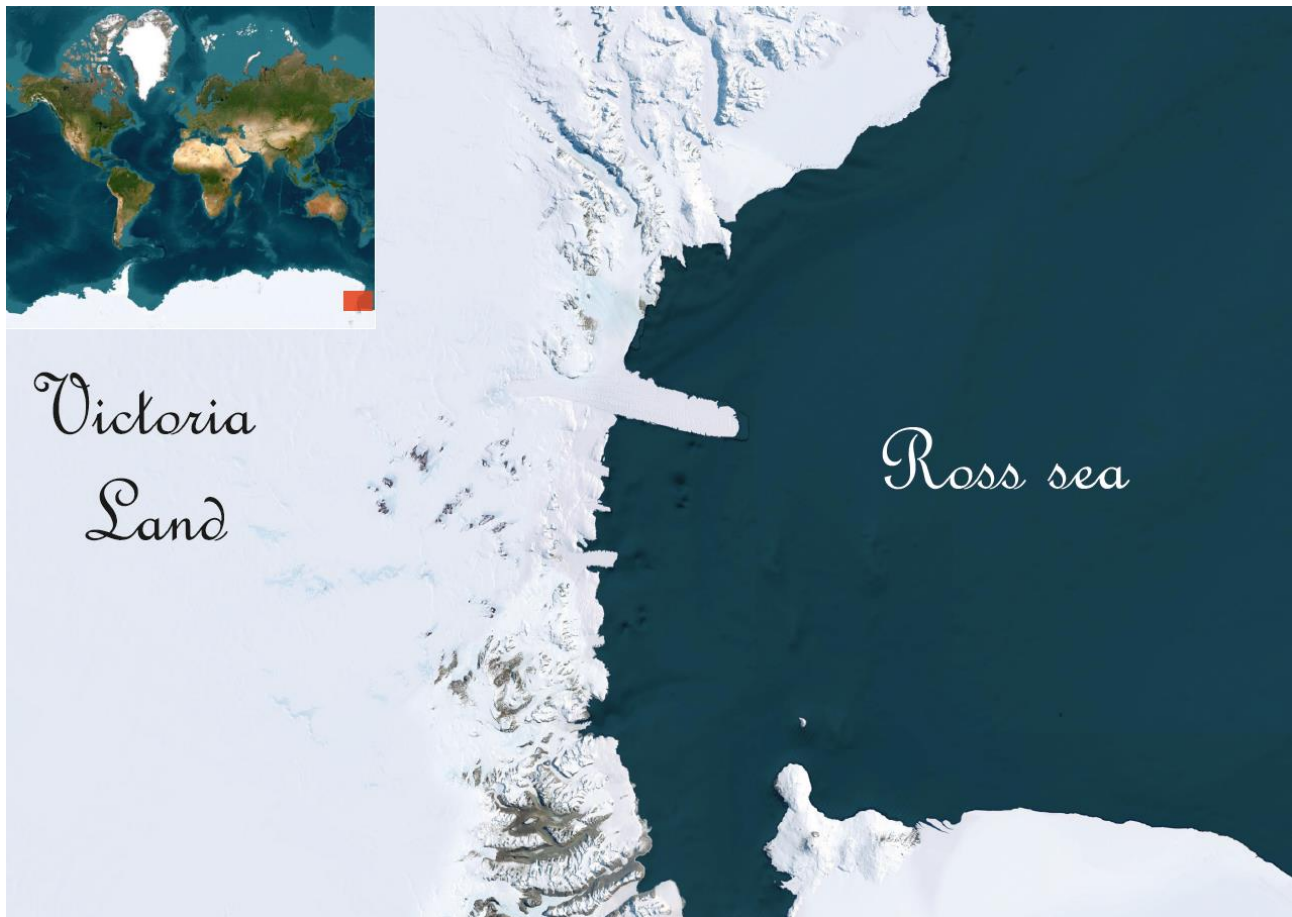


Figure 1.2 Map of Victoria Land, east Antarctica.

The climate of Northern Victoria Land is typical of the coastal areas of Continental Antarctica: the average monthly temperature fluctuates between -2°C and -5°C during January, and can reach $-26^{\circ}\text{C}/-30^{\circ}\text{C}$ during the coldest month (August), with annual averages between $-17^{\circ}\text{C}/-19^{\circ}\text{C}$. Precipitation, occurring only in the form of snow, is rather scarce, with annual averages ranging between 100 and 200 mm. Air humidity, on the other hand, tends to increase during the summer season, thanks to the humid air masses from the sea (Grigioni et al., 1992). The Northern Victoria Land includes the territory of the Terra Nova Bay, which is the windiest area in the region. It is characterized by particularly intense winds, known as katabatic, funnelled into valleys at speeds reaching approximately 160 kilometres per hour (Kurtz & Bromwich, 1985).

In the more interior zones, due to the distance from the sea and the increase in altitude, conditions become much harsher, and temperatures drop significantly. In some high-altitude mountainous areas, temperatures rarely exceed -30°C , even in the height of the austral summer. In the Northern Victoria Land, periodically there are ice-free areas, mainly represented by zones along the coasts and mountain

peaks; the rocky substrate here is quite diverse and includes sandstones, granites, volcanic rocks, basalts, etc. (Orombelli, 1986; Zucconi et al. 2016).

1.2 Lichen symbiosis

1.2.1 Morphology

A lichen is an organism formed by two or more symbiotic components, the most common of which are an autotrophic organism such as a cyanobacterium or an alga (photobiont) and a heterotrophic one, a fungus (mycobiont). Typically, over 98% of lichenized fungi fall within the phylum *Ascomycota*, while the remaining fungal species are classified under *Basidiomycota*. The photobiont includes green algae of the species *Trebouxia* and *Trentepohlia*. Some lichens are associated with cyanobacteria belonging to the genus *Nostoc* (referred to as blue-green algae) found in specialized structures called cephalodia. Therefore, a lichen can be considered an "ecosystem" where various constituent organisms collaborate (Farrar, 1976). The alga performs photosynthesis, providing carbohydrates to the fungus. Meanwhile, the upper, thick cortical layer and associated hyphae can protect algal cells from rapid water loss and intense radiation (Rundel, 1988). There are three main types of lichen growth forms: the *crustose* type, characterized by a thin, closely adhering layer to the substrate; the *foliose* type, consisting of a flattened thallus that develops parallel to the substrate with variously incised margins, resembling a leaf; the *fruticose* type, in which the lichen thallus is attached to the substrate at a single point (Fig 3).

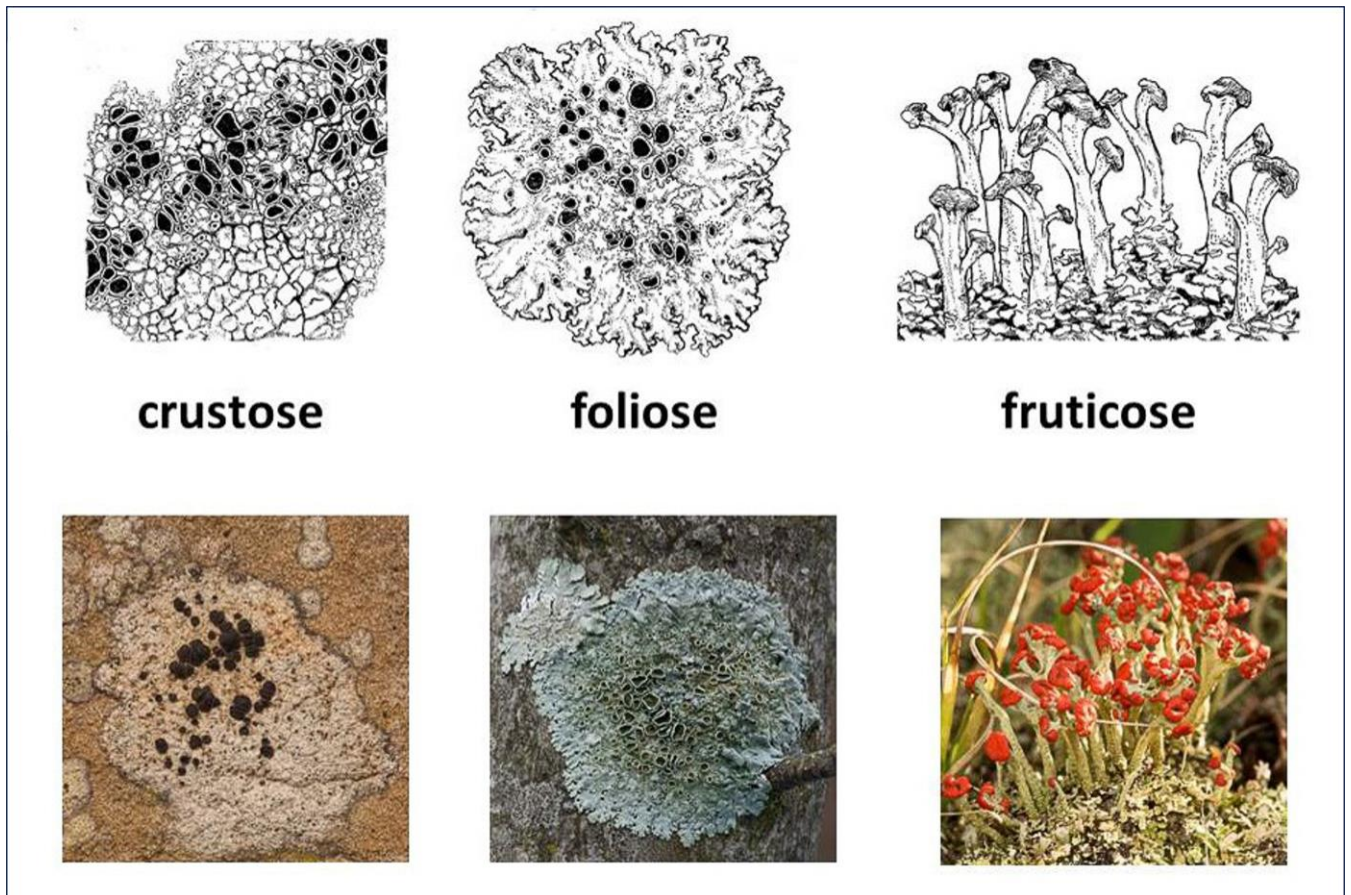


Figure 1.3. Three lichen growth forms: crustose, foliose and fruticose.

Most habitats where lichens are common feature a mixture of all three-growth forms. Another growth form includes an intermediate category between crustose and foliose lichens, known as placoid. The largely crustose thallus has distinct marginal lobes, as seen in some species of *Buellia* and *Caloplaca*. In the "gelatinous" form, algal cells are generally distributed, as observed in *Collema* and *Leptogium* (Piervittori et al., 1998). In cross-section, a typical lichen is primarily composed of fungal tissue, but in the upper cortical layers, eukaryotic algal cells are present. In simpler and more primitive structures, typical of gelatinous and crustose lichens, the fungal filaments, called hyphae, and algal or cyanobacterial cells are uniformly arranged throughout the lichen's body, spanning its entire thickness (homomerous thallus, typical of gelatinous lichens). This organization contrasts with a more advanced and stratified anatomical structure known as a heteromerous arrangement. It is composed of an upper cortical layer where the fungus, with its hyphae, forms a protective sheath (upper cortical state), while algal cells are located inside to build a well-defined layer. Below this, even more internally, various products from lichen activity accumulate between the strands of a loose fungal network (medulla). The succession of layers depends on the considered morphology (crustose,

foliose, or fruticose). The anchoring of lichens occurs through various modalities depending on their anatomical and morphological characteristics, as well as the specific structure of the substrate (soil, rock, tree bark). In lichens lacking a lower protective layer, the hyphae of the innermost layer of the thallus directly, perform this function, growing into the surface irregularities of the substrate and providing the thallus with a large number of attachment points. In some cases, crustose species can develop branched rhizines (*Squamarina*) or delicate networks (*Psora*) that tend to incorporate soil particles. In most foliose lichens, fixation to the substrate occurs through branching structures called rhizines. Some lichens do not attach to the underlying surface, facilitating reproduction and dispersion through a specific strategy: the hygroscopic gradient. In their dehydrated state, they temporarily halt their functions, tend to curl, and can be easily transported by the wind. Upon water absorption, they return to their fundamental state, expose the upper surface to the sun, and resume their functional activities (Piervittori et al., 1998).

1.2.2 The lichen as a microcosm of biodiversity

The traditional view of lichens as a stable mutualistic symbiosis between a fungus and one or more green algae or cyanobacteria has always been debated; it has been more than ever challenged by the discovery of numerous additional microorganisms that potentially emerge as obligate participants in the symbiosis (Grube et al., 2016). The symbiotic concept of a 'lichen' must consider the wide range of associated microscopic organisms. It could, therefore, be considered an example of a 'holobiont,' with a dominant fungus and an inclusive microbiota (Simon et al., 2019). Therefore, we can redefine the symbiosis of lichen as follows: 'A lichen is a self-sustaining ecosystem formed by the interaction of a dwelling fungus and an extracellular arrangement of one or more photosynthetic partners, along with an indeterminate number of other microscopic organisms' (Honegger, 2012). Lichens are persistent organisms that can vary morphologically, providing a wide variety of small-scale niches for various microorganisms, especially fungi and bacteria. Lichenicolous fungi are all those fungi that live inside or on the surface of lichens, excluding the fungus that forms the thallus itself (called mycobiont). So far, more than 1,800 species of lichenicolous fungi have been described, but there are likely many more yet to be discovered, meaning their precise number is still unknown and currently underestimated (Muggia & Grube, 2018). The fungi associated with lichens have been the subject of numerous studies for a long time, but only recently has greater attention been given to the ubiquitous presence of bacteria within the thalli of lichens (Grube et al., 2016). Typically, *Alphaproteobacteria* dominate the bacterial communities found in lichens, but other bacterial groups such as *Acidobacteria*, *Actinobacteria*, and *Betaproteobacteria* are also present (Grube et al., 2009;

Selbmann et al., 2010). The bacterial communities associated with lichens studied so far have been shown to be host-specific in their composition (Grube et al., 2009; Selbmann et al., 2010). A clear shift in the microbiome profile is observed when the parasitic lichen *Diploschistes muscorum* infects and eventually supplants *Cladonia symphycarpa*, forming an autonomous thallus (Wedin et al., 2016). During this transition, the population of *Alphaproteobacteria* dominating the bacterial community of *Cladonia* decreases, while the number of *Betaproteobacteria* or *Chloroflexota* significantly increases when *Diploschistes* takes over, supporting the idea that the bacterial community is highly specific to lichen species (Grube & Berg, 2009). These microorganisms, therefore, can play a significant role in the diversification and adaptation of lichens to different environments. This hotspot could be the key to understanding how lichens can survive in extreme environments and adapt to diverse conditions (Smith et al., 2016).

1.2.3 The Antarctic lichens

In the Antarctic territories, vascular plants have not developed effectively to withstand low temperatures. The extremely cold temperatures act as a limiting factor for the survival of vascular plants, making it challenging to develop the biological processes necessary for survival and growth. Additionally, the Antarctic soil is characterized by a deep cover of ice and snow, hindering the growth of plant roots in the ground. Plants require a suitably supportive and permeable soil to absorb the nutrients and water necessary for growth. Given the prevalence of frozen soil, such favorable conditions are absent. However, in the milder coastal zones of Antarctica, some vascular plants are present, but only two species (*Deschampsia antarctica* and *Colobanthus quitensis*) contribute to the region's flora. In the coldest Antarctic regions, lichens represent the most diverse and abundant group of organisms, especially in ice-free areas.

Since there are no vascular plants, lichens play a fundamental role as primary producers in the ecosystem. Despite the adverse and hostile living conditions, which pose a challenge for other organisms due to the high level of environmental stress, lichens adapt and persist (Nienow & Friedmann, 1993). Antarctic endolithic microorganisms adapt to various ecological niches within the lithic substrate, where they find a source of nutrients and protection. They can be found living beneath rock surfaces, in the interstitial habitats of weathered siliceous rocks exposed to atmospheric agents, as well as in the porous spaces between mineral grains in porous rocks. The most studied substrate is certainly sandstone, where the communities are referred to as cryptoendolithic. (Friedmann 1982; Selbmann et al. 2017; Coleine et al. 2018).

According to the most recent review of Antarctic lichen species, it is estimated that at least 30% of them are endemic (Smith, 2000). The adaptive radiation and speciation of these endemic species mainly occurred during the postglacial Holocene period of 10,000 years, although in some regions of the continent, it might have occurred in shorter periods following deglaciation (Seppelt, 1995). Additional speciations may have occurred during previous interglacial phases or before the Oligocene. Some oases may have remained ice-free for periods of several million years, such as the McMurdo Dry Valleys (Friedmann & Weed, 1987), or nunataks where species diversity is low. Most endemic species are found in the maritime part of Antarctica, and the sub Antarctic region exhibits an even higher percentage of endemism (Hertel, 1987).

Together with bryophytes, lichens play a crucial role in the biomass of Antarctic ecosystems, both continental and maritime. This implies their pivotal role in shaping community structure, akin to the function of trees in a forest, as indicated by Smith (1984, 1990a) and Kappen (1993a). The adaptations of Antarctic lichens often mirror those observed in alpine species (Ahmadjian, 1970). Reproduction commonly occurs through vegetative diaspores, such as soredia, rather than lichenization, and early development is markedly slow (Armstrong, 2021). Thickening of the cortical layer commonly transpires with the addition of "dead" layers of hyphae and structures, ensuring robust substrate attachment and wind resistance (Dodge, 1973). There is a dominance of crustose species, and some of them develop using a "stalked" or "cushion-shaped" growth typical of strongly humid maritime regions (Kappen, 1988). The crustose lichen typically forms a thin thallus, which is either completely attached to the substrate on its entire lower surface or concealed in the uppermost layer of the rock. The common genera of lichens that thrive in Antarctica include *Acarospora*, *Buellia*, *Candellariella*, *Lecidea*, *Lecanora*, *Rhizocarpon*, *Rinodina*, *Umbilicaria*, *Xanthoria*, *Rusavskia*, *Turgidosculum*, and *Usnea* (Lamb, 1964; Dodge, 1973). The niches provided by rocks for microbial growth are diverse such as sandstone, granite and dolerite (Selbmann et al., 2017; Coleine et al., 2018; Golubic et al., 1981). The first, starting from the surface, is the epilithic habitat consisting of the rock surface itself. An example is *Buellia frigida*, the most widespread and abundant of all Antarctic continental endemic lichens. The crustose epilithic thallus forms rosettes that can join to create extensive sheets. It has a defined shape, with radial marginal lobes and an areolate center. The color of the thallus varies from grey-white to almost black, with darker marginal lobes. The upper cortical layer consists of dark cells with thick walls of the same diameter, while the medulla is composed of loosely arranged, thin-walled hyphae. In shaded habitats, it forms green thalli due to the absence of the black pigment. The photobiont is an alga of the genus *Trebouxia*, in a layer of variable thickness beneath the cortical layer. Many species of *Acarospora* are recognizable for their characteristic yellow pigmentation, primarily due to rhizocarpic acid. Yellow *Acarosporaceae* in Antarctica are confined to two genera,

Acarospora and *Pleopsidium*. Two species of *Acarospora*, *A. gwynnii* and *A. flavocordia*, are endemic to Antarctica, but a third species, known as *Pleopsidium chlorophanum* (Castello & Nimis, 1994), is a widely distributed cosmopolitan lichen. These species exhibit yellow pigmentation and are found in areas exposed to high insolation during the summer seasons.

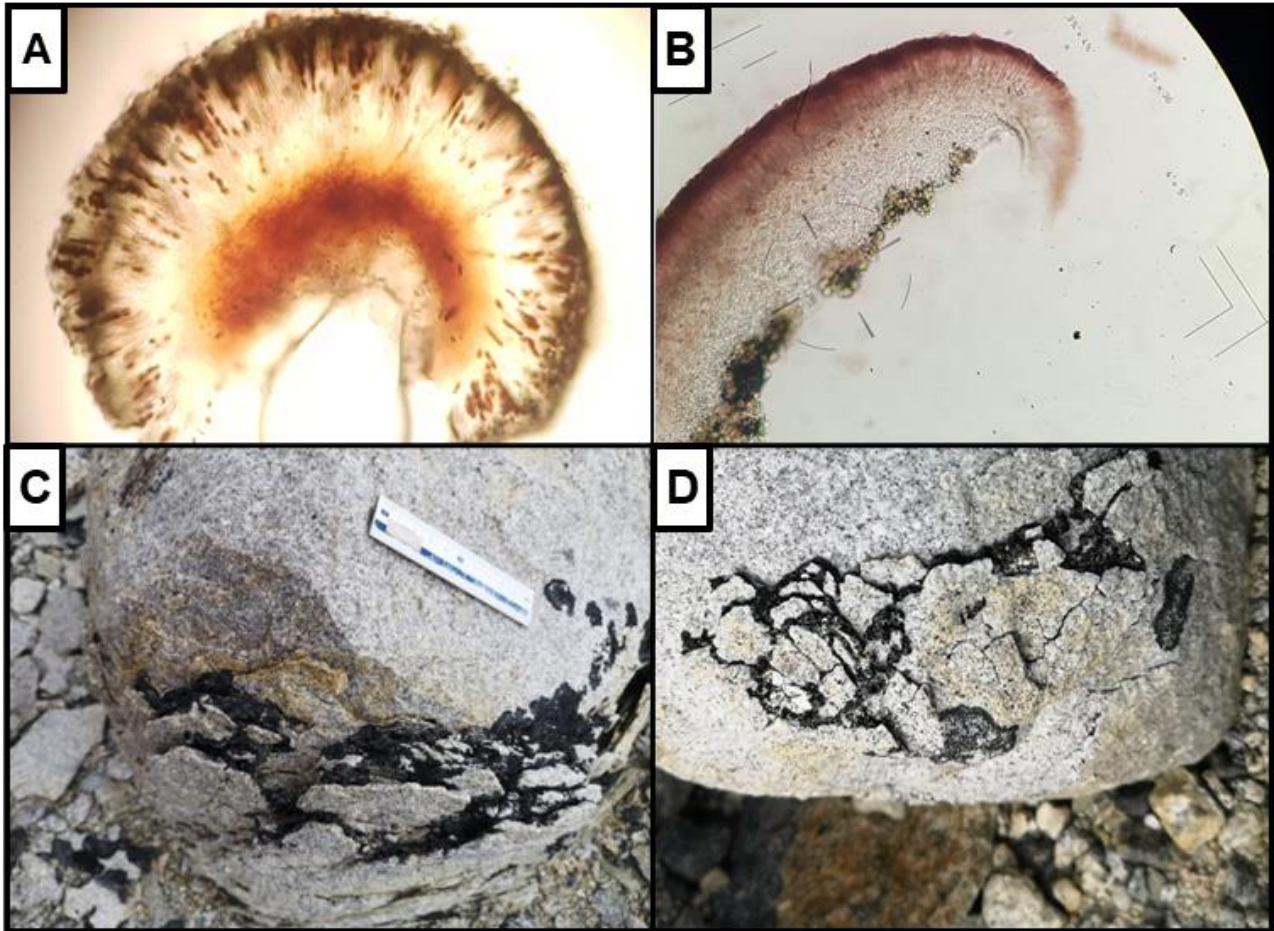


Figure 1.4. *Lecidea cancriformis* and *buellia frigida* morphology and ecology. **A)** cross-section of *Buellia frigida* apothecium; **B)** cross-section of *Lecidea cancriformis* apothecium; **C,D)** *L. cancriformis* and *B. frigida* share the same ecological niche; *L. cancriformis* successfully penetrates the granite rock creating a niche sheltered from external conditions; this is exploited by *B. frigida*.

The typical habitats for endolithic species, such as *Lecidea cancriformis* and *Rhizoplaca melanophthalma*, are usually rocky areas with low calcium content (e.g., basalt, granite, schist). *R. melanophthalma* has a thallus of light ochre color, pulvinate scales with numerous apothecia that can take on a blackish-greenish hue. Often, the thallus is scarcely visible, and only the apothecia are seen (Castello et al., 2010). The coloration of this species can be easily confused with *Lecidea cancriformis*, one of the most common endemic species in the Granite Harbour. Its thallus consists of adnate or pulvinate areoles, with a color ranging from light grey. The sub-surface layer associated with it is made up of long white rhizomorphic structures. The apothecium is sessile and has a dark

color; moreover, it can reach a length of 2 mm, with a diameter of 1.5 mm (De Los Rios et al., 2005). Another similar endemic species is *Lecanora fuscobrunnea*, which appears to be one of the very few capable of forming a superficial thallus in the McMurdo Dry Valleys of McMurdo. The latter is characterized by rather dark thalli and exhibits some peculiar anatomical adaptations. The hymenia are sometimes poorly developed in the ascomata, but even in cases where hyaline ascospores form readily, the tips of the paraphyses can become short and constricted at the septa. Consequently, the hymenial surface may be covered by rounded melanized cells that could function as mitotic spores (Obermayer & Poelt, 1992). Another example of an endemic Antarctic lichen is the *Lecanora physciella*, which grows on inland rocks at higher altitudes in the sun exposed habitat and is usually associated with *Himantormia lugubris* (Hue), *Ochrolechia parella*, *Lecanora polytropa*, *Lecidea* spp., *Rhizocarpon* spp., and *Usnea aurantiaco-atra*. It is characterized by a thallus forming a more or less continuous rosette-like crust, distinctly cracked into smaller areoles, elongated at the edge of thallus; dark yellow to almost brownish or with slightly greenish tinge; prothallus distinct and black. Apothecia (0.3) 0.4–0.6 mm in diameter, slightly immersed in thallus; margin black, distinct or excluded; discs black, bare. Apothecial anatomy: thalline margin excluded; parathecium distinct; epihymenium brownish green; hypothecium distinct; paraphyses simple, end cell not enlarged (Śliwa & Olech, 2002).

An example of a cosmopolitan lichen also found abundantly in Antarctica is the *Rusavskia elegans*. *R. elegans* (formerly *Xanthoria elegans*) is a lichenized fungus belonging to the genus *Rusavskia* and the family *Teloschistaceae*. Distinguished by its vibrant orange or red pigmentation, this species typically thrives on rocks, frequently in proximity to bird or rodent perches. With a circumpolar and alpine distribution, it holds the distinction of being among the initial lichens employed in the lichenometry technique for rock-face dating. This species thrives on various rock types, encompassing both calcareous and siliceous substrates, occasionally spreading over moss, litter, or rock surfaces. Its habitat ranges from exposed to somewhat sheltered sites, commonly in proximity to bird or small-mammal droppings (McCune & Geiser, 1997). Notably, it has demonstrated adaptability to both natural and man-made surfaces, spanning from the sea-water spray zone to boreal forests and continental grasslands (Hill, 1984; Hinds, 1995; Hinds & Hinds, 1993). Impressively, it can flourish in regions with less than 6 centimetres of annual precipitation and exhibits resilience, with the capacity to endure submersion in streams for a significant portion of the growing season (Fahselt & Krol, 1989). *R. elegans* has an extremely broad circumpolar and alpine distribution and is found on all continents except Australia (Almborn, 1987). It is widespread in Antarctic regions (Castello, 1995). The thallus of this lichen exhibits a foliose structure, resembling leaves, although the central parts may adopt a nearly crustose appearance. It is relatively small, typically measuring

less than 5 cm in width, with lobes narrower than 2 mm, ranging from appressed to loosely appressed. The upper surface displays various shades of orange, while the lower surface is white, corticate, featuring short and sparsely distributed hapters (attachment structures produced by some lichens) (McCune & Geiser, 1997). Notably, it lacks vegetative propagules such as soredia and isidia, but frequently develops apothecia. Its thalli have been described as swollen and orange-yellow in streams, compact orange on boulders, or dark orange-red on the driest rock faces (Castello, 1995).

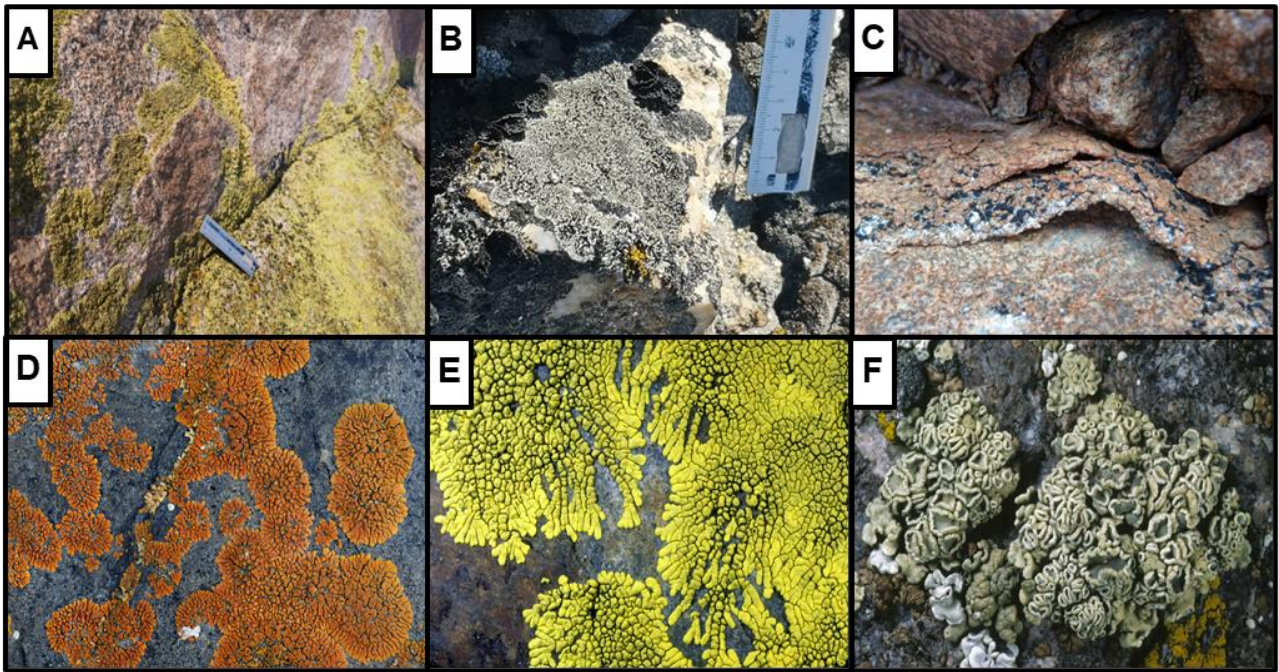


Figure 1.5 A selection of six Antarctic lichens studied in this thesis; **A)** *A. flavocordia* **B)** *L. physciella* **C)** *L. fuscobrunnea* **D)** *R. elegans* **E)** *P. chlorophanum* **F)** *R. melanophthalma*.

1.2.4. Endolithic communities

Endolithism represents one of the most remarkable and efficient adaptations to the extreme conditions in Antarctica, particularly in the inner ice-free areas of the continent (Friedmann, 1982; Coleine et al., 2018). It refers to the colonization of microorganisms within the pores, crevices, and fractures of rocks (Coleine et al., 2018; Zucconi et al., 2016). These microorganisms, encompassing bacteria, fungi, and algae, flourish in the challenging environments of Antarctica, particularly in areas devoid of surface vegetation and subjected to severe environmental factors such as low temperatures, intense UV radiation, and limited water availability (Onofri et al., 2004; Ruise et al., 2007). Endolithic communities play pivotal roles in nutrient cycling, rock weathering, and soil formation in Antarctica (Amarelle et al., 2019). Endoliths can be inhabitants residing within cracks and fissures (chasmoendoliths), occupying spaces between mineral grains (cryptoendoliths) in general associated

with the sandstone rocks, or actively penetrating and perforating the rock substrate (euendoliths) (Golubic et al., 1981; Nienow & Friedmann, 1993). Along the coasts, they thrive as epilithic organisms, while in the interior regions of the continent, they are also found as endoliths. Cryptoendolithic communities inhabit microscopic pores, either natural or excavated beneath the rock surface (Nienow & Friedmann, 1993), reaching depths of up to 10 mm (Ruisi et al., 2007; Friedmann & Sun, 2005), are distinguished by five typologies: three dominated by prokaryotes (*Gloeocapsa*, *Hormathonema-Gloeocapsa*, and *Chroococcidiopsis*) and two dominated by eukaryotes (lichens and *Hemicloris antarctica*) (Friedmann, 1982; Nienow & Friedmann, 1993).

1.2.4.1 Lichen-dominated cryptoendolithic communities in sandstones

The sandstone associated endolithic communities dominated by lichens are by far the most studied in Antarctica. Indeed, the research by Selbmann et al. (2017) demonstrated that sandstone represents the optimal substrate for endolithic colonization. Cryptendolithic lichens, unlike epilithic ones, lack a specific shape and morphology and do not produce sexual reproductive structures. This makes taxonomic identification very challenging, unless through molecular analysis. The internal colonization of the rock leads to a precise stratification where three zones can be distinguished (Friedmann, 1982). Briefly, the black zone is represented by non-lichenized black fungi, namely meristematic fungi. Meristematic growth is an additional adaptation to stressful conditions; it is a type of growth in which cells are closely packed together, thus limiting contact with the external environment (Friedmann, 1993; Onofri et al., 1999; Selbmann et al., 2005). The highly melanised cell walls of these organism protect the microbial components of the communities in the layers below from the harmful effects of intense solar exposition. The with zone is dominated by lichenized hyaline fungi and algal cells of the phycobiont dominate the white zone. The green zone is dominated by a plentiful amount of green algae, often belonging to the genus *Trebouxia*, along with colorless hyphae arranged around rock crystals (Friedmann, 1982). In some cases, an additional blue/green layer due to the presence of heterotrophic bacteria can be observed.



Figure 1.6. Sandstone colonized by cryptoendolithic communities, Linnaeus Terrace, McMurdo Dry Valleys, Southern Victoria Land (Picture by Laura Selbmann).

1.2.6 Endolithic communities in granites

Granite is an intrusive felsic igneous rock, primarily composed of feldspar, quartz, and mica. It crystallises from silica-rich magmas found kilometres deep within the Earth's crust and is subsequently brought to the surface through a series of processes. It is characterised by high hardness and low porosity, making it challenging not only for erosion by exogenous agents but also for the establishment of microbial communities. Therefore, the endolithic granite niche is unique and entirely different from sandstone, both in terms of physical and geochemical properties (Yung et al., 2014). While epilithic lichens find granite to be an optimal surface for their settlement, endolithic communities are still undersampled and understudied. The communities that best represent endolithic colonization in granite are chasmoendolithic communities. Unlike cryptoendolithic communities, which grow within airspaces present in porous sedimentary rocks to find protected niche from the external environment, chasmoendolithic communities occupy pre-existing fissures and cracks in the rock and further mechanically cracking them (Friedmann, 1982). Unlike what happens in sandstones, endolithic colonization in granites does not lead to a clear stratification of the rock; sometimes, beneath the laminar layer of the rock, it is possible to see layers, partially distinguishable from each

other, of pink, green, and black coloration due to the presence of microorganisms, which constitute the community (Yung et al., 2014; De los Ríos et al., 2005; Hall et al., 2008).

Unlike the extensively studied communities associated with sandstone, those inhabiting granite substrates have received less attention, representing an underexplored frontier in Antarctic ecology. To date, only a recent study reported that at lower elevations granite rocks often support microbial communities primarily dominated by cyanobacteria (Larsen et al., 2023).

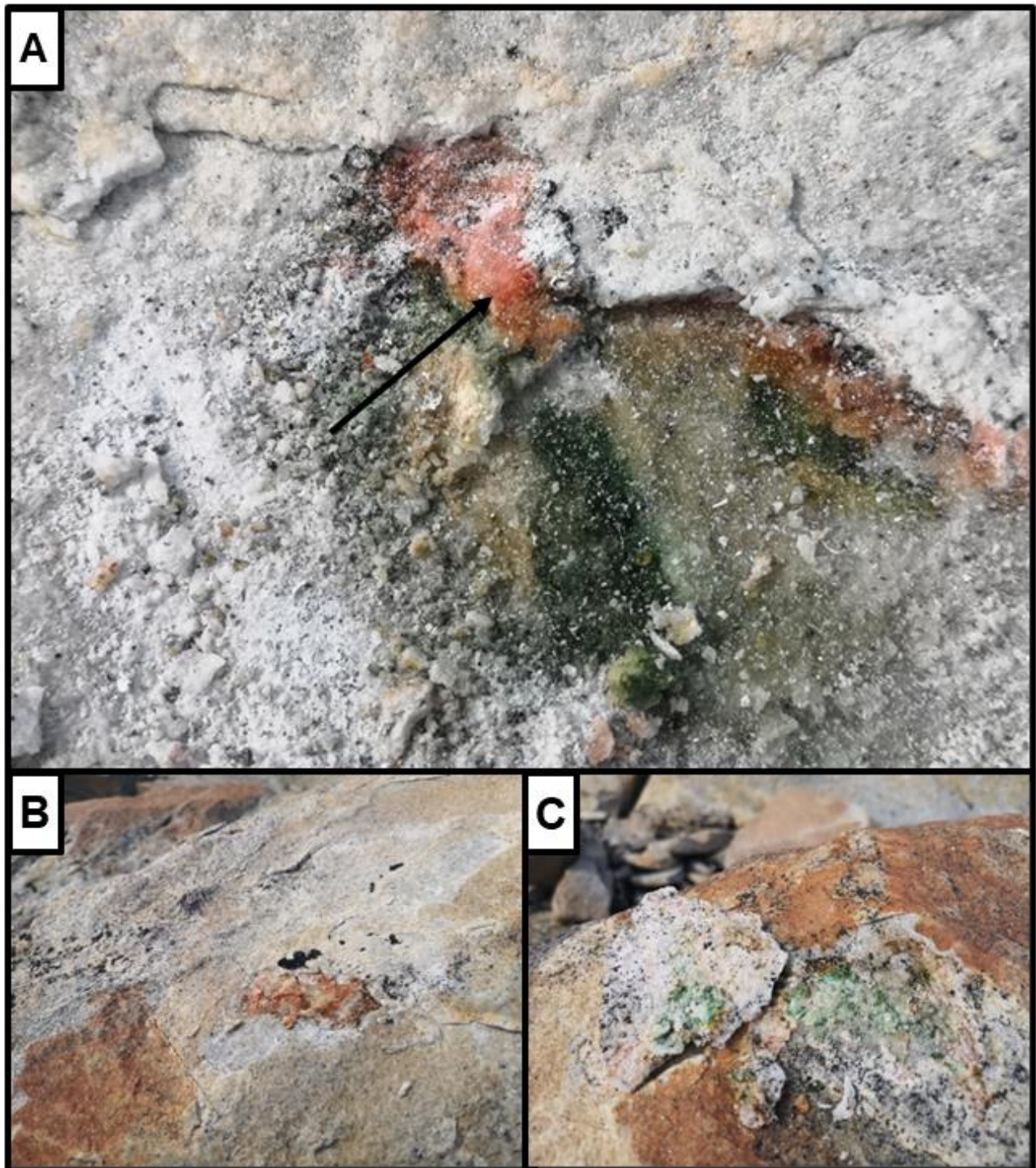


Figure 1.7 Endolithic communities associated with the granitic substrate. Starr Nunatak, Northern Victoria Land, Antarctica; A) Example of an endolithic community typical of granites where the arrow indicates the pink-orangish layer inhabited by cyanobacteria. B) The appearance of an endolithic community before excision by chisel. C) The appearance of the community immediately after excision (Pictures by Laura Selbmann).

1.4 Aim of the thesis and Chapters description

This PhD thesis is in the frame of the “THALLI” project funded by the “Italian National Program for Antarctic Research” (PNRA). By assessing the microbiota (fungi and bacteria) of 8 lichen species (5 endemic and 3 cosmopolitan), deriving from 14 different sites located in Victoria Land, East Antarctica, we aimed to: *i*) define the diversity of microbial taxa associated with the lichen symbioses in Antarctica; *ii*) identify the drivers shaping microbial assemblages in Antarctic lichens; *iii*) compare the diversity and composition between endolithic and epilithic type of ecology; *iv*) isolate, characterise and describe potential new fungal and algal taxa.

Specifically, in **Chapter 1**, we introduced Antarctica, focusing on Victoria Land's extreme climate and its selective pressure on organisms and introducing lichen symbiosis, highlighting the selected endemic and cosmopolitan lichen species (*Buellia frigida*, *Lecidea cancriformis*, *Lecanora fuscobrunnea*, *Acarospora flavocordia*, *Rusavskia elegans*, *Rhizoplaca melanophthalma*, and *Lecanora physciella*). In **Chapter 2**, we analysed the selected Antarctic lichen-associated microbial communities using amplicon-sequencing analysis of 8 lichens (5 endemic and 3 cosmopolitan) from 14 sites in Northern Victoria Land, including both bacteria and fungi. To define the fungal and bacterial diversity associated to the lichen thalli, i.e. the myco- and microbiota, respectively and verify which factor was determinant for shaping the associated assemblages, and eventually, determine the presence of a potential core of taxa that are uniquely associated either with cosmopolitan or endemic lichen species. In **Chapter 3**, we compared epilithic lichens with their ecological counterpart, the endoliths in granites collected in the same areas. For this study, we considered 8 sites, characterised by granite rock substrate, in the Northern Victoria Land. Both epilithic lichens (endemic and cosmopolitan) and complex endolithic communities dominated these sites. The aim was to concern the role of rocks and lichens as substrates in shaping of microbial assemblages associated when the epi- and endolithic colonizations co-exist in the same environment. In **Chapter 4**, we examined by culture-dependent approaches and phylogenetic analysis the Antarctic epilithic thalli of the selected lichen species to explore their potential as niches for fungal and algal species diversification. In **Chapter 5**, we provided a synthesis and conclusions of previous chapters.

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CHAPTER 2. Intrathalline fungal and bacterial diversity is uncovered in Antarctic lichen symbioses

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Abstract

Although the Antarctic continent represents one of the most hostile environments on earth, microbial life has adapted to cope with these extreme conditions. Lichens, in particular, are one of the most successful groups of organisms in Antarctica, where they serve as unique niches for microbial diversification. In the present study, we have selected eight epilithic lichen species growing in Victoria Land (i.e., three cosmopolitan and five endemic to Antarctica) to describe, in detail with amplicon sequencing approach the diversity of the associated fungal and bacterial communities. We found that the lichen mycobiota is predominantly composed of *Ascomycota* belonging to the classes *Chaetothyriomycetes* and *Dothideomycetes*, while a few key representative taxa were recognized as basidiomycetous yeasts. On the other hand, bacteria associated with lichens were represented by *Pseudomonadota*, *Cyanobacteria* and *Bacteroidota* in which psychrophilic genera were identified. The microbiota was diverse among the lichen species and their variation is driven by the lichen forming-fungus, i.e. the mycobiont, and/or by their endemic or cosmopolitan distribution. There was, in fact, a strong association of the microbial communities linked to the lichen itself, rather than to the specific characteristics of the collecting site. The lichen thallus, thus, plays an important role in microbial diversification and may potentially act as a selective biodiversity filter in which different fungal and bacterial communities thrive in it.

Keywords: biodiversity, community composition, endemism, microbiota, Victoria Land.

2.1 Introduction

Antarctica represents one of the most hostile environments on Earth (Øvstedal and Lewis Smith 2001; Selbmann et al., 2013). Here, vascular plants do not settle and are replaced by cryptogamic communities composed of mosses and, above all, lichens. Mosses and lichens spread along the coasts on rocks and soil, but lichens are the only ones able to settle even throughout the interior areas of the continent. As a result, lichens represent an important group of organisms in this extreme environment, with a high percentage of endemism (33% - 50% in the continental Antarctica): 130 endemic lichen species have been reported so far from a total Antarctic flora of 393 species (Øvstedal and Lewis Smith 2001; Castello and Nimis 1997, 2000).

Lichens are indeed notable examples of self-sustaining, long-living, symbiotic systems which derive from the mutualistic associations between biotrophic fungi (the mycobionts) and phototrophic green microalgae or cyanobacteria (the photobionts, i.e. chlorobionts and the cyanobionts, respectively; Hawksworth and Honegger, 1994). In addition, lichen thalli host a multiplicity of other microorganisms (Honegger, 2012; Hawksworth and Grube, 2020). The two main lichen symbionts have co-evolved into peculiar phenotypes –building up three-dimensional structures, i.e. the lichen thalli– and have been able to adapt to the harshest environments on Earth (Lutzoni et al., 2001; Onofri et al., 2007). Despite the ecological roles of the lichen-inhabiting fungi (i.e. the lichen mycobiota; Fernandez-Mendoza et al., 2017) and bacteria are still largely unknown, their presence has been correlated with the growth form and the ecology of the lichen hosts (Harutyunyan et al., 2008; Arnold et al., 2009; U'Ren et al., 2010, 2012, 2014; Muggia et al., 2016; Fernandez-Mendoza et al., 2017; Muggia and Grube, 2018; Smith et al., 2020; Spribille, 2018). So far, these lichen-associated microorganisms were supposed to be active players for the ecological success of lichen symbioses, also in extreme environments (Muggia and Grube, 2018; Grube et al., 2009, 2015; Grube and Berg, 2009), but still it has not been possible to report on the practical evidence of their potential roles.

To date, studies have mainly focused on not-lichenized fungi and bacteria associated to the endolithic communities, and were based on culture isolation and genetic identification of the grown strains (i.e., de la Torre et al. 2003; Selbmann et al., 2005, 2008; Egidi et al. 2014). More recently, thanks to the advent of high throughput sequencing techniques (HTS; Ji et al., 2013; Smith and Peay 2014; Tedersoo et al., 2015), a more accurate census of the viral, eukaryotic and prokaryotic assemblages of Antarctic microbial communities has been reached (de la Torre et al., 2003; Archer et al., 2017; Coleine et al. 2018a,b, 2019, 2020, 2021; Albanese et al., 2021; Mezzasoma et al., 2022; Ettinger et al., 2023). On the other hand, only two studies have focused on microbial diversity from epilithic lichens in Antarctica. These correspond to the first pioneer study from Selbmann et al. (2013), who

considered 13 species of epilithic lichens, collected in Northern and Southern Victoria Land. Later, Santiago et al. (2015) reported ascomycetes, basidiomycetes and taxa formerly recognized as zygomycetes from multiple thalli of the two endemic, macrolichen species *Usnea antarctica* and *U. aurantiaco-atra* from the Antarctic Peninsula.

To address this knowledge gap, in the present study we collected Antarctic epilithic lichens, both endemic and cosmopolitan, with the main aim to characterize by HTS their inhabiting microbial diversity comprising microfungi and bacteria. In particular, we studied five Antarctic endemic and three world wide distributed (hereon referred to as ‘cosmopolitan’) lichen species from 14 localities in the Victoria Land to: *i*) define the fungal and bacterial diversity associated to the lichen thalli ; *ii*) verify which factor is determinant for shaping the associated assemblages; *iii*) determine the presence of a potential core of taxa that are uniquely associated either with cosmopolitan or endemic lichen species.

2.2 Material and methods

2.2.1 Study area and sampling

The sampling campaign took place during the 37° Antarctic expedition November 2021- February 2022. In this campaign, 14 selected localities have been visited in the northern Victoria Land, within the south latitudinal range from the 72° to 77° parallel (Table 1). In each locality, when present and feasible to collect, at least three individuals of each selected lichen species were sampled (Table 1). There are cases in which the lichens were extremely rare, and thus less than three thalli for each occurring species were collected (Table 1). The samples were aseptically collected with the rock substrate using a geological hammer, placed into sterile plastic bags, and stored dry at -20 °C, also during the transportation to the laboratories, until downstream analyses.

Table 1. Sampling information of localities and the collected lichen species. The number of thalli collected from each site is shown in parentheses.

Locality	Coordinates	Endemic lichens	Cosmopolitan lichens
Botany Bay	77°00'00"S 162°35'00"E	<i>B. frigida</i> (6), <i>L. cancriformis</i> (6)	<i>R. elegans</i> (4)
Inexpressible Island	74°53'21.5"S 163°44'43.8"E	<i>B. frigida</i> (8), <i>L. cancriformis</i> (5)	<i>R. elegans</i> (6)
Vegetation Island	74°47'03"S 163° 39'35"E	<i>B. frigida</i> (5), <i>L. physciella</i> (3)	<i>P. chlorophanum</i> (3), <i>R. elegans</i> (6)
Prior Island	75°41'30"S 162°52'43"E	<i>B. frigida</i> (6), <i>L. cancriformis</i> (5),	
Key Island	74°04'13"S 165°18'57"E	<i>A. flavocordia</i> (12), <i>B. frigida</i> (5), <i>L. cancriformis</i> (7), <i>L. physciella</i> (7)	<i>R. elegans</i> (7), <i>R. melanophthalma</i> (1)
Starr Nunatak	75°53'54.7"S 162°35'34.4"E	<i>B. frigida</i> (11), <i>L. cancriformis</i> (15)	<i>R. elegans</i> (12)
Mt Keinath	74°55'S 164°00'E	<i>L. cancriformis</i> (1)	<i>P. chlorophanum</i> (6)
Battleship Promontory (McMurdo Dry Valleys)	76°54'S 160°50'00.0"E	<i>L. fuscobrunnea</i> (7)	
Edmonson Point	74°19,902'S 165°08,223'E		<i>R. elegans</i> (6)

Random Hills site 2	74°0.1'0.15''S 164°21'23.4''E	<i>A. flavocordia</i> (8), <i>B. frigida</i> (4), <i>L. cancriformis</i> (6)	<i>R. elegans</i> (5),
Random Hills site 3	74°0.0'10.9''S 164°28'56.4''E	<i>A. flavocordia</i> (1), <i>B. frigida</i> (6)	<i>P. chlorophanum</i> (7), <i>R. elegans</i> (6), <i>R. melanophthalma</i> (2)
Random Hills site 4	74°0.1'42.2''S 164°44'44.5''E	<i>A. flavocordia</i> (11), <i>B. frigida</i> (5), <i>L. physciella</i> (8)	<i>R. elegans</i> (6), <i>R. melanophthalma</i> (21)
Random Hills site 5	74°0.4'33.4''S 164°48'32.5''E	<i>A. flavocordia</i> (8), <i>L. physciella</i> (15)	<i>R. melanophthalma</i> (3)
Pudding Butte	75°51'30.2"S 159°58'25.7" E	<i>L. fuscobrunnea</i> (2)	
Red Castle Ridge	72°26'00''S 169°57'00''E	<i>B. frigida</i> (5), <i>L. cancriformis</i> (5)	<i>R. elegans</i> (7)
Campo Icaro	74°42'33.5"S 164°05'45.9"E	<i>B. frigida</i> (6), <i>L. cancriformis</i> (6)	
Boulder Clay	74°44'6.8''S 164°02'22.50''E	<i>L. fuscobrunnea</i> (1)	<i>P. chlorophanum</i> (16)

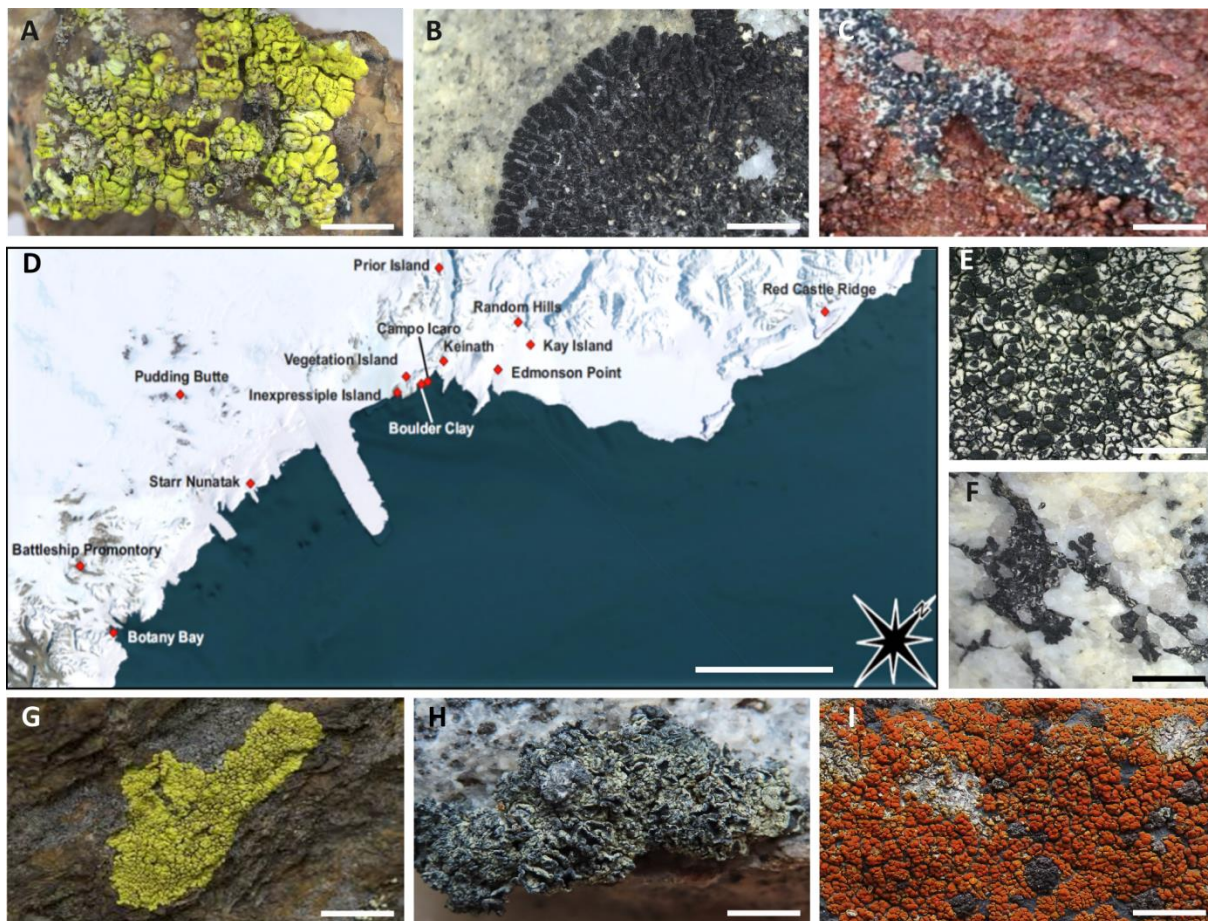


Figure 2.1. Lichen species and sampling area. **A)** *A. flavocordia*; **B)** *B. frigida*; **C)** *L. fuscobrunnea*, **D)** collecting sites in Victoria Land (Antarctica) visited for the sampling of the eight lichen species here analyzed; **E)** *L. physciella*; **F)** *L. cancriformis*; **G)** *P. chlorophanum*; **H)** *R. melanophthalma*; **I)** *R. elegans*. Scale bars: A-C, E-H) 1cm; I) 2 cm.

The collected and analyzed lichen species are all chlorolichens (i.e., forming symbiosis with green trebouxoid photobiont): the endemic Antarctic *Acarospora flavocordia*, *Buellia frigida*, *Lecanora fuscobrunnea*, *Lecanora physciella*, *Lecidea cancriformis*, and the cosmopolitan *Pleopsidium chlorophanum*, *Rhizoplaca melanophthalma*, *Rusavskia elegans*. The lichen species were chosen based on literature knowledge and their availability at the specific sampling sites.

2.2.2 DNA extraction, amplification and sequencing of fungal and bacterial communities

For the amplicon sequencing analyses, up to three thalli of each species from each locality were used. The amplicon sequencing analysis was performed on a total of 137 environmental samples belonging to the eight lichen species (*A. flavocordia*, *B. frigida*, *L. fuscobrunnea*, *L. physciella*, *L. cancriformis*,

P. chlorophanum, *R. melanophthalma*, *R. elegans*; Table 1). Metagenomic DNA extraction was performed on a 0.5 cm² fragment of the thallus, devoid of any symptoms of external infection or damage, which was removed from the substrate using a sterile razor blade and transferred into a 1.5 ml reaction tube. The fragments underwent a series of washes: they were rinsed three times for 15 min with sterile water, followed by a 30 min cleaning step using a 2% Tween 80 solution. A final wash was performed for 15 min with sterile water. The DNA extraction from the cleaned fragments followed the CTAB protocol of Cubero et al. (1999), with minor adjustments. Potential contaminants during the DNA extraction process were checked by establishing negative control samples for each lichen species. These negative controls consisted of a 1.5 ml tube kept open during the whole extraction process and processed in the same way as the lichen samples for the amplification and sequencing steps.

To study the diversity of fungal communities, the nuclear ribosomal internal transcribed spacer 1 region (ITS1) was targeted. The ITS1 region was amplified using the barcoded primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC; White et al., 1990; Smith and Peay, 2014). The PCR amplification protocol for the ITS1 fragments was run with the following conditions: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 sec, annealing at 55 °C for 45 sec, extension at 72 °C for 1 min, and a final extension step at 72 °C for 5 min.

The V4 variable region of the small ribosomal subunit 16S rDNA was used to assess bacterial diversity. This variable region was amplified using the primers F515 (GTGCCAGCMGCCGCGGTAA) and R806 (GGACTACHVGGGTWTCTAAT) as described by Caporaso et al. (2012). The PCR amplification of the V4 variable region was performed with the following protocol: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 sec, annealing at 50 °C for 1 min, extension at 72 °C for 90 sec, and a final extension step at 72 °C for 10 mins.

Samples were then sequenced in pair ends (2x300 bp) using the Illumina MiSeq platform at the Edmund Mach Foundation (San Michele all'Adige, Italy).

2.2.3 Bioinformatics analyses

The samples were processed after checking for extraction contamination with Decontam (Davis et al., 2018). Raw reads were analyzed using the Amplicon ToolKit (AMPTk) for Next Generation

Sequence (NGS) v1.2.1 (Palmer et al., 2018), USEARCH (Edgar, 2010) and VSEARCH (Rognes et al., 2016). The reads were trimmed, resulting in sequences with a length of 250 bp; reads shorter than 100 bp were discarded, and chimera removal was performed using USEARCH v. 9.2.64 with default parameters. Sequence quality filtering was performed with the expected error parameter of <1.0 (Palmer et al., 2018). The dataset was clustered with DADA2 v1.6.0 using a 99% identity parameter to generate the Amplicon Sequence Variants (ASVs). Further filtering was performed in which rare ASVs (i.e. ASVs having less than five reads) and singletons were discarded and not considered for the subsequent analyses. The taxonomy assignment was performed via the UNITE and RDP (Cole et al., 2014) database, which uses the hybrid SYNTAX algorithm (Abarenkov et al., 2020; Edgar, 2010), the sequences were aligned and the taxonomy was assigned to the corresponding ASVs of the ITS and 16S.

2.2.4 Statistical analyses

To test the effectiveness of the sampling effort of the overall lichen-associated fungal and bacterial diversity in the studied area, species accumulation curves were calculated using *specaccum* function in the ‘vegan’ package (Oksanen et al., 2013).

Venn diagrams were constructed to show the number of ASVs shared by and characterizing both endemic and cosmopolitan lichen species. The Linear discriminant Effect Size (LEfSe) analysis was performed to identify those taxa that explain the differences between endemic and cosmopolitan lichens myco- and microbiota.

To explore fungal and bacteria diversity, the analyses were done by clustering the ASVs by lichen mycobiont species (eight species) and by their ecological distribution (i.e. Antarctic endemic vs. cosmopolitan). Alpha diversity was calculated with the Chao1 index, beta diversity was calculated using the Bray Curtis dissimilarity index, and PCoA analyses were performed and tested with PERMANOVA. All analyses were performed using R packages *microeco* (Liu et al., 2021) and *phyloseq* (McMurdie et al., 2013). Kruskal–Wallis and Wilcoxon tests (Kruskal and Wallis, 1952; Wilcoxon, 1945) were used to assess significant diversity in differences among lichen species, and between groups with different ecological distributions.

2.3 Results

2.3.1 Taxonomic composition of myco- and microbiota

The outcomes of sequencing returned 9,920,387 reads for fungi and 17,300,152 for bacteria, which were clustered at 99% identity and collapsed into 1,681 and 27,341 ASVs, respectively. In order to specifically investigate the lichen mycobiota, a further filtering was applied: all ASVs matching any lichen-forming fungi (i.e., any lichen mycobionts known) were excluded from the analysis (Table S1). This resulted in a final dataset that exclusively comprised fungal taxa classified as not lichen-forming fungi. The complete datasets for fungi and bacteria were finally obtained by removing chimeras, singletons, contaminants and archaeal sequences, resulting in a total of 286 fungal and 14,908 bacterial ASVs.

The species accumulation curves gradually leveled off at the end of the sampling, indicating that the number of samples analyzed approaches an adequate number to encompass the biodiversity of the studied communities, both for bacteria and fungi.

The community analyses (Figure 2.2) of the mycobiota, revealed *Ascomycota* and *Basidiomycota* as the dominant phyla (Figure 2.2A). In six (e.g., *A. flavocordia*, *B. frigida*, *L. physciella*, *L. cancriformis*, *R. melanophthalma*, *R. elegans*) out of the eight lichen species the phylum *Ascomycota* constituted the majority of ASVs, whereas the phylum *Basidiomycota* was predominant in thalli of *L. fuscobrunnea* and *P. chlorophanum*. In all examined lichen species the mycobiota clustered at the genus level showed a large number of ASVs of unassigned taxonomy. Based on relative abundances (Figure 2.2C), it emerged that the most represented genera were *Knufia* in *A. flavocordia* (37,26%), *B. frigida* (9,5%), *L. cancriformis* (26,71%), *R. elegans* (45,54%), and *Tremella* in *L. fuscobrunnea* (33,21%), *P. chlorophanum* (51,6%), *R. melanophthalma* (13%).

The analyses of the bacterial communities revealed *Pseudomonadota*, *Cyanobacteria* and *Bacteroidota* as the dominant phyla in all the lichen species (Figure 2.2B). Looking at the relative abundance of bacteria at the genus level (Figure 2.2D), we found that most of the genera were undefined. The most abundant genera for each species were: *Hymenobacter* in *R. elegans* (9,04%), *B. frigida* (8,44%), *L. cancriformis* (7,48%); *Acidisoma* in *R. melanophthalma* (6,52%) and *L. physciella* (1,1%); *Armatomonas* in *L. fuscobrunnea* (4,65%) and *A. flavocordia* (0,7%); *Mucilaginibacter* in *P. chlorophanum* (2,73%).

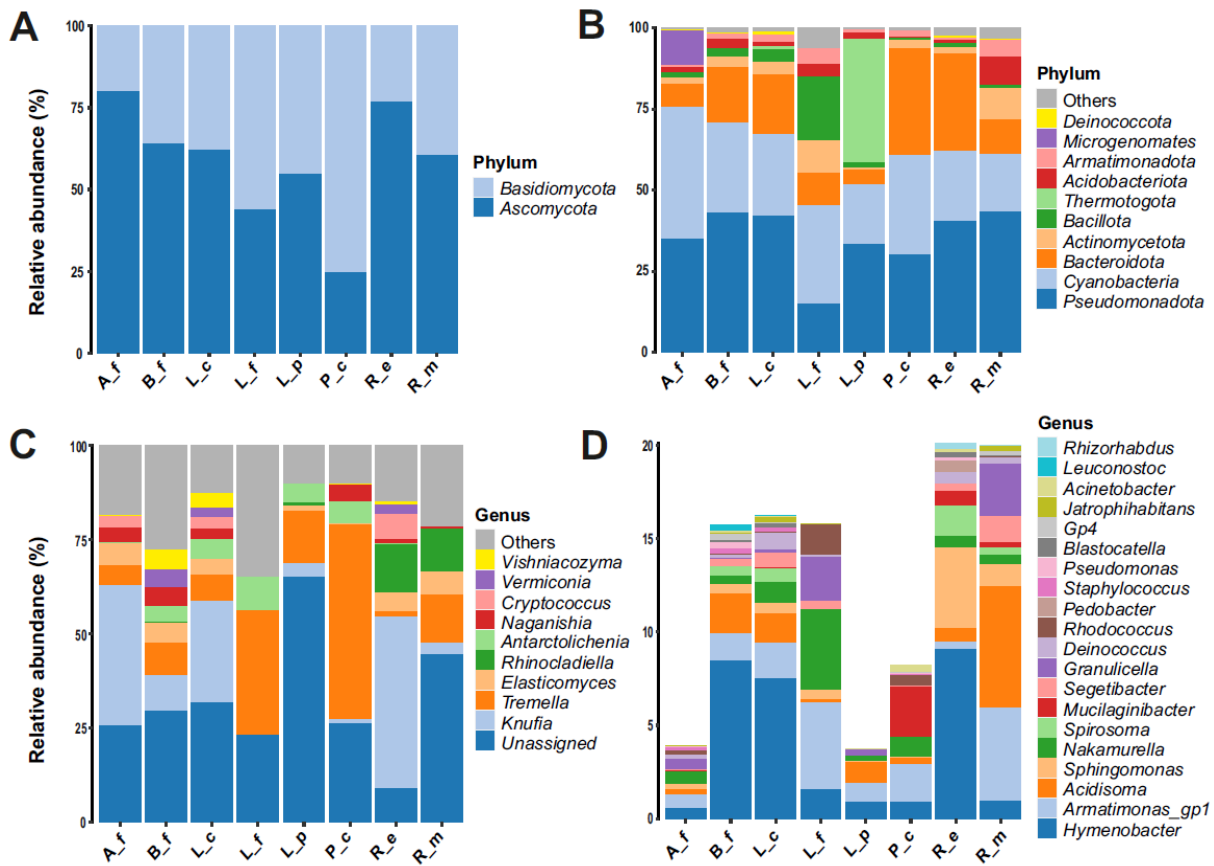


Figure 2.2. Relative abundance plots of fungi (**A, C**) and bacteria (**B, D**) reported for each lichen species. Lichen species names were abbreviated as follow: *Acarospora flavocordia* (A_f), *Buellia frigida* (B_f), *Lecidea cancriformis* (L_c), *Lecanora fuscobrunnea* (L_f), *Lecanora physciella* (L_p), *Pleopsidium chlorophanum* (P_c), *Rusavskia elegans* (R_e), *Rhizoplaca melanophthalma* (R_m). Abundance data are all shown in percentage; D panel is up to 20% because unidentified genera are not shown.

2.3.2 Alpha diversity analyses

The Chao1 indexes for fungal and bacterial diversity are shown in Figure 3. Mycobiota of *A. flavocordia* (Chao1 = 40.1) and *B. frigida* (Chao1 = 37.9) presented the highest Chao1 values among the lichen species, while *L. fuscobrunnea* (Chao1 = 6.33) displayed the lowest value (Figure 2.3A). The Kruskal-Wallis Dunn test, conducted for multiple comparisons within lichen species, revealed significant statistical differences between *L. fuscobrunnea* and *A. flavocordia*, *B. frigida*, and *L. cancriformis* (p-value < 0.001). Moreover, statistical differences were observed between *P. chlorophanum* and *A. flavocordia* (p-value < 0.05), *B. frigida* (p-value < 0.01), and *L. cancriformis*

(p-value < 0.05). *R. elegans* displayed statistical differences with *A. flavocordia* (p-value < 0.05), *B. frigida* (p-value < 0.01), and *L. cancriformis* (p-value < 0.05).

Looking at the bacterial assemblages, *L. cancriformis* and *B. frigida* displayed the highest Chao1 values (1,736 and 1,594, respectively), while *L. fuscobrunnea* (Chao1 = 833) the lowest (Figure 2.3B). *L. cancriformis* and *B. frigida* were the only two lichens that displayed statistical differences against the other species. *L. cancriformis* showed statistical differences (p-value < 0.001) with *A. flavocordia*, *P. chlorophanum* and *L. fuscobrunnea*; with *R. elegans* (p-value < 0.01), and with *L. physciella* (p-value < 0.05). *B. frigida* shows statistical differences (p-value < 0.001) with *A. flavocordia* and *P. chlorophanum*, with *L. fuscobrunnea* (p-value < 0.01), and with *L. physciella* (p-value < 0.05; Figure 2.3B).

2.3.3 Beta diversity analyses

The Bray-Curtis (incidence and relative abundance data) distance was used to assess the beta diversity and the differences in fungal and bacterial assemblages among lichen species. The results were presented through PCoA plots along with PERMANOVA statistical analysis. The effects of the lichen mycobiont species and ecological distribution (i.e., endemic vs. cosmopolitan) were found to be significant (p-value < 0.001), although the ecological distribution seemed to have a lesser impact on the community ordination than that of the lichen mycobiont (Figure 2.3C, 2.3D).

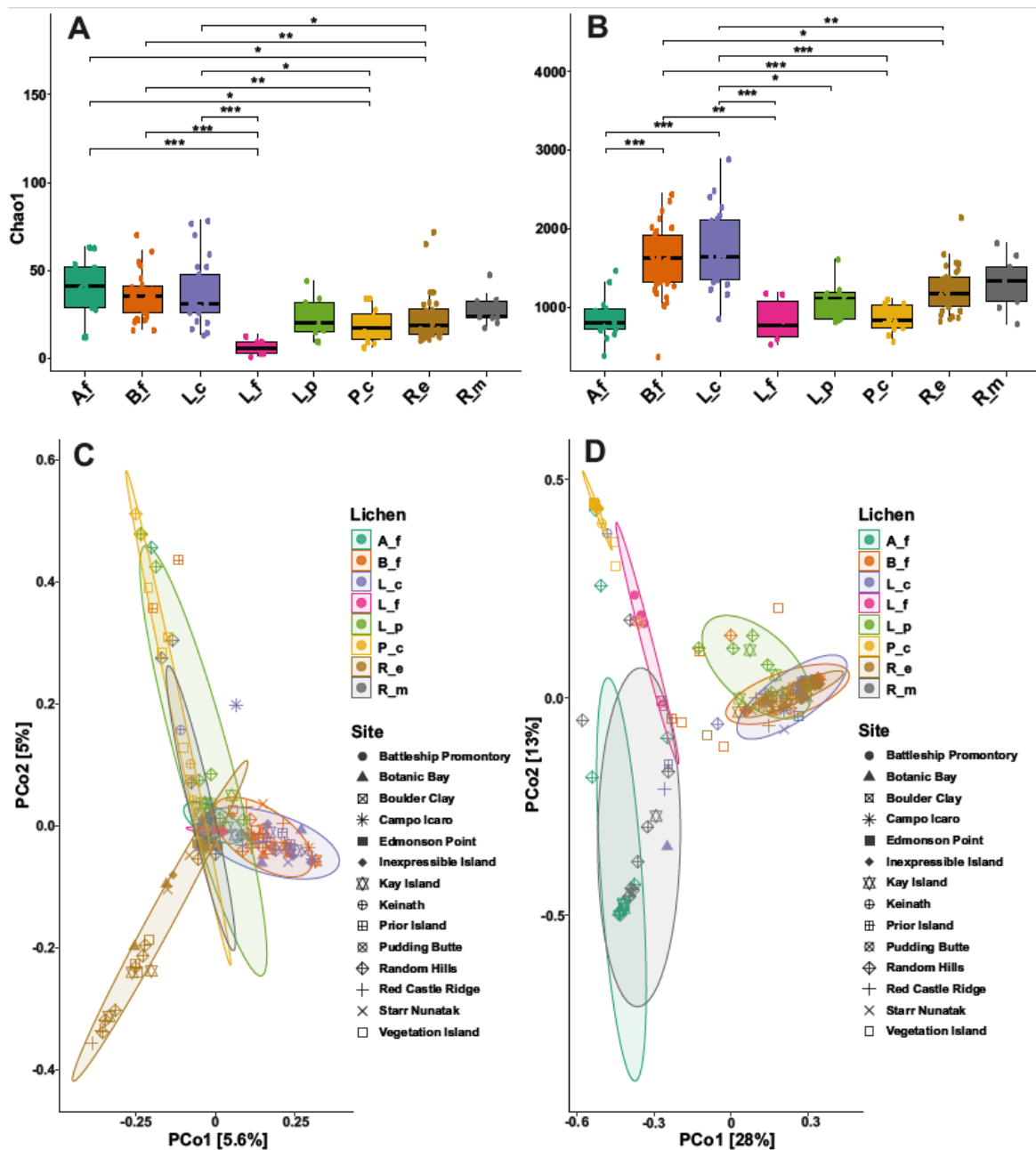


Figure 2.3. A, B) Alpha diversity and Chao1 index plots of fungal (A) and bacteria (B) communities. Statistical differences between groups, tested by Kruskal-Wallis test were marked with *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. C, D) Beta diversity and principal component analysis (PCoA) based on Bray-Curtis distance including ellipse of 95% confidence interval of fungal (C) and bacteria (D) communities. On the axes and legends lichen species names were abbreviated as follow: *Acarospora flavocordia* (A_f), *Buellia frigida* (B_f), *Lecidea cancriformis* (L_c), *Lecanora fuscobrunnea* (L_f), *Lecanora physciella* (L_p), *Pleopsidium chlorophanum* (P_c), *Rusavskia elegans* (R_e), *Rhizoplaca melanophthalma* (R_m).

2.3.4 Cosmopolitan vs. endemic lichen species

When the lichen species were grouped according to their endemic and cosmopolitan distribution, no significant differences in the relative abundances of fungal phyla were found. For fungi, in both

endemic and cosmopolitan lichens the phylum *Ascomycota* was the dominant one (63.33% and 61.6%, respectively), followed by *Basidiomycota* (36.64% in endemic and 38.39% in cosmopolitan). Other phyla such as *Blastocladiomycota*, *Mortierellomycota* and *Zoopagomycota*, were present with an abundance lower than 1% (Figure 2.4A). The genera *Vishniacozyma*, *Meristemomyces*, *Ramularia*, and *Friedmanniomyces* were more abundant in endemic lichens, while *Cystobasidium*, *Rhinocladiella*, *Dothiorella*, and *Cryptococcus* were more abundant in the cosmopolitan lichens (Figure 2.4C).

Concerning bacteria, the most abundant phyla were *Pseudomonadota*, *Cyanobacteria* and *Bacteroidota*. Specifically, *Pseudomonadota* was the main represented phylum in both endemic (38.43%) and cosmopolitan (38.37%) species. *Cyanobacteria* instead was the second most abundant phylum in endemic lichens (28.35%) and the third in cosmopolitan (23.18%), while *Bacteroidota* was the second most abundant phylum in cosmopolitan (27.09%) and the third in endemic (14.1%) lichens (Figure 2.4B). Looking at the genus level, most of the dataset is represented by unidentified genera in both the endemic (85%) and cosmopolitan lichens (80%). Instead, among the few identified genera, the most abundant were *Hymenobacter* (5.66%), *Armatimonas* (1.67%), *Acidisoma* (1.41%) and *Spirosoma* (1.03%) in endemic lichens, and *Hymenobacter* (5.69%), *Sphingomonas* (2.75%), *Acidisoma* (1.67%), *Armatimonas* (1.58%) and *Mucilaginibacter* (1.16%) in cosmopolitan lichens. All the other genera were present as less than 1% (Figure 2.4D).

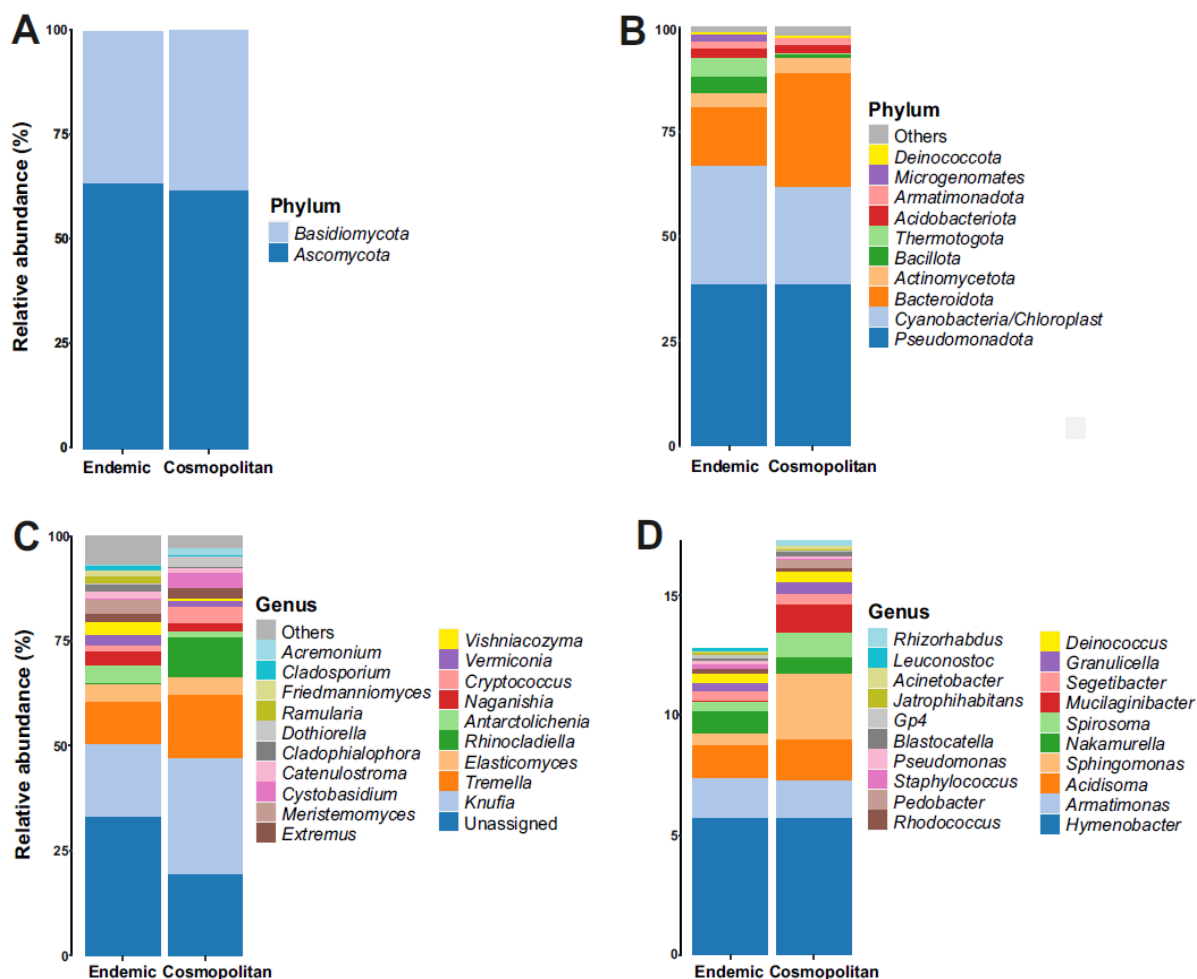


Figure 2.4. Relative abundance plots according to lichen distribution, i.e. endemic vs cosmopolitan species: composition of phyla (A) and genera (C) of fungi; composition of phyla (B) and genera (D) of bacteria.

2.3.5 Cosmopolitan vs endemic diversity

The Chao1 index indicated that the fungal and bacterial diversity values were higher for the endemic than for the cosmopolitan lichens (p-value < 0.001; Figure 2.5A). The Wilcoxon test conducted on the analysis indicated a significant difference in alpha diversity between the two lichen groups only regarding the bacterial diversity (p-value < 0.05; Figure 2.5D).

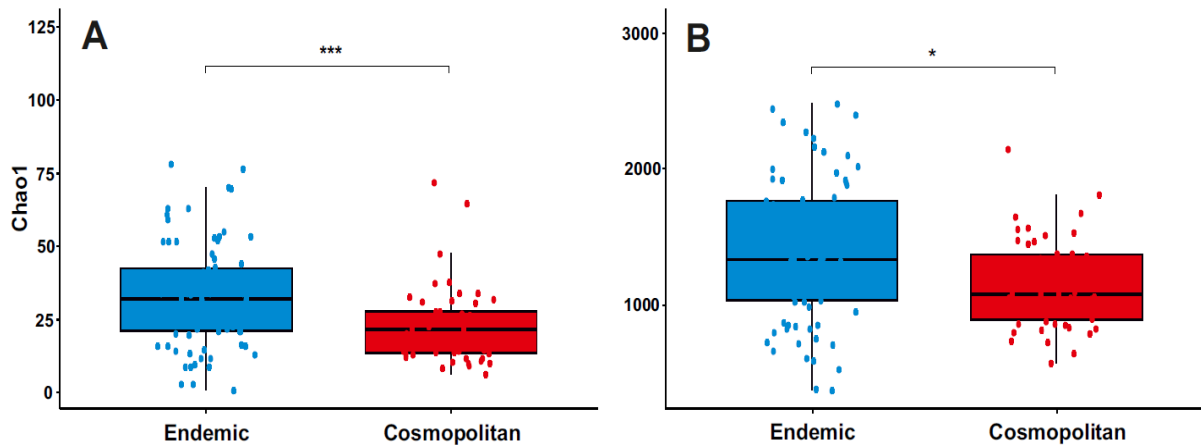


Figure 2.5. Comparison of alpha diversity using Chao1 index of fungal (A) and bacterial (B) diversity between endemic and cosmopolitan lichens. The statistic support was tested by Wilcoxon test and is indicated as *** $p < 0.001$, * $p < 0.05$.

2.3.6 Shared ASVs and species indicator analysis

The Venn diagrams (Figure 2.6A, 2.6B) visually presented the fungal and bacterial ASVs that characterized the endemic and cosmopolitan lichens. The shared core of the mycobiota was composed of 137 fungal ASVs, which accounted for 48.9% of the total species composition identified. These ASVs corresponded to 54 genera, with *Knufia*, *Tremella*, and *Naganisha* being the most abundant ones. One-hundred-six (37.9%) ASVs were exclusively found in endemic lichens, representing 33 genera; among these, *Friedmanniomyces*, *Exophiala*, *Ramularia*, and *Meyerozyma* were the four most abundant genera. Cosmopolitan lichens had 37 unique ASVs, belonging to seven genera, which constituted 13.2% of the total composition.

The shared microbiota (Figure 6B), comprised 5,650 ASVs, accounting for 37.9 % of the total abundance of the dataset. Yet, 6,989 ASVs were exclusive of endemic lichens, accounting for 46.9 % of the abundance of the total dataset; on the other hand, 2,269 ASVs were found peculiar for the cosmopolitan lichens, accounting for 15.2 % of the total abundance.

The species indicator analyses uncovered genera and species that displayed a strong association with specific lichen species and ecological distributions. A statistically significant association was found between *A. flavocordia* and four ASVs belonging to the genera *Cladophialophora*, *Knufia*, and the family *Tremellaceae*. *L. fuscobrunnea* and *P. chlorophanum* were each associated with one ASV, which belonged to the genera *Aspergillus* and *Tremella*, respectively. Additionally, *R. melanophthalma* and *R. elegans* were statistically associated with two ASVs, corresponding to the genera *Dothiorella* and *Knufia*, respectively. Regarding representative taxa in the ecological distribution cluster, significant statistical associations were observed between cosmopolitan lichens

and 13 ASVs found in the genera *Knufia*, *Rhinochadiella*, *Tremella*, *Extremus*, *Cystobasidium*, *Aspergillus*, and *Pseudoeurotium*. Endemic lichens, on the other hand, had 22 ASVs belonging to 13 genera, with the most statistically significant ones being *Vishniacozyma*, *Knufia*, *Antarctolichenia*, *Friedmanniomyces*, *Thelebolus*, *Ramularia*, *Cystobasidium*, *Tilletiopsis* and *Acremonium*.

The effect size of the separation between the endemic and cosmopolitan fungal and bacterial diversity analyses showed taxa with the highest discriminatory power between the endemic and cosmopolitan groups of lichen species. The statistical significant results (LDA >2, p.value <0,001). The fungal taxa with higher segregative values were the members of the class *Dothideomycetes*, the genus *Rhinochadiella*, the order *Capnodiales*, and the family *Herpotrichiellaceae* (Figure 2.6C). Regarding the bacterial diversity, the most notable were represented by the phyla *Bacillota* and *Bacteroidota*, and the *Clostridia* and *Flavobacteria* classes (Figure 2.6D).

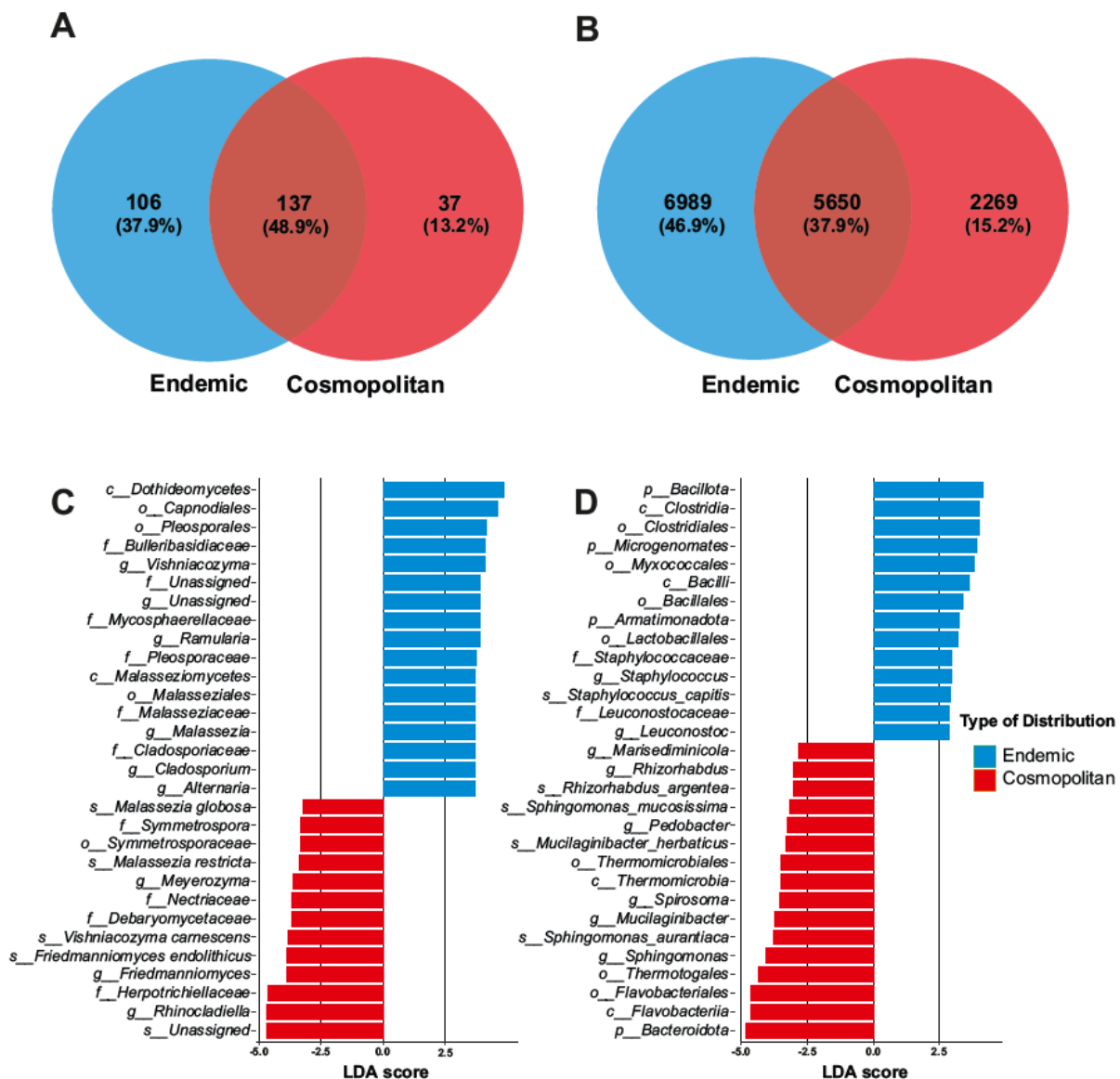


Figure 2.6. Shared ASVs detected by the LefSe analysis. **A, B**) Venn diagrams showing the shared number of ASVs between endemic and cosmopolitan lichens for the fungal **(A)** and bacterial **(B)** diversity. **C, D**) LefSe between endemic and cosmopolitan lichens relative to the fungal **(C)** and bacterial diversity **(D)**. The 30 taxa with the highest LDA score are shown.

2.4 Discussion

In the present study, we have uncovered the diversity of fungi and bacteria associated within thalli of eight lichen species growing in the Victoria Land, which themselves represent peculiar microniches and may act as cradles for the diversification of microbial life (Selbman et al., 2008; Arnold et al., 2009; Santiago et al., 2015). While substantial progress has been made in investigating the diversity of fungi and bacteria inhabiting lichen thalli at boreal and arctic latitudes (Cardinale et. al., 2006; Liba et al., 2006; Selbmann et al., 2010; Lee et al., 2014; Grube et al., 2009; Hodkinson and Lutzoni 2009; Bates et. al., 2011; Spiribille et al., 2020; Timling et al., 2014; Klarenberg et al., 2020), so far there have been fewer possibilities to analyze the intrathalline diversity of lichens from Antarctica (Grimm et al., 2021).

2.4.1 The lichen mycobiota: composition and diversity

In general, the lichen-associated fungal diversity in the investigated species predominantly comprised *Ascomycota* taxa, and these results are in line with previous studies which have either analyzed lichen-dominated endolithic communities (Selbmann et al., 2005, 2008; Egidi et al., 2014, Coleine et al., 2018a), or fruticose lichens growing on soils with different deglaciation times (Beck et al., 2023), as well as the diversity of moss-associated fungi in the ice-free coastal outcrops in Antarctica (Cox et al., 2016; Ji et al., 2016; Yung et al., 2014; Hirose et al., 2016). The prevalence of *Chaetothyriomycetes* and *Dothideomycetes* is another important outcome that supports the frequent occurrence of these groups of fungi in epilithic lichens form dry and cold habitats (Muggia et al., 2016; Muggia and Gube 2018; Cometto et al., 2023, 2024), and as common representatives in lichen-dominated endolithic communities (Selbmann et al., 2005, 2008; Egidi et al., 2014). *Knufia* (*Chaetothyriales*) is reported here as the most abundant genus, characterized by its capacity to adapt to different ecologies, such as rock-inhabiting fungus in extremophilic conditions (Isola et al., 2016) or as a lichen-associated taxon (Untereiner et al., 2011; Cometto et al., 2023).

The ASVs analysis at the genus level revealed the presence of a recently described fungal species, *Antarctolichenia onofrii*, which was originally isolated multiple times from Antarctic rocks as a rock-

dwelling fungus (Muggia et al., 2021). This species is a meristematic melanized fungus and was shown to be a peculiar representative of the Antarctic mycobiota; it also emerges here as part of the lichen mycobiota in Antarctica, as it has never been found elsewhere (i.e., from rock or lichen thalli from other geographic origins). *Antarctolichenia onofrii*, albeit clearly free-living, still maintains a loose relation to and interdependence with algae, being unable to propagate in axenic culture for a long time; its constant occurrence in nature with lichens (both epilithic and endolithic) support the hypothesis for a possible bridge between free living (rock associated) and symbiotic life-style.

Our findings, however, also unveil a significant presence of *Basidiomycota* in all examined thalli, even though their abundances were in general much lower than *Ascomycota*. Notably, only in the case of the two lichens *P. chlorophanum* and *L. fuscobrunnea*, a higher abundance of *Basidiomycota* was detected. *Basidiomycota* were found to be particularly abundant in the Antarctic region only in two studies, conducted in the waters of the Antarctic Peninsula (Garmendia et al., 2021) and in snow-ice near the Italian-French station Concordia, respectively (Stoppiello et al., 2023). In lichens from other regions in the world, *Basidiomycota* have also been shown to be quite ubiquitous but in much lower amounts than *Ascomycota*, and rarely showed specificity for the host lichen (Smith et al., 2020; Lendemer et al., 2019; Cometto et al., 2022). Three most abundant basidiomycetous yeast genera were recovered in both the Antarctic endemic and the cosmopolitan lichens, i.e., *Tremella*, *Vishniacozyma* and *Naganishia*. These three genera are known to be extremophilic yeasts (Buzzini et al., 2018), which are even able to develop pathogenicity toward humans (Rush et al., 2023; Nizovoy et al., 2021). Environmental strains of *Tremella*, *Vishniacozyma* and *Naganishia* were found also in the two cosmopolitan lichens *T. atra* and *R. melanophthalma* collected across the other six continents (Cometto et al., 2022, 2024); however, only *R. melanophthalma* was included in the present study, as it was found in the sampling localities. The genus *Tremella* emerges here as it is a well-known lichen parasite (Millanes et al., 2011), which has been studied because of its phenotypic traits on many host lichen thalli (Millanes et al., 2012, 2014, 2015). Here we present an additional case of its occurrence as cryptic species within the thalli, as no symptoms of its infection could be found on any of the analyzed lichen specimens. Cometto et al. (2022, 2024) already reported *Tremella* as cryptically present in lichens and succeeded in isolating it in culture (Cometto et al. 2022), as well as we did (De Carolis et al., in preparation). The high abundance of basidiomycetous yeasts compared to ascomycetous yeasts in Antarctic lichens could be due to the different organization of the yeast cells. Indeed, the thicker cell wall and presence of capsules in basidiomycetous yeasts may represent an adaptive advantage in the Antarctic environment (Garmendia et al., 2021; Stoppiello et al., 2023), as well as their tendency to reside in the lichen thalli as resting cells/spores.

Eventually species diversity analysis performed between the mycobiota of endemic and cosmopolitan lichens revealed that a few fungal genera that are endemic to Antarctica are also elements of only the endemic lichen mycobiota. One example is *Friedmanniomyces*, which has been frequently reported as a component of endolithic communities (Onofri et al., 1999).

2.4.2 The lichen-associated bacterial communities: composition and diversity

Pseudomonadota, *Cyanobacteria* and *Bacteroidota* were recovered as the most abundant bacterial phyla. *Pseudomonadota* is in general widely distributed in different habitats on Earth (Nemergut et al., 2011) and in bioaerosols in the atmosphere (Fang et al., 2020 and Tang et al., 2018), while *Bacteroidota* were also already found as predominant phyla among bacteria living in Antarctic lakes (Michaud et al., 2012). According to previous studies, it was found that heterotrophic *Pseudomonadota* and *Bacteroidota* coexist with Antarctic cyanobacteria (Cornet et al., 2018), in particular in glaciers and cold deserts in Antarctica, e.g. in the King George and James Ross Islands, in the Arctic (Alaska and Greenland) and in Asia (Himalaya Mountains, Pamir, Then-Shan and Qilian; Segawa et al., 2017). *Cyanobacteria* was the second most abundant phylum, likely for their ability to survive in extreme climatic conditions thanks to their molecular adaptations (Vincent, 2007). Some cyanobacteria are known for their ability to fix carbon, molecular nitrogen and to mobilize inorganic phosphate from organic phosphates (Pandey et al., 2004). In addition, they are the only known prokaryotes capable of performing oxygenic photosynthesis, even at extreme temperatures, often associated with aerobic nitrogen fixation. To adapt to low temperatures, cyanobacteria accumulate polyunsaturated fatty acids and short-chain fatty acids (D'Amico et al., 2006; Zakhia et al., 2008). These modifications allow them to maintain an active metabolism even at temperatures below -20 °C (Psenner and Sattler, 1998). Moreover, in the cold soils and rocks of Antarctica it has been observed that trace gas oxidation can produce sufficient energy not only to sustain persistence, but also to fuel the Calvin-Benson-Bassham cycle (CBB) in a subset of resident bacterial taxa through the process of atmospheric chemosynthesis (Williams et al., 2024; Coleine et al., 2024; Ray et al., 2022; Ji et al., 2017).

The most frequently isolated genera from East Antarctica, but also in the present research, include *Hymenobacter*, *Acidisoma*, *Armatimona/ Armatimonadetes* and *Sphingomonas* (Lambrechts et al., 2019; Peeters et al., 2011; Benaud et al., 2022; Pudasaini et al., 2017). These bacteria are all presented as chemoheterotrophic, strictly aerobic and UV-resistant. These findings are consistent with their ability to survive the harsh environmental conditions of the region. In particular, members belonging

to *Acidisoma* are among the most abundant, as also evidenced by previous studies of endolithic communities (Coleine et. al., 2019). This genus has rarely been observed in the ice-free areas of Victoria Land, but is common in cold regions such as the Alps (Nakai et al., 2013), suggesting that taxa belonging to this group may have evolved unique adaptation for cold resistance. *Acidisoma* species are acidophilic and psychrotolerant heterotrophic bacteria. They can use a wide range of organic substrates, particularly carbohydrates and amino acids (Belova et al., 2009). The predominant genus analyzed was *Hymenobacter*, which is found in several Arctic coastal glaciers. Glaciers, indeed, have been identified as a possible vehicle for bacterial transport to downstream marine waters. Several taxa belonging to this group have been retrieved in the McMurdo Dry Valleys in Antarctica (Hirsch et al., 1998). In addition, it has also been identified in coastal ice samples in southern Shetland (García-Lopez et al., 2021). These locations are all oligotrophic, which could suggest that the genus *Hymenobacter* owns the genetic setup to grow using a limited number of carbon sources, such as some sugars, sugar alcohols, organic acids, and some amino acids (Marizcurrena et al., 2019). On the other hand, the chemoheterotrophic genus *Armatimonas* has not been reported in Antarctic areas but has been found in alpine glaciers (Tolotti, 2020). These bacteria are members of the phylum *Armatimonadetes*, which is still poorly studied. Many of the *Armatimonas* bacteria have a wide range of enzymes that can be activated at different temperatures, allowing them to use the available resources (Tamaki et al., 2011).

A taxonomic peculiarity concerns the phylum *Thermotogota*, which comprises chemoorganotrophic bacteria capable of metabolizing sugars and various polymers. They are hyperthermophilic and are found in extremely hot environments. In the course of this study, they were found in large quantities for the first time in a thallus of *L. physciella*, thus emerging as a significant difference of the composition of the microbial communities of the analyzed lichen dataset. Earlier studies have found *Thermotogota* in the saline minerals of the glacier in the Boulder Clay area, a few kilometers from the Italian Mario Zucchelli station in Victoria Land further North (Azzaro et al., 2022).

2.4.3 Lichen symbioses select their own myco- and bacterial diversity

The low Chao1 values for the mycobiota approached those found in the mycobiota of two lichen species collected worldwide in extreme, high altitudinal environments (*R. melanophthalma* and *Tephromela atra*) by Cometto et al. (2024). On the other hand, Chao1 values were relatively high for the bacteria and showed extremely significant differences in the composition of the communities (e.g. Hodkinson et. al., 2012; Bates et. al., 2011; Cardinale et. al., 2006) suggesting that a considerable

proportion of the lichen-associated bacteria are specific for the lichen species. Indeed, the statistically significant differences that we found between the lichen myco- and bacterial diversity among the analyzed species, suggest that the lichen itself influences most the diversity associated with the thalli. This is supported further by the PCo A analysis concerning beta-diversity, in which the mycobiont species most explained the community variation. Instead, the 'site' factor contributed less to the overall variation in the fungal and bacterial community, contrasting the results reported previously by Coleine et. al. (2019). This observation may be explained by the fact that in the lichen symbioses the mycobionts (more than the photobionts) shape, also in unique ways, the phenotype of the lichen thallus itself, with their morpho-anatomical structures and the production of secondary metabolites. However, our analyses showed that the investigated lichen species, although differing only slightly in their external phenotypes, being most of them crustose epilithic (with the exception of the fruticose *R. melanophthalma*, and the partially foliose *R. elegans*), and either Antarctic endemics or cosmopolitans, shared only part of their myco- and bacterial diversity. Lichens, thus, seem to act as environmental filters for microorganisms in general (Hodkinson et al., 2012; Cardinale et al., 2006; Almendras et al., 2018) but also in Antarctica. Interestingly, the influence of the mycobiont on the whole microbial diversity does not emerge when only different populations of a single or a few lichen species are compared (Cometto et al. 2024), or when only few specimens would be investigated for a certain species. It seems, instead, that when a wide spectrum of species is collected in different localities, though under the same (or highly similar) environmental conditions, the mycobiont identity, together with the key ecological traits of its entire lichen symbiosis, lets emerge the intrathalline fungal and bacterial diversity.

2.4.4 Core species lack in endemic and cosmopolitan lichens

In this study, we did not discern a core group of species shared between endemic and cosmopolitan lichens. Rather, there are fungal and bacterial taxa that are more predominant but not unique, in either one or the other group of lichen species. There are only a few taxa, e.g. *Friedmanniomyces* or *Bacteroidota*, for which we could assume that their stronger association with endemic lichens would relate to the physical and/or biotic barriers that limit the dispersal of the endemic lichen species outside the continent. This would promote the independent evolution of isolated populations of the lichens themselves and their associated fungi and bacteria (Olden et al., 2004; Armstrong, 2017). Prolonged isolation could be a structuring factor for the biogeography of microorganisms in Antarctica, and this could explain why only a few certain genera are detected as more abundant in endemic than in cosmopolitan lichens.

2.5 Conclusions

This study reports in detail the comparison of fungal and bacteria diversity associated with lichen thalli in Antarctica, by considering either Antarctic endemic or cosmopolitan species. We found a higher biodiversity associated with endemic than with cosmopolitan lichen species representing also some endemic microbial taxa. This could be due to physical or biotic barriers that limit the dispersal of species and thus promote the independent evolution of isolated microbial assemblages.

Yet, also in this type of environment, we report a high percentage of reads that cannot be assigned to any known taxon. These unidentified organisms may potentially represent species in their course of evolution, and forthcoming more extended analyses and comparison with further species and updated databases may reveal in the future the presence of many taxa new to science.

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Authors’ contribution:

Conceptualization: L. Muggia, L. Selbmann, C. Coleine, M. Tretiach; Data curation, Formal analysis, Methodology, Roles/Writing - original draft: G. A. Stoppiello, R. De Carolis, L. Muggia, L. Selbmann, C. Coleine; Funding acquisition: L. Muggia, L. Selbmann; Investigation, Writing - Review & Editing: A.G. Stoppiello, R. De Carolis, L. Muggia, L. Selbmann, C. Coleine, M. Tretiach; Project administration: L. Muggia, L. Selbmann; Resources, Supervision: L. Muggia, L. Selbmann; Software: A.G. Stoppiello, R. De Carolis, C. Coleine; Validation: L. Muggia, L. Selbmann, M. Tretiach; Visualization: A.G. Stoppiello, R. De Carolis.

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Chapter 3. Ecological niche drives fungal and bacterial diversity in Antarctic endolithic and epilithic communities inhabiting granites

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Abstract

The Antarctic continent harbors unique ecosystems, including diverse microbial communities inhabiting rocky substrates. Despite extensive research on Antarctic microbial ecology, a gap in knowledge persists regarding the comparative analysis of microbial communities associated with Antarctic granite and epilithic lichen environments. This study addresses the lack of research on both endolithic communities in granite and epilithic communities represented by lichens in Antarctica. Moreover, this study represents the first comparative analysis between these two ecologies, providing valuable insights into their microbial diversity and composition. We addressed this gap by conducting a comprehensive comparative analysis of microbial communities colonizing granite surfaces in Antarctica, focusing on both endolithic and epilithic niches. Through high-throughput sequencing approaches, we characterized the fungal and bacterial diversity associated with these environments. Our results revealed distinct differences between endolithic and epilithic communities in terms of diversity and composition. Endolithic communities exhibited higher microbial diversity, likely influenced by a combination of environmental factors and the complex interplay between lichens and other microorganisms. Moreover, cyanobacteria were found to play a significant role in endolithic communities, demonstrating their adaptability to low-light conditions and their importance in nutrient cycling. Overall, our findings shed light on the ecological dynamics of microbial communities in extreme environments and highlight the importance of further research to elucidate their roles in Antarctic ecosystems.

Keywords: Antarctica, metabarcoding, lichens, microbial diversity, rocks

3.1 Introduction

Endolithism is one of the most amazing and efficient adaptations to extreme conditions in Antarctica (Coleine et al. 2018), referring to the colonization of microorganisms within the pores, cracks, and fissures of rocks (Coleine et al. 2018; Zucconi et al. 2016). These microorganisms, including bacteria, fungi, and algae, thrive in the extreme conditions of Antarctica, particularly in regions where the surface is devoid of vegetation and subjected to harsh environmental conditions such as low temperatures, high UV radiation, and limited water availability (Onofri et al. 2004; Ruisi et al. 2007). Endolithic communities, defined chasmo-, crypto-, and euendolithic according to the growth mode within rocks (Golubic et al., 1981), play crucial roles in nutrient cycling, weathering of rocks, and the formation of soil in Antarctica (Amarelle et al. 2019).

Epilithism, on the other hand, pertains to the colonization of organisms on the surface of rocks. In Antarctica, this can include various organisms such as lichens and mosses that are adapted to survive in the harsh polar environment (Onofri et al. 2007). Among these organisms, lichens constitute the most widely distributed and functionally adapted organisms, constituting preferential niches for microbial diversification and contributing to the overall biodiversity of Antarctic ecosystems (Stoppiello et al. 2024; Selbmann et al. 2013).

Overall, under harsher conditions epilithic communities find a more buffered niche inside rocks and endoliths dominate (Friedmann et al. 1982); where the environmental conditions are less severe endo- and epilithic colonization may coexist: many lichens, for instance, are frequently partially epilithic and partially endolithic (De Los Rios et al. 2005). Even in the McMurdo Dry Valleys in the inner areas of the continent, certain endolithic lichens may develop epilithic sexual structures in sheltered niches. In Victoria Land, unlike the extensively studied communities associated with sandstone, those inhabiting granite substrates have received comparatively less attention in research endeavors, representing an underexplored frontier in Antarctic ecology. A few studies have compared endolithic communities in granite versus sandstone and examined specific lichens that penetrate substrates or studied the microbiome associated to the antarctic lichens (Larsen et al. 2024; Archer et al. 2017; De Los Rios et al. 2005; Stoppiello et al. 2024); however, there is currently a gap concerning the role of rocks and lichens as substrates in shaping of microbial assemblages associated when the epi- and endolithic colonizations co-exist in the same environment. Such an investigation could provide valuable insights into their composition and mutual influence and could help to untangle such divergent ecological stratification.

To address this knowledge gap, we collected endolithically colonized granites from eight sites in Victoria Land during the XXXVII Italian Antarctic Expedition. The microbial diversity inhabiting these granites, comprising microfungi and bacteria, was compared to data recently derived from six epilithic lichen communities collected in the same sites using high-throughput sequencing (HTS) (Stoppiello et al. 2024).

3.2 Materials and Methods

3.2.1 Study area and sampling

The sampling campaign took place during the 37° Antarctic expedition November 2021- February 2022. In this campaign, 8 selected localities have been visited in the Northern Victoria Land, within the south latitudinal range from the 72° to 77°; in each locality at least three individuals of available lichen species were sampled (Table 1) for a total of 90 samples collected. The samples were aseptically collected with the rock substrate using a geological hammer, placed into sterile plastic bags, and stored at -20 °C, also during the transportation to the laboratories at University of Tuscia, Italy until downstream analyses.

Table 3.1. Sampling information of localities and the collected lichen species. The number of thalli collected from each site is shown in parentheses.

Locality	Coordinates	Endolithic communities samples	Epilithic communities (number of samples collected)
Botany Bay	77°00'00"S 162°35'00"E	3	<i>B. frigida</i> (3), <i>L. cancriformis</i> (3), <i>R. elegans</i> (3)
Inexpressible Island	74°53'21.5"S 163°44'43.8"E	3	<i>B. frigida</i> (3), <i>L. cancriformis</i> (3), <i>R. elegans</i> (3)
Vegetation Island	74°47'03"S 163°39'35"E	3	<i>B. frigida</i> (5), <i>P. chlorophanum</i> (3), <i>R. elegans</i> (3)

Prior Island	75°41'30''S 162°52'43''E	3	<i>B. frigida</i> (3), <i>L. cancriformis</i> (3)
Key Island	74°04'13''S 165°18'57''E	3	<i>R. elegans</i> (3), <i>A. flavocordia</i> (3), <i>B. frigida</i> (3), <i>L. cancriformis</i> (3), <i>L. physciella</i> (3)
Starr Nunatak	75°53'54.7''S 162°35'34.4''E	3	<i>B. frigida</i> (3), <i>L. cancriformis</i> (3), <i>R. elegans</i> (3)
Mt Keinath	74°55'S 164°00'E	3	<i>L. cancriformis</i> (1), <i>P. chlorophanum</i> (3)
Campo Icaro	74°42'33.5"S 164°05'45.9"E	3	<i>B. frigida</i> (3), <i>L. cancriformis</i> (3)

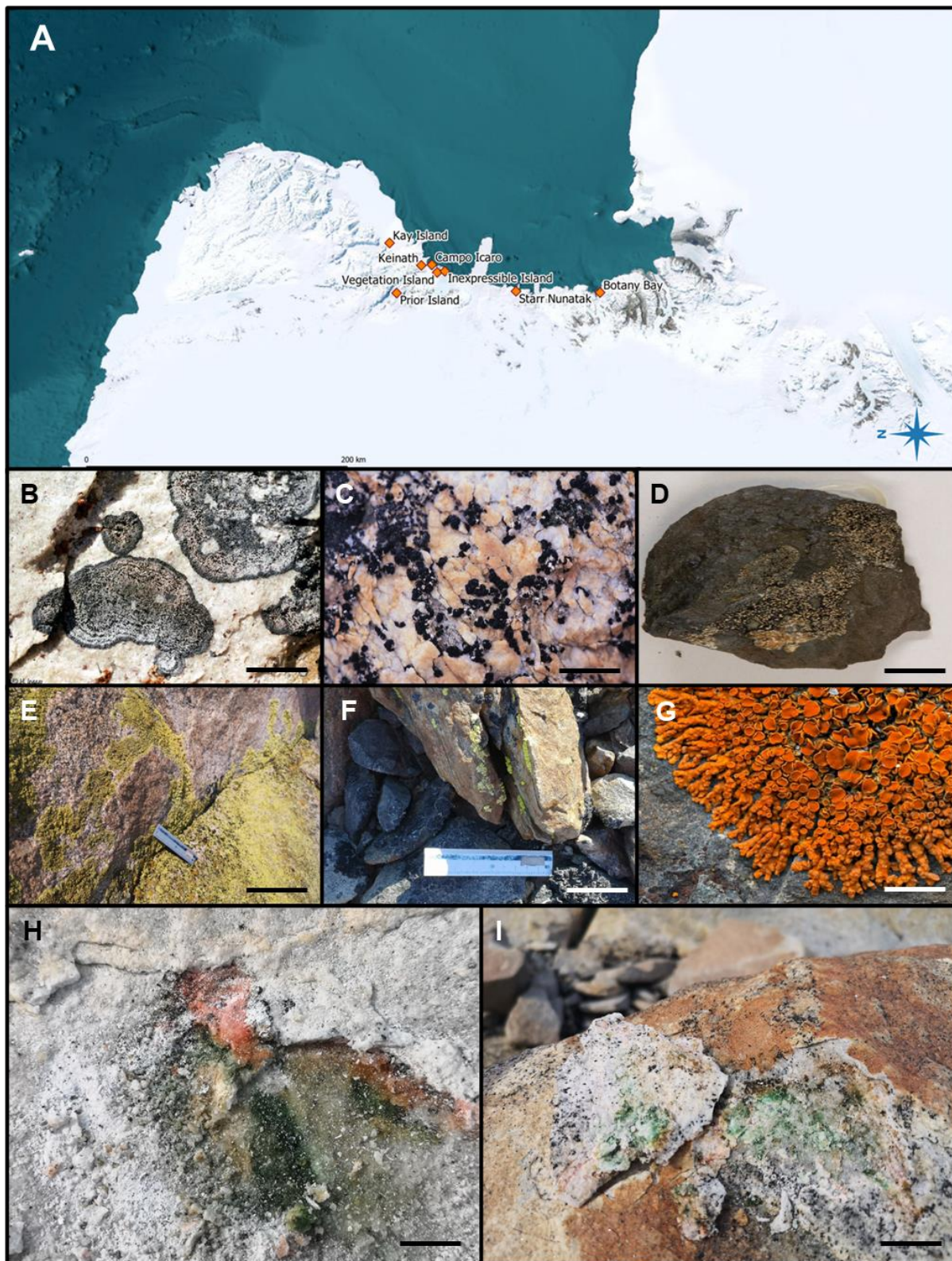


Figure 3.1. Sampling area lichen species. **A)** collecting sites in Victoria Land (Antarctica) visited for the sampling of the six lichen species and the endolithically colonized granites here analyzed; **B)** *B. frigida*; **C)** *L. cancriformis*, **D)** *L. physciella*; **E)** *A. flavocordia* **F)** *P. chlorophanum*; **G)** *R. elegans*. **H,I)** Endolithic granite associated communities. Scale bars: A) 200 km; B,G) 2 cm ; C, H,I) 1 cm; D) 3 cm; F) 5 cm; E) 10 cm

Endoliths were selected by eye according to their degree of colonisation. The collected and analyzed lichen species are all chlorolichens (i.e., forming symbiosis with green trebouxoid photobiont): *Acarospora flavocordia*, *Buellia frigida*, *Lecanora physciella*, *Lecidea cancriformis*, *Pleopsidium chlorophanum*, *Rusavskia elegans*. The lichen species were chosen based on literature knowledge and their availability at the specific sampling sites.

3.2.2 DNA extraction, amplification and sequencing of fungal and bacterial communities

Total community DNA was extracted from 0.5 g of powdered rocks using DNeasy PowerSoil PowerLyzer Kit (Qiagen, German); for epilithic associated communities see Stoppiello et al. 2024. The amplicon sequencing analysis was performed on a total of 27 environmental samples belonging to the colonized granites from 8 different sites; for the 6 lichens for a total of 66 samples see Stoppiello et al 2024. Potential contaminants during the DNA extraction process were monitored by including negative control samples for each granite sample. These negative controls comprised 1.5 ml tubes left open throughout the extraction procedure and treated in the same manner as the samples during amplification and sequencing, DNA extracted was diluted in 40µl of water .

To study the diversity of fungal communities, the nuclear ribosomal internal transcribed spacer 1 region (ITS1) was targeted. The ITS1 region was amplified using the barcoded primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC; White et al., 1990; Smith and Peay, 2014). The V4 variable region of the small ribosomal subunit 16S rDNA was used to assess bacterial diversity. This variable region was amplified using the primers F515 (GTGCCAGCMGCCGCGGTAA) and R806 (GGACTACHVGGGTWTCTAAT) as described by Caporaso et al. (2012). The PCR steps for the fragment's amplification were carried out as in Stoppiello et al, (2024). Samples were then sequenced in pair ends (2x300 bp) using the Illumina MiSeq platform at the Edmund Mach Foundation (San Michele all'Adige, Italy).

3.2.3 Bioinformatics

The samples were treated after checking for extraction contamination with Decontam (Davis et al., 2018). Raw reads were analyzed using the Amplicon ToolKit (AMPTk) for Next Generation Sequence (NGS) v1.2.1 (Palmer et al., 2018), USEARCH (Edgar, 2010) and VSEARCH (Rognes et al., 2016). The reads were trimmed, resulting in sequences with a length of 250 bp; reads shorter than 100 bp were discarded, and chimera removal was performed using USEARCH v. 9.2.64 with default

parameters. Sequence quality filtering was performed with the expected error parameter of <1.0 (Palmer et al., 2018). The dataset was clustered with DADA2 v1.6.0 using a 99% identity parameter to generate the Amplicon Sequence Variants (ASVs). Further filtering was performed in which rare ASVs (i.e. ASVs having less than five reads) and singletons were discarded and not considered for the subsequent analyses. The taxonomy assignment was performed via the UNITE and RDP (Cole et al., 2014) database, which uses the hybrid SYNTAX algorithm (Abarenkov et al., 2020; Edgar, 2010), the sequences were aligned and the taxonomy was assigned to the corresponding ASVs of the ITS and 16S.

3.2.4 Statistical analyses

To explore fungal and bacteria diversity, the analyses were done by clustering the ASVs grouped by their type of ecology (i.e. Endolithic vs. Epilithic). Alpha diversity was calculated with the Observed, Chao1, Shannon and Simpson indexes Beta diversity was calculated using the Jaccard dissimilarity index, and PCoA analyses were performed and tested with PERMANOVA using the false discovery rate (fdr) statistical method. All analyses were performed using R packages microeco (Liu et al., 2021) and phyloseq (McMurdie et al., 2013). Kruskal–Wallis and Wilcoxon tests (Kruskal and Wallis, 1952; Wilcoxon, 1945) were used to assess significant diversity in differences among ecology types.

To comprehend the microbial community composition and correlation across the two ecology types we performed the Random Forest (Beck and Foster 2014; Yatsunenko et al. 2012). Random Forest is a machine learning technique used for classification and regression tasks. It involves creating an ensemble of decision trees trained on random subsets of the data. The "mean decrease Gini" measures the importance of variables in the model by assessing how much they contribute to reducing impurity during tree construction. A higher mean decrease Gini value indicates greater variable importance in the model.

3.3. Results

3.3.1 Bacteria and fungi taxonomic structure among endolithic and epilithic type of ecology

The amplicon sequencing analysis was performed on a total of 90 environmental samples, of which 24 belonging to endolithic communities and 66 to the epilithic lichen species (*A. flavocordia*, *B. frigida*, *L. physciella*, *L. cancriformis*, *P. chlorophanum*, *R. elegans*; Table 3.1). Three samples were used as a negative extraction protocol for the endoliths, after checking for extraction contamination

with Decontam (Davis et al., 2018), we carried on with the analysis. data were treated equally with the epilithic counterpart as in Stoppiello et al. 2024.

The sequencing results yielded 8,480,704 reads for fungi and 10,650,734 reads for bacteria, which were clustered at 99% identity and collapsed into 1,287 and 5,675 ASVs, respectively, after removing chimeras, singletons, contaminants and archaeal sequences.

To focus specifically on the epilithic growth, additional filtering was conducted: all ASVs matching any known lichen-forming fungi (i.e., any lichen mycobionts) were excluded from the analysis. This resulted in a final dataset consisting exclusively of fungal taxa classified as non-lichen mycobionts.

3.3.2 Fungal taxonomy

The community analyses of the fungi revealed *Ascomycota* and *Basidiomycota* as the dominant phyla (fig 3.2A). In both the type of ecology the phylum *Ascomycota* constituted the majority of ASVs. The most frequent class in both groups was found to be *Lecanoromycetes*, which accounts for 78.5% of the endolithic and 53.5% of the epilithic. The class *Lecanoromycetes* represented by 4 main orders in endoliths and 3 in lichens composed of (36% and 31% *Caliciales*), (18% and 0% *Lecideales*), (17% and 8% *Lecanorales*) and (4% and 5% *Acarosporales*) respectively. Two other orders present were the *Lichenostigmatales* and *Chaetothyriales* respectively with 9% and 10% in the lichens and 0.4% and 1.7% in endoliths. The most commonly found families between two groups are *Caliciaceae* 34%, *Lecideaceae* 18% and *Lecanoraceae* 15% for the endoliths and *Caliciaceae* 28%, *Trichomeriaceae* 9.5% and *Phaeococcomycetaceae* 9% for the lichens (sup. 1C). Genera most present in endoliths were *Buellia* 32%, *Lecidea* 18% and *Lecanora* 8% and *Buellia* 27%, *Antarctolichenia* 95% and *Knufia* 9.5 % (fig. 3.2B). Species most commonly found in endoliths are *B. frigida* 31%, *L. cancriiformis* 14% and *L. fuscobrunnea* 4%. On the other hand, in the epilithic ecological counterpart the most abundant were *B. frigida* 25%, *Antarctolichenia onofrii* 8.5% and *Caloplaca rouxii* 3.7 % (fig 3.2C).

3.3.3 Bacterial taxonomy

The bacterial communities encompass a diverse array of phyla within the entire dataset. Notably, *Actinomycetota* were predominant in the endolithic communities, comprising 35% of the total, while *Pseudomonadota* prevails in the epilithic communities, constituting 45.7%. Additionally, *Cyanobacteriota* 18%, *Pseudomonadota* 17% and *Bacteroidota* 14% were prominent constituents of the endolithic communities, whereas *Bacteroidota* 32%, *Actinomycetota* 8.4%, and *Armatimonadaeota* 4%, feature prominently in the epilithic communities. At the taxonomic class level, Actinobacteria dominated the endolithic communities, constituting 34.7% of the microbial

composition, albeit representing 8.4% in the epilithic communities. Conversely, *Alphaproteobacteria* prevailed in the epilithic communities, comprising 39% of the microbial population, while representing 11.1% in the endolithic counterparts. Additionally, Cyanobacteria, *Sphingobacteria*, and *Cytophagia* were notable in the endolithic communities, with relative abundances ranging from 18.7% to 4.3%, whereas *Armatimonadia*, *Sphingobacteriia*, and *Cytophagia* were prominent in the epilithic communities, ranging from 18.3% to 3.8% (fig 3.2D). At the order taxonomic level, *Actinomycetales* represent the predominant taxa in endolithic communities, comprising 21.1% of the microbial population, followed by *Rubrobacterales*, *Armatimonadales*, *Sphingobacteriales*. Conversely, in epilithic communities, Rhodospirillales emerged as the most abundant order, constituting 34.6% of the microbial composition, succeeded by *Sphingomonadales*, *Armatimonadales*, *Actinomycetales*. In endolithic communities, although the *Rubrobacteraceae* family was the most abundant (8.9%), other families *Trueperaceae*, *Sphingobacteriaceae*, and *Acetobacteraceae* all with 5% of abundance) In epilithic communities, the most abundant family was *Acetobacteraceae* (34.6%), followed by *Cytophagaceae* 18%, *Sphingobacteriaceae* 12% and *Armatimonadaceae* 4%. The latter taxonomic rank analyzed was genus. The most abundant genera attributable to endoliths were *Rubrobacter* 9%, *Truepera* 5% and *Nakamurella* 2%. The most abundant genera related to the lichens were *Hymenobacter* 17%, *Acidisoma* and *Armatimonas* both with 4% (fig. 3.2E).

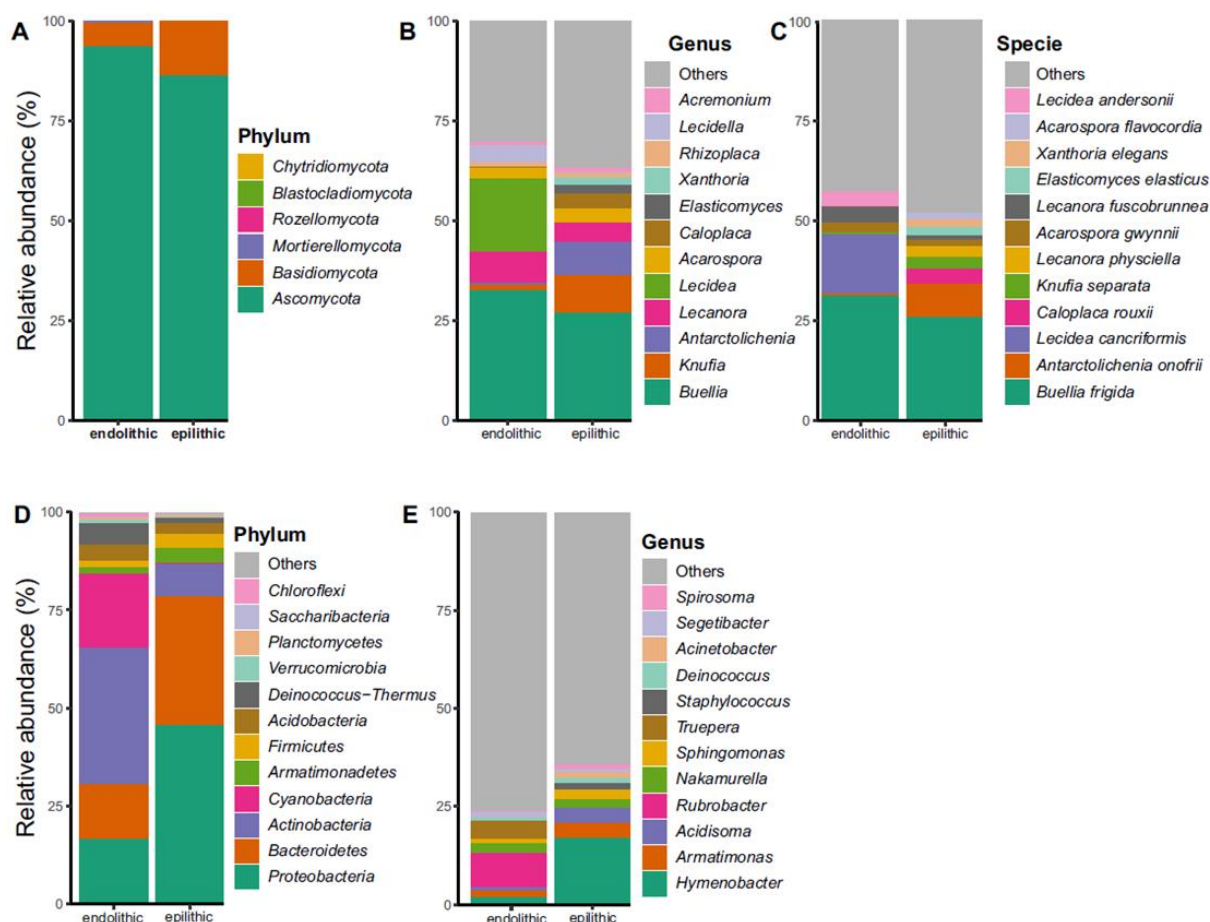


Figure 3.2. Relative abundance plots of fungi (A-C) and bacteria (D, E) reported for each type of ecology (Endolithic and Epilithic).

3.3.4 Unique fungal and bacterial taxa differentiate the microbial communities associated to granites and epilithic niches.

Several fungal species were dominant only in granite-associated communities (p-value < 0.001); for instance, lichens as the species *Lecidea cancriformis*, *Carbonea vorticosa*, *L. andersonii*, *Lecidella carpathica* and *L. grenii* and basidiomycetous yeasts *Jaminalaea angkorensis*, *Cystobasidium laringis*, *Naganishia vishniacii*, and *N. friedmannii*. The lichen species micobyonts were particularly abundant in granite samples (Fig. 3.3A). Analyzing the bacterial component reveals genera associated with both ecological types. Endolithic communities exhibit a greater diversity of genera, including *Ewingella*, *Euzebya*, *Truepera* and *Rubrobacter*. Four predominant bacterial genera result associated with epilithic communities such as *Corynebacterium*, *Methylobacterium*, *Staphylococcus* and *Acinetobacter* (fig 3B).

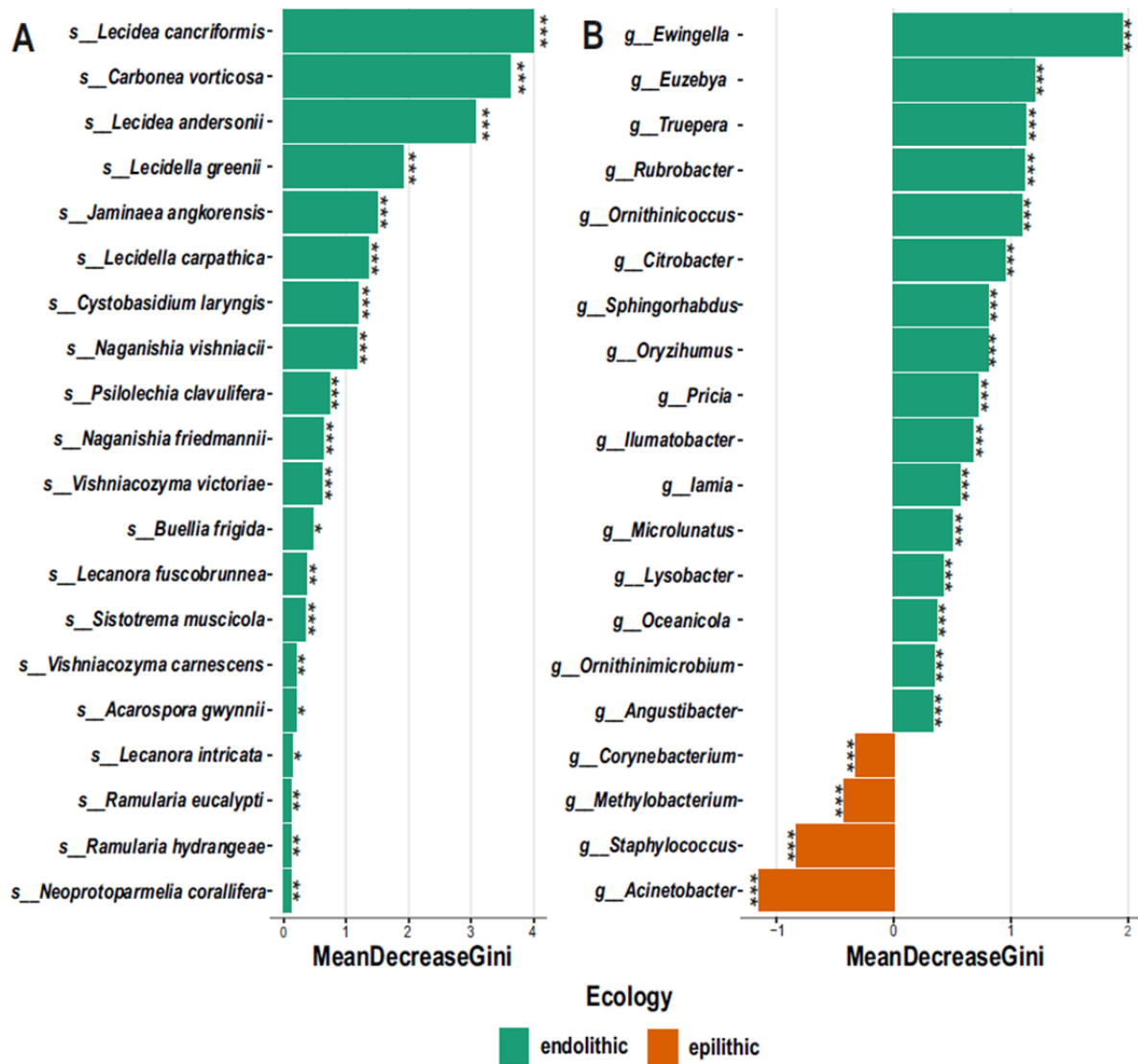


Figure 3.3. Taxonomic and community composition using Random Forest method of endolithic vs epilithic ecology, light blue and orange, respectively. **(A)** fungal species associated only with endolithic type of ecology; **(B)** bacterial genera associated with both the type of ecology. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001. Top 20 taxa are shown.

3.3.5 Alpha diversity analysis.

The Chao1 index for fungal and bacterial diversity are visualized in Figure 3.4. Endoliths presented the highest Chao1 values among the type of ecology, for both the microorganism assemblages compared to lichens. The Wilcoxon test, conducted for paired comparisons within groups, revealed significant statistical differences between types of ecology in both the assemblages (p-value < 0.001). Shannon index indicates that the biodiversity was higher in endoliths than in lichens (p-value < 0.001) only for the bacterial component. The same occurs when considering the Simpson index (Fig. 3.4).

Specifically, it is statistically significantly higher (p-value < 0.001) in endoliths compared to the epilithic counterpart only concerning bacteria.

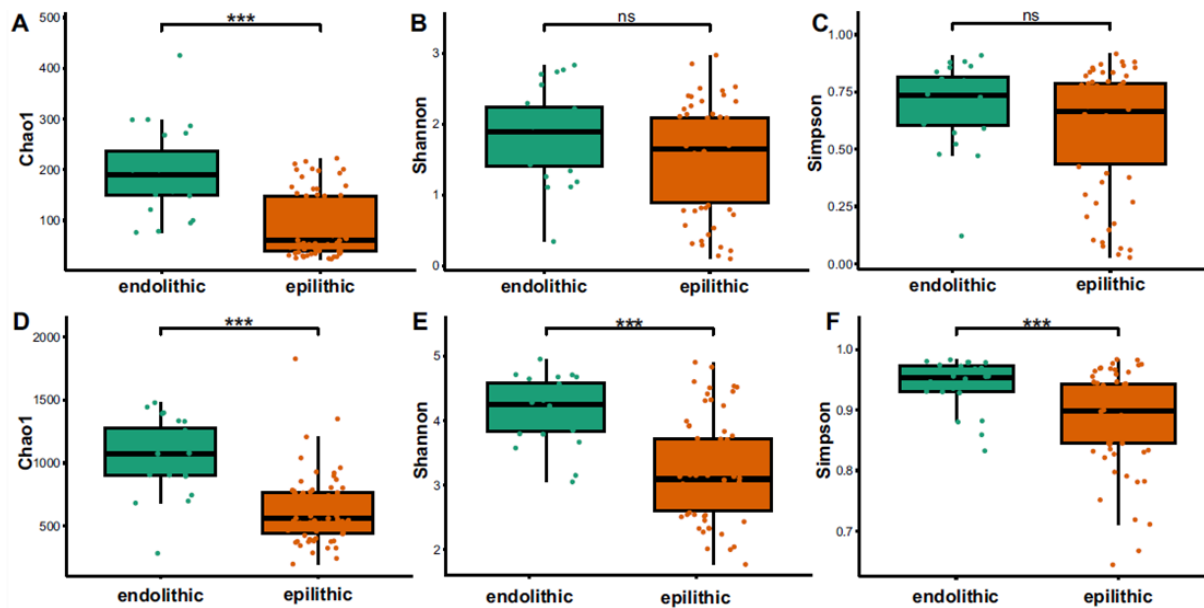


Figure 3.4. Comparison of alpha diversity using Chao1, Shannon and Simpson indexes of fungal (A-C) and bacterial (D-F) diversity between endolithic and epilithic type of ecology. The statistic support was tested by Wilcoxon test and is indicated as *** $p < 0.001$. ns = not statistically significant.

3.3.6 Beta diversity analysis.

The Jaccard and unweighted UniFrac distances were used to assess the beta diversity and the differences in fungal and bacterial assemblages among the 2 different types of ecology. The results were presented through PCoA plots along with PERMANOVA statistical analysis. The effects of the type of ecology (i.e., endolithic vs. epilithic) were found to be significant (p-value < 0.001), although the geographical distribution seemed to have a lesser impact on the community ordination than that of the ecology (Fig. 3.5). Statistics are shown in detail in supplementay.

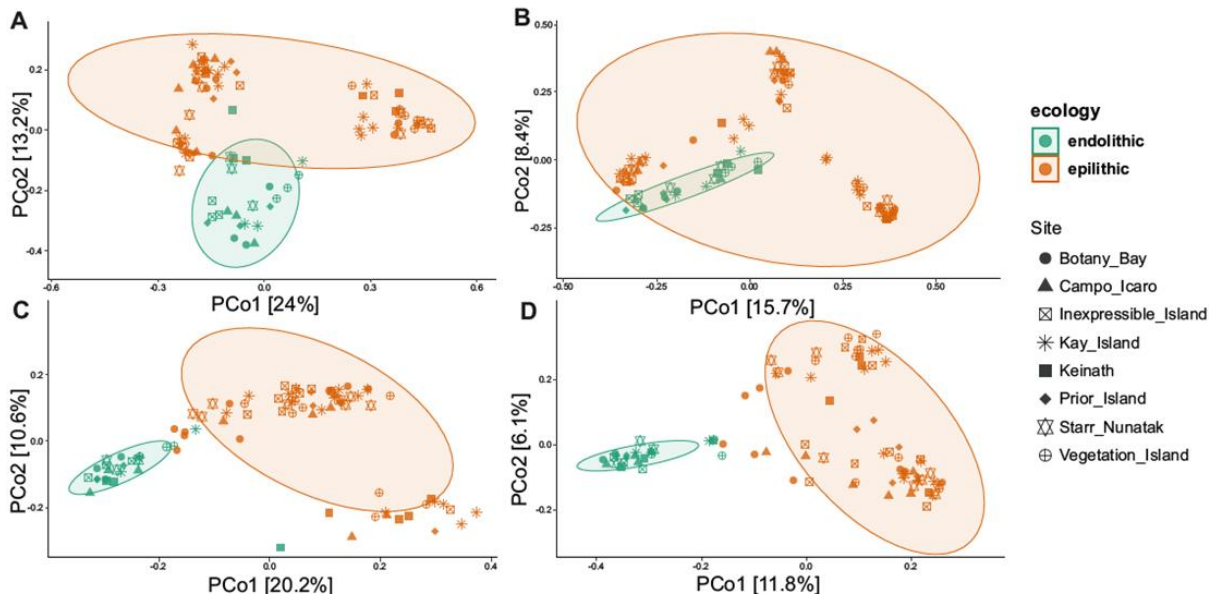


Figure 3.5. Beta diversity and principal component analysis (PCoA) based on unweighted UniFrac and Jaccard distance including ellipse of 95% confidence interval. Unweighted UniFrac distance for fungi (A) and bacteria (C); Jaccard distance for fungi (B) and bacteria (D).

3.4 Discussions

3.4.1 Fungal taxa associated with Endolithic and Epilithic ecologies.

fungal diversity associated both with endolithic communities and epilithic lichens in the examined species primarily consisted of *Ascomycota* taxa (fig. 3.2A), aligning with previous investigations focusing on lichen-dominated endolithic communities and lichen associated communities inhabiting sandstones (Selbmann et al., 2005, 2008; Egidi et al., 2014; Coleine et al., 2018a, Stoppiello et al. 2024). The most prevalent class identified within endolithic organisms is *Lecanoromycetes*, observed in comparable percentages within the cryptoendolithic communities of Victoria Land (Coleine et al., 2018) which included *Caliciales*, *Lecideales*, *Lecanorales* *Acarosporales*. Conversely, associated with epilithic lichens, excluding the mycobiont, were present the orders *Chaetothyriales*, *Tremellales*, and *Lichenostigmatales* reinforcing the evidence about the frequent presence of these fungal groups in epilithic lichens from dry and cold habitats (Muggia et al., 2016; Muggia and Grube 2018; Cometto et al., 2023, 2024; Stoppiello et al. 2024). At finer taxonomic level, it becomes evident that the predominant fungal community associated with endoliths primarily comprised *Buellia*, *Lecidea*, *Acarospora*, *Lecidella*, and *Carbonea* (fig. 3.2B). These lichenized fungi have been also previously identified as major components of cryptoendolithic communities in sandstone, specifically *Carbonea*, *Lecidella*, and *Acarospora* (Coleine et al. 2021, 2022; Ruprecht et al. 2010; Selbmann et al. 2013). Moreover, *Lecidea* and *Buellia* have been microscopically detected in Antarctic granites (De Los Rios et al., 2005). *L. cancriformis*, *L. andersonii*, *L. carpathica*, *L.grenii*, *C. vorticosa*, *L.*

fuscobrunnea were also strongly correlated with the RandomForest method to the endolithic ecology (fig. 3.3), corroborating once again the primary and dominant role of lichens in rocks-inhabiting communities. Notably, this study marks the first instance of their identification as all constituents of the predominant endolithic community associated with granite in Antarctica. Other taxa, although not dominant in terms of abundance, represented by basidiomycetoid yeasts, correlated with the endolithic communities, represented by species such as *Jaminaea angkorensis*, *Cystobasidium laringis*, *Naganishia vishniacii*, *Naganishia friedmannii*, *Vishniacozyma victoriae* (fig. 3.3A). Basidiomycetes yeasts are an important component of the lichen mycobiota and also have been isolated from endolithic communities (Spribille et al. 2016, 2018; Cometto et al. 2022; Coleine et al. 2018), except for *J. angkorensis*. This species of basidiomycetoid yeast is phylogenetically closely related to the genus *Rhodotorula*, which has been extensively isolated from Antarctica (Pavlova et al. 2001; Wang et al. 2015). *N. friedmannii* and *N. vishniacii* have been extensively isolated from both endolithic communities and lichens in Antarctica (Coleine et al. 2021b; Selbmann et al. 2017; Vishniac, 1985). *C. laringis* has been reported from cold environments in the Arctic, as well as from soils and ice samples collected at Concordia Station in Antarctica (Turchetti et al. 2018; Tsuji et al. 2016; Stoppiello et al. 2023). *Cystobasidium* species isolated from cold environments share a psychrophilic or psychrotolerant attitude and are able to survive the temperature of their natural habitat. *V. victoriae* was one of the most frequently isolated species in both polar and subpolar environments (Sannino et al. 2023; Tsuji et al. 2020).

Knufia, in Antarctica, is typically isolated to rocks (Coleine et al. 2021); however, in this study, we found it particularly abundant in epilithic communities. *Antarctolichenia* and *Elasticomyces*, on the other hand, corroborate the previous data of isolation as they are found in Antarctic lichens (Stoppiello et al. 2024; Muggia et al. 2021; Egidi et al. 2014).

3.4.2 Bacterial taxa associated with Endolithic and Epilithic ecologies.

Pseudomonadota, *Actinomycetota* and *Bacteroidota* were recovered as the most abundant bacterial phyla of both epi- and endolithic ecologies (fig. 3.2D). *Pseudomonadota* is in general widely distributed in different habitats on Earth (Nemergut et al., 2011), *Bacteroidota* were also already found as predominant phylum in Antarctic lakes (Michaud et al., 2012) and *Actinomycetota* were isolated from Antarctic soils (Silva et al. 2020; Araujo et al. 2020). Furthermore, all these phyla were found in Antarctic lichens (Stoppiello et al. 2024). *Cyanobacteria* represented one of the most abundant phyla in granites from our dataset, whereas it is almost absent in epilithic communities. The phylum *Cyanobacteria* was already reported as one of the most abundant in endolithic communities

in granites (Archer et al. 2017; Larsen et al. 2023). Some cyanobacteria are known for their ability to fix carbon, molecular nitrogen and to mobilize inorganic phosphate from organic phosphates (Pandey et al., 2004). These microorganisms, possibly, could play a crucial role in organizing these chemical elements and rendering them available within the community, while the epilithic communities exposed to the external environment might utilize a readily available source of organic nitrogen directly from the guano of marine birds (Schnug et al. 2018). *Cyanobacteria*, capable of photosynthesis even under low solar incidence, require water for photosynthetic processes. Although liquid water may not be available year-round, studies have shown that the humidity present in the environment, condensing within rock crevices, aids in sustaining basal activity (Büdel et al. 2008; Cowan & Taw, 2004). Therefore, a possible explanation for their abundance in granites could be the more buffered microenvironment offered by the substrate. Lichens, on the other hand, have their own unique ecological niche and microbial composition (Stoppiello et al. 2024), which may not favour the establishment and proliferation of cyanobacteria. Additionally, the presence of other microorganisms within lichen thalli, such as fungal partners and other bacteria, may also influence the colonization and survival of cyanobacteria and their variability (Wirtz et al. 2003). At the genus level, the most abundant genera found in epilithic communities were *Hymenobacter* and *Armatimonas*, consistent with the previous study by Stoppiello et al. (2024). Regarding the endolithic communities, the most abundant genera found were *Truepera* and *Rubrobacter*. *Truepera* is a genus of radioresistant bacteria previously isolated in the Lake Wellman area in Antarctica (Aislabie et al. 2013). Another radioresistant bacterial genus is *Rubrobacter* previously reported from Victoria Land soils of Wright Valley (Aislabie et al. 2006). Based on this data, the endolithic niche appears to favor the selection of radioresistant bacteria. These genera, is also predominant in granite habitats, serve as indicators of this ecological niche (fig. 3B). Both genera exhibit radioresistance even under high levels of UV irradiation and have been isolated from Antarctic regions such as Wright Valley and Lake Wellmann (Aislabie et al. 2006, 2013). Given their radioresistant nature and ability to withstand UV radiation, these genera demonstrate an ecological adaptation to the extreme conditions found within endolithic niches, characterized by limited sunlight exposure. Their presence underscores their resilience and survival capacity in such hostile environments (Mezzasoma et al. 2022; Qu et al. 2020). As a consequence, natural selection likely exerted significant evolutionary pressure on them and this allowed them to play a pivotal role within the community. The genera *Acinetobacter* and *Staphylococcus* were correlated with epilithic communities. *Acinetobacter* is a bacterial genus frequently encountered across diverse regions of Antarctica, spanning soil and ice. These microorganisms exhibit remarkable adaptability and resilience in extreme environments typified by cold temperatures, intense UV radiation, and scarce nutrient resources (Tamang et al. 2023; Marcoleta

et al. 2022). *Staphylococcus* is widespread in nature and occupies a variety of niches. Both *Acinetobacter* and *Staphylococcus* have been found abundantly in bird guano. Specifically, *Staphylococcus* has been isolated from Antarctic seabird guano (Vrbovska et al. 2020; Dahiru & Enabulele, 2015). This finding lends further support to the previously hypothesized role of bird guano in providing organic nitrogen, thereby supporting the proliferation of cyanobacteria in endolithic communities, where physical access to nitrogen is more challenging.

3.4.3 Endolithic and epilithic ecology select specific microbial diversity.

The ecological significance of these findings lies in the observed disparity in microbial diversity and richness between endolithic and epilithic communities. Endoliths exhibit higher microbial diversity and species richness compared to epiliths, indicating a more biodiverse ecosystem within the rock substrates. This difference may be explained by more stable and sheltered microenvironments provided by the rocks, fostering favourable conditions for microbial colonization and proliferation (Coleine et al. 2018, 2019). In specific terms, the data demonstrate elevated Chao1 index in endolithic communities, denoting greater species richness, in contrast to epilithic counterparts. Moreover, Shannon index, reflective of species diversity and distribution, is higher in endolithic communities, particularly concerning bacterial diversity. Conversely, Simpson index, indicating species evenness, manifest higher values in endolithic communities regarding bacterial composition, suggesting a more uniform distribution of bacterial species within the rock substrates (Selbmann et al 2017; Coleine et al. 2018).

Within the endolithic community, multiple lichen species are present, albeit with varying relative abundances. The elevated diversity may be attributed to the colonization of numerous lichen species within the rock crevices, thus fostering a more complex community compared to the aggregate diversity of the epilithic lichens under study. This finding is further substantiated by beta diversity analyses, which unveiled significant disparities in both composition (presence-absence) and phylogenetic similarity between the two groups, underscoring how these distinct niches drive the evolution and adaptation of entirely different microbial communities. However, some degree of overlap was observed, particularly in fungi, reinforcing the intimate association that the fungal assemblages share with the epilithic lichen species under consideration. In bacteria, an even more pronounced clustering is observed. This may be attributed to the lichen thallus inside the rock acting as a hotspot for diversification, leading to a higher number of bacterial organisms being associated with greater diversity and abundance of fungal species (Stoppiello et al. 2024).

3.5 Conclusions

In conclusion, our study sheds light on the microbial communities thriving within the niches of Antarctic granite and epilithic habitats. We observed distinct patterns of diversity and composition between these two ecological niches, with endolithic communities exhibiting higher microbial diversity, particularly among bacteria. The prevalence of lichens in both habitats suggests their pivotal role in shaping these communities, while the presence of specific fungal and bacterial taxa further highlights their adaptation to extreme environmental conditions. Moreover, our findings underscore the potential influence of external factors in shaping microbial communities, as in the case of cyanobacteria, particularly in providing organic nitrogen sources. One intriguing aspect that warrants further investigation is the potential role of radioresistance in shaping microbial communities within the Antarctic endolithic niche. While our study has revealed higher microbial diversity in endolithic communities compared to lichen-dominated habitats, future research could explore whether this trend correlates with the prevalence of radioresistant species. Overall, this study enhances our understanding of microbial ecology in polar regions and underscores the importance of further research to elucidate the mechanisms driving microbial diversity and adaptation in extreme environments.

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CHAPTER 4. Culturable fungi and algae associated to Antarctic epilithic lichen thalli.

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Abstract

Lichen thalli are niches for microorganisms, including microfungi, microalgae and non-photosynthetic bacteria, which form communities of variable composition, often shaped by the environmental conditions under which the lichen thalli develop. In Antarctica lichens represent an important group of organisms characterized by a high percentage of endemism, which have specialized to grow on rocks, as the predominant substrate for colonization. Here Antarctic epilithic thalli of five endemic and three cosmopolitan lichen species have been investigated for their potential role as fungal species hotspots. The culturable fraction of the Antarctic lichen-associated fungi and algae was uncovered with the aim to isolate as many culturable species as possible, and find whether taxa are strictly connected to the Antarctic environment or are cosmopolitan species that particularly associate to lichens in harsh, extreme environments. Over 300 fungal and algal inocula grew in culture, among which we recognized species previously described as well as five new fungal species of ascomycetes and Basidiomycetes in the genera *Dactylospora*, *Knufia*, *Petrophila*, *Pseudeurotium*, and *Kurtzmanomyces*. These species seem to select lichens as their preferred niche in Antarctica and worldwide. Indeed, the major representatives of the lichen mycobiota are fungi that do not show any

specificity toward a particular lichen host species, rather toward the lichen thallus in general as a structure in which spores, yeast cells and mycelia fragments thrive or rest. In Antarctica, where living conditions on rock surfaces are far more selective than elsewhere, lichen thalli would be even more exploited as suitable niches by other fungi for their evolution and diversification.

Keywords

Basidiomycetes, diversity, endolichenic, Dothideomycetes, Eurotiomycetes, photobiont, phylogeny

4.1 Introduction

Lichens are well-known self-sustaining and long-living symbioses in which a main fungal partner, i.e. the mycobiont, associates with a population of the main photosynthetic partners, i.e. the photobionts (green algae, cyanobacteria or both), to build as a stable structure the lichen thallus (Hawksworth and Honegger, 1984). Within lichen thalli a multiplicity of microorganisms, including microfungi, microalgae and non-photosynthetic bacteria are housed (Hawksworth and Grube, 2020) and form microbial communities of variable composition, often shaped by the environmental conditions under which the lichen thalli develop (e.g. U'Ren et al., 2010, 2012; Grube et al., 2015; Moya et al., 2017; Molins et al., 2021; Smith et al., 2020; Cometto et al., under review). These lichen-associated microbial communities frequently occur cryptically, and in the past few years their species diversity has been better understood by DNA metabarcoding studies and culture isolations (Arnold et al., 2009; Fernandez Mendoza et al., 2017; Muggia and Grube, 2018; Banchi et al., 2018; Yang et al., 2020; Beck et al., 2023). For that it concerns the eukaryotic fraction, the lichen-associated fungi, represent a wide array of major lineages in Ascomycota and Basidiomycota, and include both filamentous and yeast species (Spribille et al., 2018; Muggia and Grube, 2018; Cometto et al., 2022). The lichen-associated microalgae, instead, are majorly represented by the classes Trebouxiophyceae and Ulvophyceae (Moya et al., 2017; Molins et al., 2021). In the last decade, metabarcoding studies have been applied to uncover in a comprehensive way this microbial taxonomic diversity cryptically occurring in lichens (U'Ren et al., 2010; Spribille et al., 2016; Fernández-Mendoza et al., 2017; Zhang et al., 2015; Banchi et al., 2018; Smith et al., 2020; Muggia and Grube, 2018; Beck et al., 2023).

However, the isolation of culturable strains and their maintenance in vitro is essential to integrate the knowledge on the morphological diversity of these cryptic communities. So far, an increasing number of studies have aimed at elucidating the diversity of the cryptically lichen-associated fungi and algae either with one or the other approach, but only a pair of studies have combined both the metabarcoding and culture-dependent approaches (Yang et al., 2020; Cometto et al., under revision). Thanks to the successful isolation in culture of several strains coming from different geographic origins, new species have been formally recognized (Černajová and Skaloud, 2019; Muggia et al., 2021b; Cometto et al., 2023; Chang et al., 2023). In particular, within the subclass Chaetothyriomycetidae (Eurotiomycetes) the family Pleostigmataceae (including three *Pleostigma* species; Muggia et al., 2021b), the species *Melanina gunde-cimermanniae* (Muggia et al., 2021b), ten new *Cladophialophora* (Cometto et al., 2023; Chang et al., 2023) and one new *Paracaldophialophora* species (Cometto et al., 2023) were described. Most of these species were isolated from lichens from very dry, cold and hot habitats and high altitude/alpine environments, hinting to the fact that lichens

from extreme environments may serve as ecological niches and cradles of species diversification (Harutyunyan et al., 2008; Arnold et al., 2009; Quan et al., 2020, 2023).

Environmental conditions which are considered the most extreme on Earth and at the limit of life are those found in Antarctica, where only microbial communities composed by rock-inhabiting lichenized and not-lichenized fungi and algae can outcompete and settle even throughout the most dry and cold interior areas of the continent (Øvstedal and Lewis Smith 2001; Selbmann et al., 2013a). In Antarctica, as well as at high altitudes, species must be able to cope with continuous fluctuations of temperatures, humidity/draught, salinity, oligotrophy, deposition of inorganic and organic nutrients, and high ultraviolet (UV) radiation (Gorbushina, 2007; Onofri et al., 2007; Selbmann et al., 2015; Coleine et al., 2021b; De Los Ríos et al., 2014). Though, in Antarctica lichens represent an important group of organisms characterized by a high percentage of endemism (33%-50% in the continental Antarctica) with a total of 130 endemic species (Øvstedal and Lewis Smith, 2001; Castello and Nimis, 1997, 2000). Here, lichens have specialized to grow on rocks, as the predominant substrate for colonization (Nienow and Friedmann, 1993), building either a crustose, thin, epilithic thallus, or strictly endolithic, with the thallus developing inside the rock substrate when conditions become incompatible with epilithic life (De los Ríos et al., 2005; species with foliose and fruticose growth forms are mostly found in coastal habitats). In Antarctica these so formed “cryptoendolithic lichen-dominated communities” (Friedmann, 1982; de la Torre et al., 2003), have already been characterized for their fungal and bacterial composition and diversity (Selbmann et al., 2005, 2008; Muggia et al., 2021a), as they represent an environment much more buffered from the environmental extremes (Casero et al., 2021; Perez-Fernandez et al., 2022) and where microorganisms can diversify.

However, the Antarctic epilithic thalli have not been investigated for their potential role as species hotspots since recently, when five endemic Antarctic (*Acarospora flavocordia*, *Buellia frigida*, *Lecanora fuscobrunnea*, *Lecanora physciella*, *Lecidea cancriformis*) and three cosmopolitan (*Pleopsidium chlorophanum*, *Rhizoplaca melanophthalma*, *Rusavskia elegans*) lichen species have been selected for an amplicon sequencing studies tackling their associated fungal and bacterial diversity (Stoppiello et al. under revision). Here we have integrated the molecular analyses of Stoppiello et al. (under revision) by uncovering the culturable fraction of the lichen-associated fungi and algae (photobionts included) from Antarctica. We applied a culture approach aiming at isolating as many culturable specie as possible, to find whether taxa are strictly connected to the Antarctic environment or are cosmopolitan species that particularly associate to lichens in harsh, extreme conditions, as those recently described from thalli growing at high altitudes and in dry, highly irradiated habitats worldwide (Muggia et al., 2021b, Cometto et al., 2023).

4.2 Material and methods

4.2.1 Sampling

The sampling campaign took place during the Antarctic expedition 2021-2022. A total of 14 localities have been selected in the northern Victoria Land that in the latitudinal range from 72° to 77° parallel (; see chapter 2, Table 1). If present, at least three samples of eight selected species were collected at each site. The samples were aseptically removed using a geological hammer, collected in plastic sterile bags, transported and then stored at -20 °C in the laboratories (at the University of Tuscia and University of Trieste, Italy), until downstream analyses. The lichens chosen for the study were epilithic species with a crustose to sub-foliose and sub-fruticose thallus, i.e., the five Antarctic endemics *Acarospora flavocordia*, *Buellia frigida*, *Lecanora fuscobrunnea*, *Lecanora physciella*, *Lecidea cancriformis*, and three cosmopolitan species *Pleopsidium chlorophanum*, *Rizoplaca melanophthalma* (sub-fruticose thallus), and *Rusavskia elegans* (sub-foliose thallus). Lichen species were chosen based on bibliographic knowledge and their availability at the specific sampling sites.

4.2.2 Isolation of fungi and algae from lichen thalli

The isolation of microfungi and microalgae associated within the lichen thalli was performed for two thalli for each lichen species for each locality following the protocol of Yamamoto et al. (2002). Approximately 2 mm² fragments of lichen thalli were dissected with a sterile razor blade. The fragments included one or two thallus areolas and a few apothecia if present. The fragments were washed three times for 15 min with sterile water, followed by 30 min of washing with 500 µl of Tween20 diluted 1:10. A final washing step was performed rinsing the thallus fragments three times for 15 min with sterile water. The clean fragments were ground in sterile water under the hood and tiny thallus fragments were picked with a sterile bamboo stick and transferred into agar tubes. Different media were used to promote the growth of as many different fungal and algal species as possible: Trebouxia medium (TM, Ahmadjian, 1987), Malt Yeast-extract (MY, Lilly and Barnett, 1951), and Bold's Basal Medium (BBM, Nichols and Bold, 1965). The media were added with chloramphenicol [25mg/ml] to reduce bacteria contamination. The tubes were incubated in a growing chamber under the conditions of 17 °C, 20 µmol × photons m⁻² × s⁻¹, with a light/dark cycle of 14/10 h, and let grow for a period of 5 up to 10 months. Isolates that after a few months were grown up to 2-5 mm, were removed from the slant agar tubes, subcultured in petri plates, and incubate for further 2-4 months to reach a sufficient biomass that could be used for their genetic and morphological characterization, as well as for preserve them as cryostock. The isolated strains are deposited and

preserved in the Culture Collection of Fungi from Extreme Environments (CCFEE), Mycological Section of the Italian Antarctic National Museum (MNA) at the University of Tuscia (Viterbo, Italy).

4.2.3 DNA extraction, amplification and sequencing

The isolates were genetically identified and DNA extraction followed the CTAB protocol of Cubero et al. (1999), with minor adjustments. The identity of all fungal strains was studied with sequences of at least two marker genes: i) the nuclear internal transcribed spacers (nucITS) and 5.8S rDNA ribosomal gene amplified with the primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990), ii) the D1/D2 domain of the 28S nuclear large ribosomal subunit (nucLSU) amplified with the primers LR0R and LR7 (Vilgalys and Hester, 1990). To better delimit phylogenetic placement of those isolates that were identified as belonging to the order Capnodiales in Dothideomycetes, we further amplified the markers RNA polymerase II (rpb2) and the nuclear small ribosomal subunit (nucSSU) using the primers fRPB2-5F (Liu et al., 1999) and fRPB2-414R (Quaedvlieg et al., 2012) and primers NuSSU-0072-5' and NuSSU-0852 (Gargas. and Taylor, 1992), respectively. 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.

The genetic identification and phylogenetic analyses of the photobionts were performed on algal sequences amplified directly from the lichen thali and algal strains isolated in cultures. The analyses were based on two marker genes, i.e., the nuclear internal transcribed spacer of the ribosomal DNA (ITS rDNA) and the large subunit of the ribulose-1,5-biphosphate carboxylase (RbcL). The ITS was amplified using the *Trebouxia*-specific primers ITS1T and ITS4T (Kroken and Taylor, 2000), the RbcL was amplified with the primers RbcL803rev and RbcL320 (Nozaki, 1995), and the applied PCR conditions followed Muggia et al. (2014b) and Muggia et al. (2008, 2010), respectively. The sequence identity of isolated algae was used to find correspondence between the sequence(s) obtained directly from the thallus DNA extraction amplified with the same photobiont specific primers.

All 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 biotek). Clean amplicons were sent for Sanger sequencing to Eurofins Genomics (Italy).

4.2.4 Phylogenetic analyses

The newly generated sequences were checked with sequences available in the GenBank database by BLAST similarity search (Altschul et al., 1990). Taxa that matched our sequences with an identity value higher than 97% were selected to study the systematic placement of the isolates with phylogenetic analyses. The first blast comparison returned the similarity of the new sequences with three classes of Ascomycetes, i.e. Dothideomycetes, Eurotiomycetes and Leotiomyces, and with two classes of Basidiomycetes represented by Microbotryomycetes and Agaricostilbomycetes, and the three orders Cystobasidiales, Philobasidiales, Tremellales. For each group, a specific dataset was built (Supplementary Material Tables S1-S8). The complete datasets encompassed a broad range of taxonomic diversity, with the inclusion of genera and families that were closely related to the new sequences, as identified in previous researches, in particular, the dataset of Dothideomycetes and Eurotiomycetes was based on Cometto et al. (2023), to better resolve phylogeny of *Dactylospora* genus (Eurotiomycetes) we added also 47 sequences retrieved from GeneBank (NCBI) database, while the dataset of Leotiomyces was based on Quijada et al. (2022). The datasets built for the classes and orders of the Basidiomycetes were based on those presented by Cometto et al. (2022).

Sequence alignments for each locus (nucITS, nucLSU, nucSSU, RPB2, RbcL) and for each fungal taxa were prepared with MAFFT v.7 (Kato and Standley, 2013) using the g-ins-I alignment strategy. All the fungal taxa and photobiont datasets underwent Maximum Likelihood (ML) and Bayesian Inference (BI) analyses on the CIPRES web portal (Miller et al., 2010). For the ML analysis, the program RaxML v. 8.2.12 (Stamatakis, 2014) with the GTRGAMMA substitution model and 1000 bootstrap replicates was used. In the case of BI, we employed Mr Bayes v. 3.2.7a (Huelsenbeck and Ronquist, 2001) and ran two parallel runs with six chains each, extending over five million generations. The analysis began with a random tree and sampled every 100th step. We discarded the initial 25% of the data as burn-in, and the corresponding posterior probabilities (PPs) were computed from the remaining trees. The ITS phylogenetic reconstruction was made for all taxa recognized (Supplementary Figures S1-S5), while the multilocus datasets were prepared as follow: i) ITS and LSU markers for Agaricostilbomycetes, Chaetothyriales, Cystobasidiales and Leotiomyces ii) ITS, nucLSU, nucSSU and RPB2 markers for Capnodiales (Dothideomycetes). The multilocus gene alignments were prepared in MEGA (Kumar et al. 2018) and then analyzed with both RAXML and BEAST with the same settings of the single locus analyses.

4.2.5 Morphological analyses

The analyses of morphological and anatomical characters have been performed on eleven fungal strains and one lineage of *Trebouxia* that was the only one representing a new lineage. The strains were analyzed using standard microscopic techniques and documented with digital photographs. Analyses and photographs were performed on about one-year-old subcultures. The following characters were studied for the fungal isolates: form of growth (filamentous vs. yeast-like), melanization of the hyphae, form and size of hyphal cells, branching of hyphae, development of conidiogenous cells and formation of conidia. Small fragments of mycelia were removed, and squashed sections were mounted in water and studied by light microscopy.

The morphological traits studied for the isolated algae were the size of the cells, presence of autospores and the chloroplast type whether detectable.

All images were acquired with a ZeissAxioCam MRc5 digital camera fitted to the microscope, digitally processed and slightly refined in sharpness and color saturation with Adobe Photoshop 7.0. Photo tables were prepared with CorelDRAW X4.

The anatomical characterization (ultrastructure of the chloroplast and type of pyrenoid) of new *Trebouxia* lineage was performed using transmission electron microscopy (TEM).

4.3 Results

4.3.1 Fungal diversity

A total of 328 fungal inocula grew as axenic isolates and were further kept in culture. After fungal growth, we proceeded with species identification generating 220 nucITS, 62 nucLSU, 12 RPB2, and 12 nucSSU new sequences (Table 1). According to the blast search analysis, the new sequences (total number reported in parenthesis) corresponded to the ascomycete classes Dothideomycetes (61), Eurotiomycetes (71), Leotiomycetes (19), and to the Basidiomycetes Agaricostilbomycetes (9), Cystobasidiales (34), Filobasidiales (14), Microbotryomycetes (2) and Tremellales (10). In general, the phylogenetic reconstructions either based on the ITS locus alone, or on multilocus datasets, were topological concordant with previous studies (such as Cometto et al., 2022, 2023 and references therein; Quan et al., 2020, 2023).

Within Dothideomycetes, the new sequences in the order Capnodiales were 43 sequences belonging to *Elasticomyces elasticus* isolated from thalli of *L. cancriformis*, *L. physciella*, *A. flavocordia*, *B. frigida*, *R. elegans*, and *R. melanophthalma* (Supplementary Figure S1). A single isolate from the thallus of *R. elegans* L4530 corresponded to the genus *Catenulostroma*, while another single isolate from the thallus *R. melanophthalma* L4599 was identified as Cladosporiales sp. A supported clade of six new sequences was identified as *Meristemomyces* spp., isolated from thalli of *L. cancriformis* and *L. fuscobrunnea*. Interestingly we found seven strains belonging to three supported clades in the family Extremaceae. One sequence, retrieved from *R. melanophthalma*, for Cladosporiales (L4599) and two new sequences (S71, S72) belonging to the new species *Petrophila complexa* (here described) and retrieved from *L. cancriformis* thalli (Supplementary Figure S1). The multi-locus phylogenetic reconstruction for Capnodiales based on the ITS, SSU, LSU and RPB2 loci (Figure 1) showed a new *Petrophila* species supported by both ML and Bayesian inferences. The strains S71 and S72 were isolated in Campo Icaro and Prior Island, respectively. Morphological observations were performed on strains (S71; Figure 11), description reported below.

Table 1. Culture strains and the new generated sequences in this work.

Isolate	Organism	Lichen species host
L4489	<i>Knufia</i> sp. Nov.	<i>Rusavskia elegans</i>
L4490	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4492	<i>Naganishia friedmannii</i>	<i>Rusavskia elegans</i>
L4493	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4494	Cystobasidiales sp.	<i>Rusavskia elegans</i>
L4495	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4496	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4497	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4498	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4500	<i>Elasticomyces elasticus</i>	<i>Rusavskia elegans</i>
L4503	<i>Naganishia friedmannii</i>	<i>Rusavskia elegans</i>
L4504	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4506	<i>Naganishia friedmannii</i>	<i>Rusavskia elegans</i>
L4508	<i>Elasticomyces elasticus</i>	<i>Rusavskia elegans</i>
L4509	<i>Elasticomyces elasticus</i>	<i>Rusavskia elegans</i>
L4510	<i>Tremella macrobasidiata</i>	<i>Rusavskia elegans</i>
L4511	<i>Tremella macrobasidiata</i>	<i>Rusavskia elegans</i>

L4512	<i>Naganishia friedmannii</i>	<i>Rusavskia elegans</i>
L4516	<i>Tremella macrobasidiata</i>	<i>Rusavskia elegans</i>
L4517	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4519	<i>Tremella macrobasidiata</i>	<i>Rusavskia elegans</i>
L4520	<i>Tremella macrobasidiata</i>	<i>Rusavskia elegans</i>
L4521	<i>Knufia</i> sp. nov.	<i>Rusavskia elegans</i>
L4523	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4524	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4526	<i>Tremella macrobasidiata</i>	<i>Rusavskia elegans</i>
L4527	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4528	<i>Paracladophialofphora</i> <i>lichenicola</i>	<i>Rusavskia elegans</i>
L4529	<i>Dactylospora</i> sp. nov.	<i>Rusavskia elegans</i>
L4530	<i>Catenulostroma</i> sp.	<i>Rusavskia elegans</i>
L4531	<i>Cystobasidium</i> sp.	<i>Rusavskia elegans</i>
L4532	<i>Dactylospora</i> sp. nov.	<i>Rusavskia elegans</i>
L4533	Yeast lineage II (sensu Milannes et al. 2011)	<i>Rusavskia elegans</i>
L4534	Yeast lineage II (sensu Milannes et al. 2011)	<i>Rusavskia elegans</i>

L4535	<i>Cystobasidium</i> sp.	<i>Rusavskia elegans</i>
L4536	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4537	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4538	<i>Cystobasidium</i> sp.	<i>Rusavskia elegans</i>
L4539	<i>Elasticomyces elasticus</i>	<i>Rusavskia elegans</i>
L4540	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4541	<i>Cladophialophora humicola</i>	<i>Rusavskia elegans</i>
L4542	<i>Cladophialophora humicola</i>	<i>Rusavskia elegans</i>
L4543	<i>Knufia</i> sp. nov	<i>Rusavskia elegans</i>
L4544	<i>Knufia</i> sp. nov	<i>Rusavskia elegans</i>
L4545	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4546	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4547	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4548	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4549	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4550	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4551	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4552	<i>Knufia separata</i>	<i>Rusavskia elegans</i>

L4553	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4554	<i>Extremaceae sp.</i>	<i>Rusavskia elegans</i>
L4555	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4556	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4557	<i>Cladophialophora humicola</i>	<i>Rizoplaca melanophthalma</i>
L4558	<i>Cladophialophora humicola</i>	<i>Rizoplaca melanophthalma</i>
L4559	<i>Cladophialophora humicola</i>	<i>Rizoplaca melanophthalma</i>
L4560	<i>Cladophialophora humicola</i>	<i>Rizoplaca melanophthalma</i>
L4561	<i>Elasticomyces elasticus</i>	<i>Rizoplaca melanophthalma</i>
L4562	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4563	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4564	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4565	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4566	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4567	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4568	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4569	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4570	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>

L4571	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4572	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4573	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4574	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4575	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4576	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4577	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4578	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4579	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4580	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4581	<i>Elasticomyces elasticus</i>	<i>Lecanora physciella</i>
L4583	<i>Elasticomyces elasticus</i>	<i>Rusavskia elegans</i>
L4584	Cystobasidiales sp.	<i>Rusavskia elegans</i>
L4588	<i>Knufia</i> sp. nov.	<i>Rusavskia elegans</i>
L4589	<i>Tremella macrobasidiata</i>	<i>Rusavskia elegans</i>
L4590	<i>Tremella macrobasidiata</i>	<i>Rusavskia elegans</i>
L4591	<i>Knufia separata</i>	<i>Rusavskia elegans</i>
L4592	<i>Tremella macrobasidiata</i>	<i>Rusavskia elegans</i>

L4593	<i>Dactylospora</i> sp. nov.	<i>Rusavskia elegans</i>
L4594	<i>Cystobasidium</i> sp.	<i>Rusavskia elegans</i>
L4595	<i>Cystobasidium</i> sp.	<i>Rusavskia elegans</i>
L4596	<i>Cystobasidium</i> sp.	<i>Rusavskia elegans</i>
L4598	<i>Cystobasidium</i> sp.	<i>Rusavskia elegans</i>
L4599	Cladosporales sp.	<i>Rizoplaca melanophthalma</i>
L4600	<i>Cystobasidium</i> sp.	<i>Rizoplaca melanophthalma</i>
L4602	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4603	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4604	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4605	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4606	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4607	<i>Elasticomyces elasticus</i>	<i>Rizoplaca melanophthalma</i>
L4608	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4609	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4610	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4611	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4612	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>

L4613	<i>Naganishia friedmannii</i>	<i>Rizoplaca melanophthalma</i>
L4614	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4616	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4617	<i>Naganishia friedmannii</i>	<i>Rizoplaca melanophthalma</i>
L4618	<i>Kurtzmanomyces</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4619	<i>Kurtzmanomyces</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4620	<i>Naganishia friedmannii</i>	<i>Rizoplaca melanophthalma</i>
L4621	<i>Naganishia friedmannii</i>	<i>Rizoplaca melanophthalma</i>
L4622	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4623	<i>Naganishia friedmannii</i>	<i>Rizoplaca melanophthalma</i>
L4624	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4625	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4626	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4627	<i>Kurtzmanomyces</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4628	<i>Kurtzmanomyces</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4630	<i>Kurtzmanomyces</i> sp. nov.	<i>Lecanora physciella</i>
L4632	<i>Pseudeurotium</i> sp. nov.	<i>Rusavskia elegans</i>
L4633	<i>Kurtzmanomyces</i> sp. nov.	<i>Rizoplaca melanophthalma</i>

L4636	<i>Pseudeurotium</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
L4638	<i>Kurtzmanomyces</i> sp. nov.	<i>Rizoplaca melanophthalma</i>
S100	<i>Phaeanellomyces</i> sp.	<i>Acarospora flavocordia</i>
S101	<i>Microbotryomycetes</i> sp.	<i>Buellia frigida</i>
S102	<i>Cystobasidium</i> sp.	<i>Acarospora flavocordia</i>
S103	<i>Naganishia friedmannii</i>	<i>Acarospora flavocordia</i>
S104	<i>Cystobasidium</i> sp.	<i>Buellia frigida</i>
S105	<i>Kurtzmanomyces</i> sp. nov.	<i>Buellia frigida</i>
S106	<i>Cystobasidium</i> sp.	<i>Acarospora flavocordia</i>
S107	<i>Cystobasidium</i> sp.	<i>Acarospora flavocordia</i>
S108	<i>Cystobasidium</i> sp.	<i>Acarospora flavocordia</i>
S10	<i>Elasticomyces elasticus</i>	<i>Acarospora flavocordia</i>
S110	<i>Cystobasidium</i> sp.	<i>Acarospora flavocordia</i>
S111	<i>Phaeanellomyces</i> sp.	<i>Acarospora flavocordia</i>
S113	<i>Cystobasidium</i> sp.	<i>Lecidea cancriformis</i>
S114	Yeast lineage II (sensu Milannes et al. 2011)	<i>Lecidea cancriformis</i>
S115	<i>Phaeanellomyces</i> sp.	<i>Acarospora flavocordia</i>
S116	<i>Cystobasidium</i> sp.	<i>Buellia frigida</i>

S117	<i>Cystobasidium</i> sp.	<i>Buellia frigida</i>
S118	<i>Cystobasidium</i> sp.	<i>Acarospora flavocordia</i>
S11	<i>Knufia separata</i>	<i>Acarospora flavocordia</i>
S120	<i>Phaeanellomyces</i> sp.	<i>Acarospora flavocordia</i>
S121	<i>Phaeanellomyces</i> sp.	<i>Acarospora flavocordia</i>
S12	<i>Knufia separata</i>	<i>Acarospora flavocordia</i>
S13	<i>Elasticomyces elasticus</i>	<i>Buellia frigida</i>
S14	<i>Elasticomyces elasticus</i>	<i>Buellia frigida</i>
S15	<i>Elasticomyces elasticus</i>	<i>Buellia frigida</i>
S16	<i>Elasticomyces elasticus</i>	<i>Buellia frigida</i>
S17	<i>Elasticomyces elasticus</i>	<i>Buellia frigida</i>
S18	<i>Elasticomyces elasticus</i>	<i>Buellia frigida</i>
S19	<i>Elasticomyces elasticus</i>	<i>Lecidea cancriformis</i>
S1	<i>Elasticomyces elasticus</i>	<i>Lecidea cancriformis</i>
S20	<i>Elasticomyces elasticus</i>	<i>Acarospora flavocordia</i>
S21	<i>Elasticomyces elasticus</i>	<i>Buellia frigida</i>
S22	<i>Elasticomyces elasticus</i>	<i>Buellia frigida</i>
S23	<i>Chaetothyriales</i> sp.	<i>Acarospora flavocordia</i>

S24	Chaetothyriales sp.	<i>Acarospora flavocordia</i>
S25	<i>Elasticomyces elasticus</i>	<i>Lecidea cancriformis</i>
S26	<i>Cladophialophora endolichenicola</i>	<i>Acarospora flavocordia</i>
S27	Extremaceae sp.	<i>Buellia frigida</i>
S28	<i>Cladophialophora endolichenicola</i>	<i>Acarospora flavocordia</i>
S2	<i>Extremus</i> sp.	<i>Acarospora flavocordia</i>
S30	Extremaceae sp.	<i>Buellia frigida</i>
S31	<i>Elasticomyces elasticus</i>	<i>Acarospora flavocordia</i>
S32	<i>Meristemomyces</i> sp.	<i>Lecidea cancriformis</i>
S33	<i>Meristemomyces</i> sp.	<i>Lecidea cancriformis</i>
S34	<i>Elasticomyces elasticus</i>	<i>Lecidea cancriformis</i>
S35	<i>Knufia separata</i>	<i>Lecidea cancriformis</i>
S36	<i>Knufia separata</i>	<i>Lecidea cancriformis</i>
S37	<i>Cladophialophora endolichenicola</i>	<i>Acarospora flavocordia</i>
S38	<i>Dactylospora</i> sp. nov.	<i>Acarospora flavocordia</i>
S39	<i>Phaeanellomyces</i> sp.	<i>Buellia frigida</i>
S3	<i>Extremus</i> sp.	<i>Acarospora flavocordia</i>

S42	<i>Dactylospora</i> sp. nov.	<i>Acarospora flavocordia</i>
S45	<i>Phaeanellomyces</i> sp.	<i>Buellia frigida</i>
S4	<i>Elasticomyces elasticus</i>	<i>Acarospora flavocordia</i>
S51	<i>Dactylospora</i> sp. nov.	<i>Acarospora flavocordia</i>
S53	<i>Dactylospora</i> sp. nov.	<i>Acarospora flavocordia</i>
S54	<i>Dactylospora</i> sp. nov.	<i>Acarospora flavocordia</i>
S55	<i>Dactylospora</i> sp. nov.	<i>Acarospora flavocordia</i>
S56	<i>Dactylospora</i> sp. nov.	<i>Acarospora flavocordia</i>
S57	<i>Dactylospora</i> sp. nov.	<i>Acarospora flavocordia</i>
S5	<i>Elasticomyces elasticus</i>	<i>Buellia frigida</i>
S60	<i>Meristemomyces</i> sp.	<i>Lecanora fuscobrunnea</i>
S61	<i>Meristemomyces</i> sp.	<i>Lecanora fuscobrunnea</i>
S64	Extremaceae sp.	<i>Buellia frigida</i>
S65	Extremaceae sp.	<i>Buellia frigida</i>
S66	<i>Meristemomyces</i> sp.	<i>Lecanora fuscobrunnea</i>
S67	<i>Meristemomyces</i> sp.	<i>Lecanora fuscobrunnea</i>
S6	<i>Cladophialophora</i> <i>endolichenicola</i>	<i>Acarospora flavocordia</i>
S71	<i>Petrophila complexa</i>	<i>Lecidea cancriformis</i>

S72	<i>Petrophila complexa</i>	<i>Lecidea cancriformis</i>
S73	<i>Paracladophialofphora lichenicola</i>	<i>Buellia frigida</i>
S74	<i>Paracladophialofphora lichenicola</i>	<i>Buellia frigida</i>
S75	<i>Cystobasidium</i> sp.	<i>Lecidea cancriformis</i>
S76	<i>Naganishia friedmannii</i>	<i>Lecidea cancriformis</i>
S77	<i>Cystobasidium</i> sp.	<i>Acarospora flavocordia</i>
S78	<i>Cystobasidium</i> sp.	<i>Lecidea cancriformis</i>
S79	<i>Cystobasidium</i> sp.	<i>Acarospora flavocordia</i>
S7	<i>Elasticomyces elasticus</i>	<i>Acarospora flavocordia</i>
S80	<i>Naganishia friedmannii</i>	<i>Acarospora flavocordia</i>
S82	<i>Naganishia friedmannii</i>	<i>Acarospora flavocordia</i>
S83	<i>Cystobasidium</i> sp.	<i>Buellia frigida</i>
S84	<i>Cystobasidium</i> sp.	<i>Buellia frigida</i>
S85	<i>Cystobasidium</i> sp.	<i>Lecidea cancriformis</i>
S86	<i>Naganishia friedmannii</i>	<i>Buellia frigida</i>
S87	<i>Cystobasidium</i> sp.	<i>Buellia frigida</i>
S89	<i>Cystobasidium</i> sp.	<i>Lecidea cancriformis</i>

S90	<i>Cystobasidium</i> sp.	<i>Acarospora flavocordia</i>
S91	<i>Kurtzmanomyces</i> sp. nov.	<i>Buellia frigida</i>
S92	<i>Phaeanellomyces</i> sp.	<i>Acarospora flavocordia</i>
S93	<i>Cystobasidium</i> sp.	<i>Acarospora flavocordia</i>
S94	<i>Cystobasidium</i> sp.	<i>Buellia frigida</i>
S95	<i>Cystobasidium</i> sp.	<i>Buellia frigida</i>
S97	<i>Cystobasidium</i> sp.	<i>Lecidea cancriformis</i>
S98	<i>Glaciozyma watsonii</i>	<i>Buellia frigida</i>

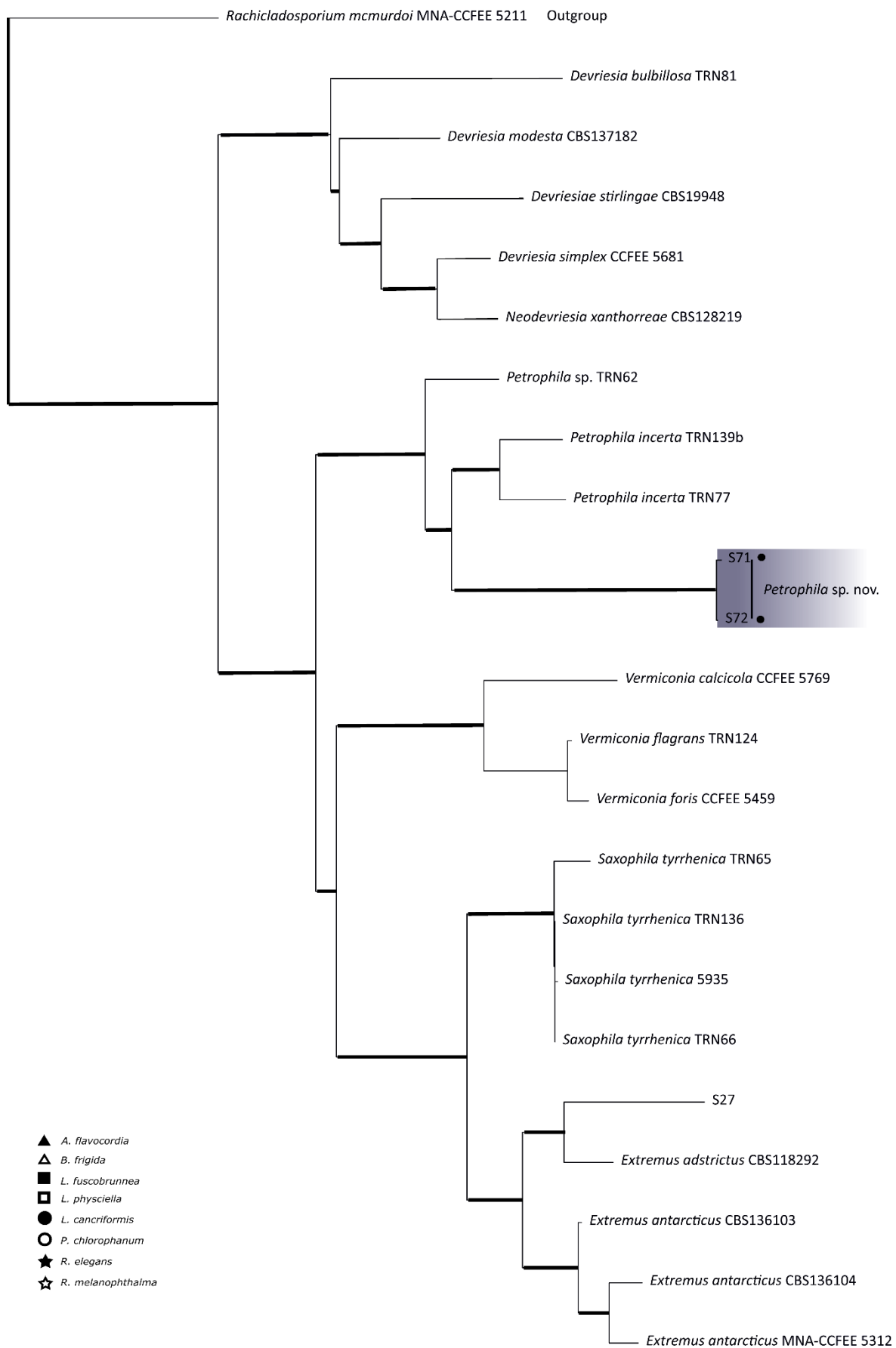


Figure 4.1. Phylogenetic inference of Dothideomycetes based on the concatenated nuclear ITS-LSU-SSU-RPB2 dataset. Branches in bold denote RAxML bootstrap support >75% and Bayesian posterior probabilities ≥ 0.9 . Newly obtained sequences are in bold and enclosed in a gray box. Symbols next to the strain number correspond to the lichen species from which they were isolated.

The phylogenetic inference estimated for Eurotiomycetes and based on the ITS locus highlighted two new lineages. The first one was identified as a *Dactylospora* species within Sclerococcales, and is represented by 12 isolates coming from three thalli of *A. flavocordia* collected at Random Hills and three thalli of *R. elegans* collected in Star Nunnatak, Edmondson point, and Random Hills (See chapter 2, Table 1). The multi-locus phylogenetic reconstruction based on the ITS and LSU loci (Figure 2), confirmed the presence of this new *Dactylospora* clade supported by both ML and Bayesian inferences. The morphological analyses were performed on four strains (L4532, L4632, S38, S51; Figure 4.12)

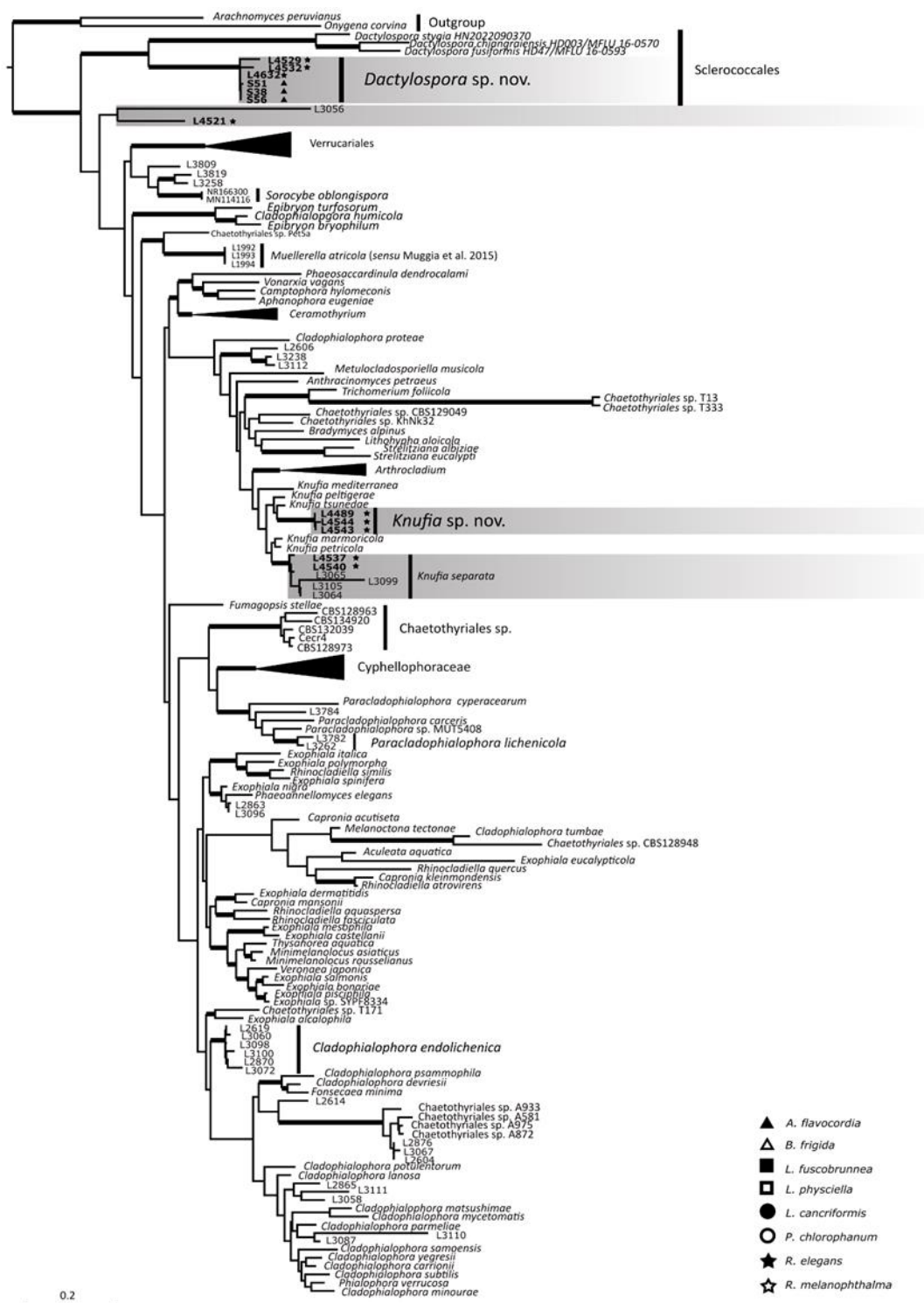


Figure 4.2. Phylogenetic inference of Chaetothyriales based on the concatenated nuclear ITS-LSU dataset. Branches in bold denote RAXML bootstrap support >75% and Bayesian posterior probabilities ≥ 0.9 . Newly obtained sequences are in bold and enclosed in a gray box. Symbols next to the strain number correspond to the lichen species from which they were isolated (see legend).

Six strains were assigned to the family Epibryaceae, isolated from two thalli of *R. elegans* and four thalli of *R. melanophthalma*. Epibryaceae is again unresolved and therefore it does not receive full support neither by the ML nor by the Bayesian analyses.

Four strains (L4489, L4543, and L4544; Figure 4.2), isolated from three thalli of *R. elegans* collected at Star Nunnatak and Random Hills, represented the second new chaetothyrialean lineage (Figure 4.2), fully supported and belonging to the genus *Knufia* (Trichomeriaceae, Chaetothyriales). In the multilocus phylogeny (Figure 4.2) this new *Knufia* sp. lineage was placed as sister taxon of *Knufia tsunetae*. Most of the strains in Chaetothyriales, i.e. a total of 25, corresponded to *Knufia separata* isolated from one thallus of *L. cancriformis* collected at Botanic Bay, one thallus from *A. flavocordia* collected at Kay Island, and nine thalli of *R. elegans* collected at Vegetation Island, Star Nunnatak and Random Hills (Figure 4.2).

We also isolated three strains of the recently described species *Paracladophialophora lichenicola* (Paracladophialophoraceae), coming from thalli of *L. cancriformis* and *B. frigida*, and four strains of *Cladophialophora endolichenica* (Herpotrichiellaceae), isolated from two thalli of *A. flavocordia*. The last eight strains of Chaetothyriales formed an own subclade within *Phaeoanellomyces elegans* and likely represent a new group of antarctic haplotypes of this species.

The last two strains S23 and S24 are so far identified as Chaetothyriales sp. within Herpotrichiellaceae and are placed unresolved in a clade with two *Capronia* and *Rhinocladiella* species and *Aculeata aquatica*.

19 strains belonged to the class Leotiomycetes, and both the single locus (Supplementary Figure S3) and the combined ITS-LSU (Figure 4.3) phylogenies placed them in a new clade close to the genera *Pseudeurotium*, *Thelebolus*, *Cleistothelebolus* and *Antarctomyces*. In particular, the combined dataset based on that of Quija et al. (2022) placed this new clade as sister of *Pseudeurotium zonatum*; all the strains were isolated from three thalli of *R. melanophthalma* collected at Random Hills. The morphological analyses carried out on two strains (L4525 and L4625; Figure 4.14).



Figure 4.3. Phylogenetic inference of Leotiomycetes based on the concatenated nuclear ITS-LSU dataset. Branches in bold denote RAxML bootstrap support >75% and Bayesian posterior probabilities ≥ 0.9 . Newly obtained sequences are in bold and enclosed in a gray box. Symbols next to the strain number correspond to the lichen species from which they were isolated (see legend).

Within the Basidiomycota, the ITS phylogenetic inference (Supplementary Figure S4) of Agaricostilbomycota identified a new clade of *Kurtzmanomyces* represented by 11 strains, fully supported and sister to *Kurtzmanomyces nectarei*. The phylogenetic placement and the statistical support of the lineage were confirmed also by the multilocus analyses (ITS and LSU; Figure 4). The strains belonging to this new clade were isolated from one thallus of *B. frigida*, one of *L. physciella*, and two of *R. melanophthalma* (Figure 4), all collected at Random Hills.

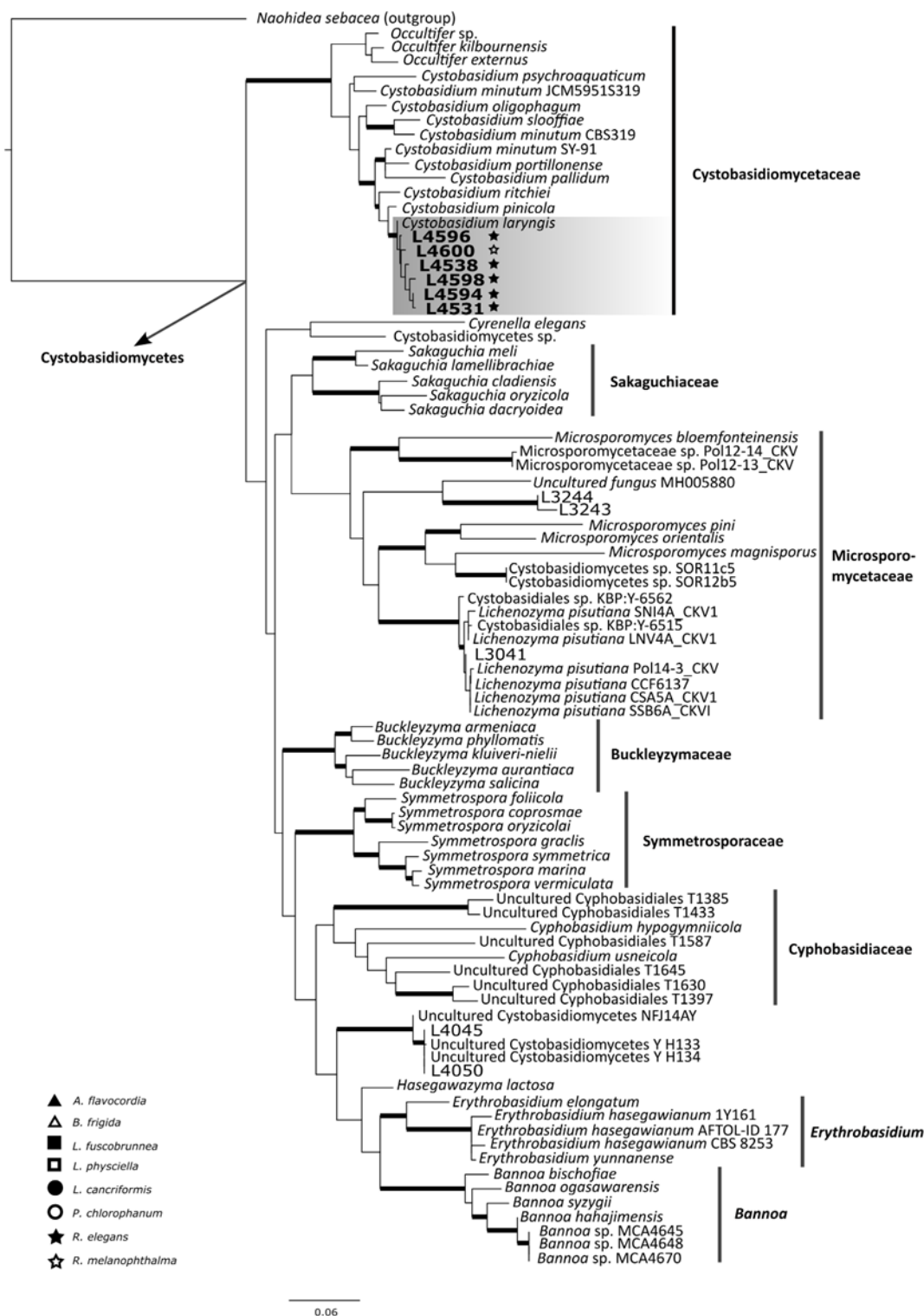


Figure 4.4. Phylogenetic inference of Agaricostilbomycetes based on the concatenated nuclear ITS-LSU dataset. Branches in bold denote RAxML bootstrap support >75% and Bayesian posterior probabilities ≥ 0.9 . Newly obtained sequences are in bold and enclosed in a gray box. Symbols next to the strain number correspond to the lichen species from which they were isolated (see legend). Agaricostilbomycetes clades are named according to Millanes et al. (2021).

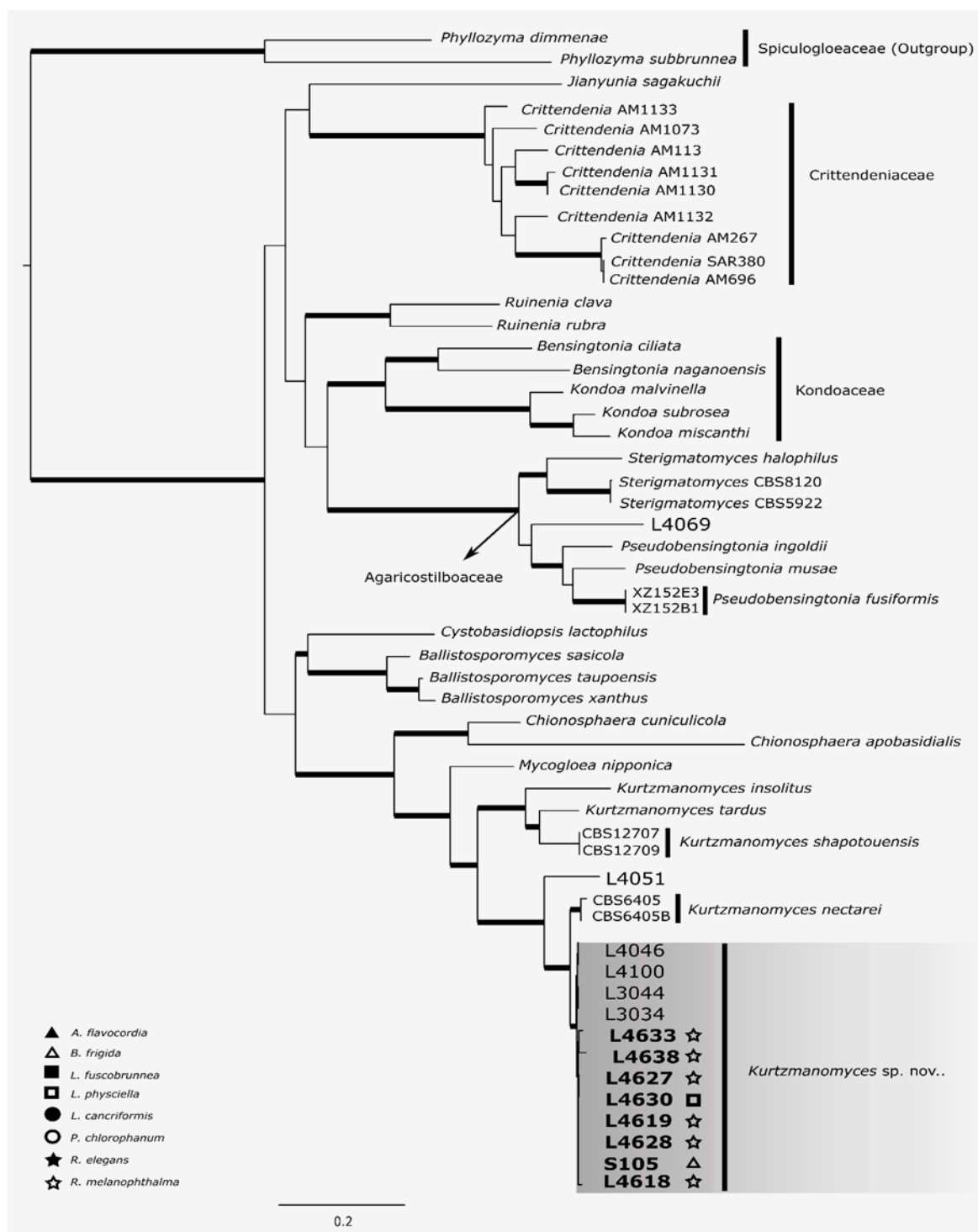


Figure 4.5. Phylogenetic inference of Cystobasidiales genus based on the concatenated nuclear ITS-LSU dataset. Branches in bold denote RAxML bootstrap support $>75\%$ and Bayesian posterior probabilities ≥ 0.9 . Newly obtained sequences are in bold and enclosed in a gray box. Cystobasidiales clades are named according to Černajová and Škaloud (2019) and Millanes et al. (2016). Symbols next to the strain number correspond to the lichen species from which they were isolated (see legend).

A total of 32 strains were identified as *Cystobasidium* (Cystobasidiales) and were isolated from the five lichen species *A. flavocordia* (10) *B. frigida* (8), *L. cancriformis* (6), *R. melanophthalma* (1), and *R. elegans* (7). Of these, 27 strains corresponded to the species *C. laryngis*, while four strains are sister to *C. ritchiei* and one to *C. pallidum*. Two further strains (L4494, L4584) isolated from *R. elegans* were identified within Cystobasidiales at a basal position within the order.

Within Filobasidiales (Figure 6) 11 strains isolated from *A. flavocordia* (1), *B. frigida* (2), *R. melanophthalma* (5), and *R. elegans* (3) were recognized as *Naganisha friedmannii* and are placed as the next relative to *Naganishia onofrii* and unresolved with *N. vaughanmartiniae*. Other two strains isolated from thalli of *L. cancriformis* and *R. elegans* were placed in a clade and partially unresolved together with *Naganishia albidosimilis*, *N. diffluens*, *N. liquefaciens* and *N. adeliensis*, and with other four strains of *N. albidosimilis* previously isolated from lichens by Cometto et al. (2022).

Only two strains isolated from *B. frigida* belonged to Microbotryomycetes (Figure 7), i.e. S101 and S98. S101 was found as sister taxon of *Heterogastridium pycnidioideum*, and could be corresponded to this species (although branches subtending these two taxa are rather long), while S98 was recognized as *Glaciozyma watsonii*, as unresolved together with many other samples of *G. watsonii*.

Ten new strains were identified within the order Tremellales (Figure 8), eight of which were isolated from *R. elegans* and are identified as *Tremella macrobasidiata*, while the other two were isolated from *L. cancriformis* and *R. elegans*, and were placed within the Yeast lineage II *sensu* Millanes et al. (2011), which still needs to be formally described.

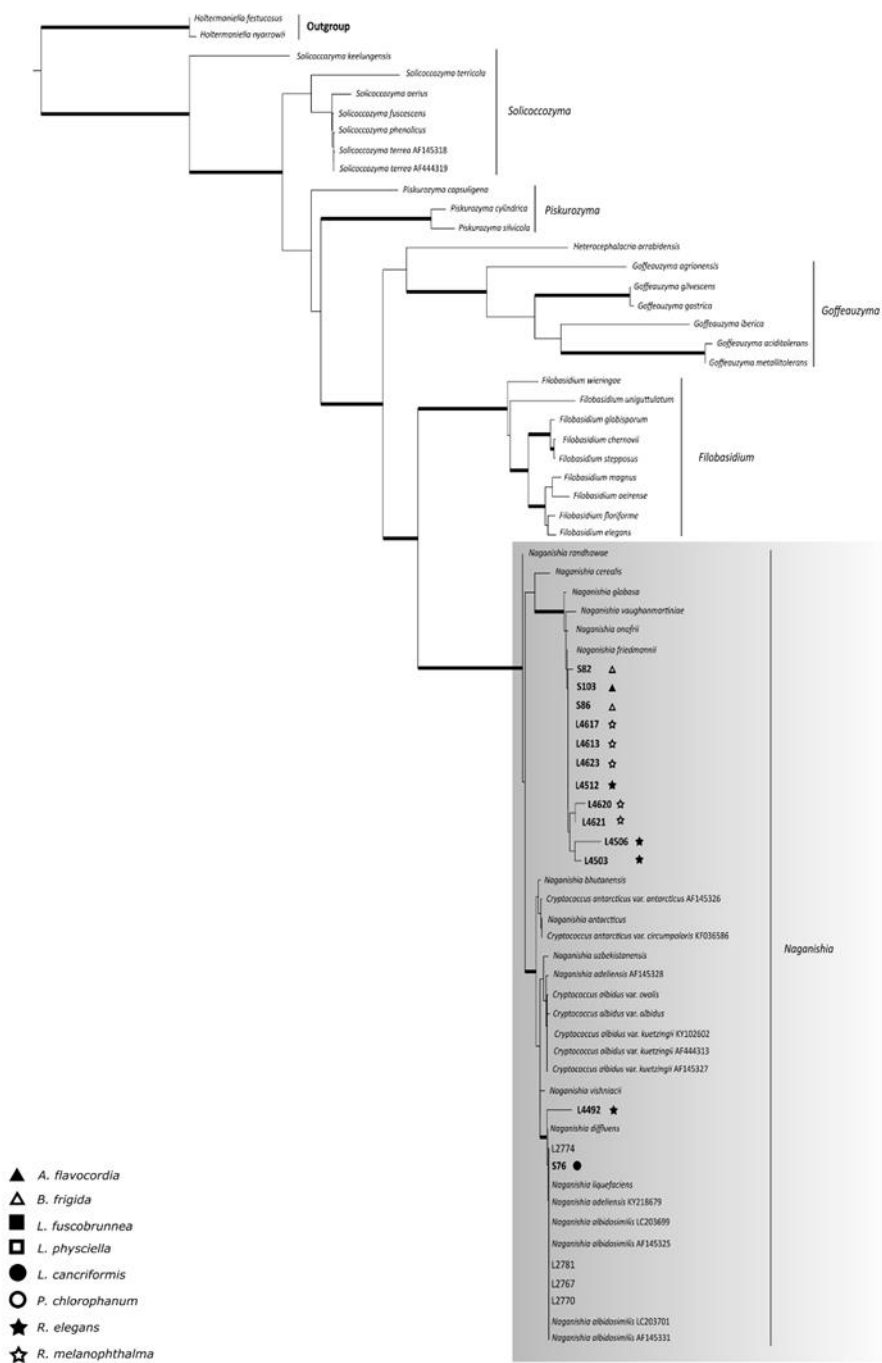


Figure 4.6. Phylogenetic inference of Filobasidiales genus based on nuclear ITS dataset. Branches in bold denote RAxML bootstrap support >75% and Bayesian posterior probabilities ≥ 0.9 . Newly obtained sequences are in bold and enclosed in a grey box. Filobasidiales clades are named according to Boekhout et al. (2011) and Liu et al. (2015a,b). Symbols next to the strain number correspond to the lichen species from which they were isolated (see legend).



Figure 4.7. Phylogenetic inference of Microbotryomycetes genus based on nuclear ITS dataset. Branches in bold denote RAxML bootstrap support >75% and Bayesian posterior probabilities ≥ 0.9 . Newly obtained sequences are in bold and enclosed in a gray box. Microbotryomycetes clades are named according to Yurkov et al. (2016) and Kachalkin et al. (2019). Symbols next to the strain number correspond to the lichen species from which they were isolated (see legend).

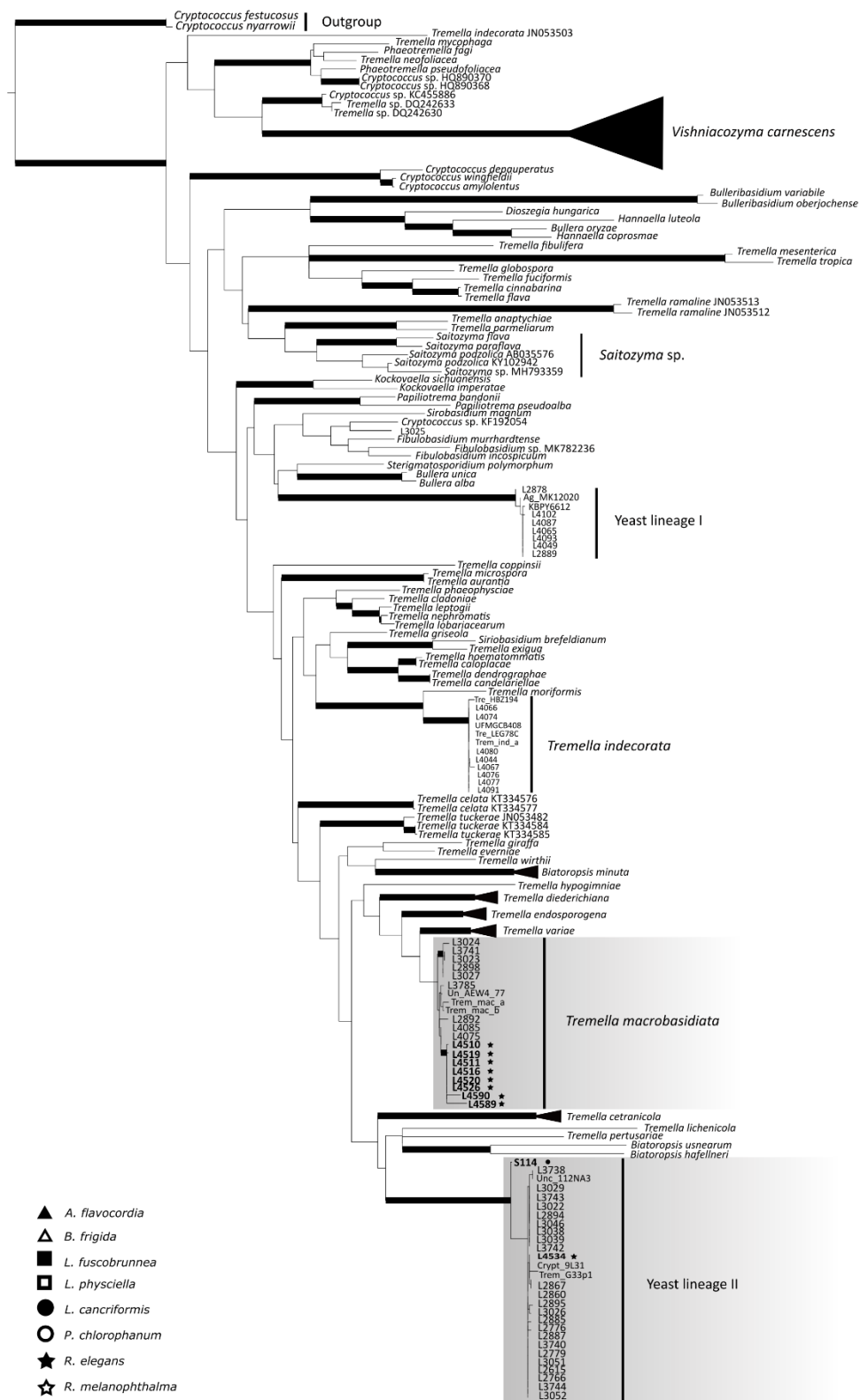


Figure 4.8. Phylogenetic inference of Tremellales genus based on nuclear ITS dataset. branches in bold denote RAxML bootstrap support >75% and Bayesian posterior probabilities ≥ 0.9 . Newly obtained sequences are in bold and enclosed in a gray box. Tremellales clades are named according to Duarte et al. (2016), Millanes et al. (2011), Scorzetti et al. (2000), and Zamora et al. (2017). Symbols next to the strain number correspond to the lichen species from which they were isolated (see legend).

4.3.2 Algal Diversity

Out of the eight lichen species studied, we were able to obtain new isolates of microalgae from *Lecanora physciella*, *Lecidea cancriformis*, *Pleopsidium chlorophanum*, *Rhizoplaca melanophthalma*, and *Rusavskia elegans*. In total, we succeeded in generating new 39 ITS and 47 RbcL sequences, which were used to identify the isolated strains and the photobionts directly amplified from the thallus samples.

The phylogenetic analysis, based on the *RbcL* locus, revealed that all the photobiont sequences amplified from the lichen thalli, belonged to the genus *Trebouxia* (Figure 9). Specifically, we found that five sequences were associated with CLADE 'I', nine to CLADE 'S', and twenty-nine to CLADE 'A'. Other 13 new sequences were obtained from the axenic algal cultures isolated from the lichen samples. Among these, ten were identified as belonging to the genus *Trebouxia*. Seven of them were isolated from *L. cancriformis* (L4648, L4644, L4657, L4647, L4655, L4645, L4656), two from *R. elegans* (L4639, L4665), and one from *R. melanophthalma* (L4652). The remaining four were associated with the genera *Myrmecia* (L4651) and *Chloroidium* (L4653, L4650, L4649). The strain L4651 was placed basal to the clade formed by *Myrmecia* sp. OB14.461, *Myrmecia* sp. SH2018 and *Myrmecia biatorellae*. The strain L4653 was closely related to *Chloroidium antarcticum*, while L4649 and L4650, isolated from the same thallus (L4440) and likely representing the same genetic strain, are sister to the clade formed by the three species *C. ellipsoideum*, *C. lichenum* and *C. angustoellipsoideum*.

To better understand the diversity inside the genus *Trebouxia* and compare it with previous studies (Muggia et al., 2020a; De Carolis et al., 2022), we further performed the phylogenetic analysis based on the ITS locus (Figure 4.10). Twenty of the *Trebouxia* sequences amplified from the thalli of *R. elegans*, *R. melanophthalma* and *L. physciella*, were identified as the lineage *Trebouxia* A02, nine sequences obtained from *P. chlorophanum* and *R. melanophthalma* were included in the lineage *Trebouxia* S02, while four sequences derived from other thalli of *R. melanophthalma* were identified as *Trebouxia impressa*. Seven ITS sequences obtained from axenic cultured strains isolated from *L. cancriformis*, formed a new, well-supported lineage closely related to *Trebouxia* A13.

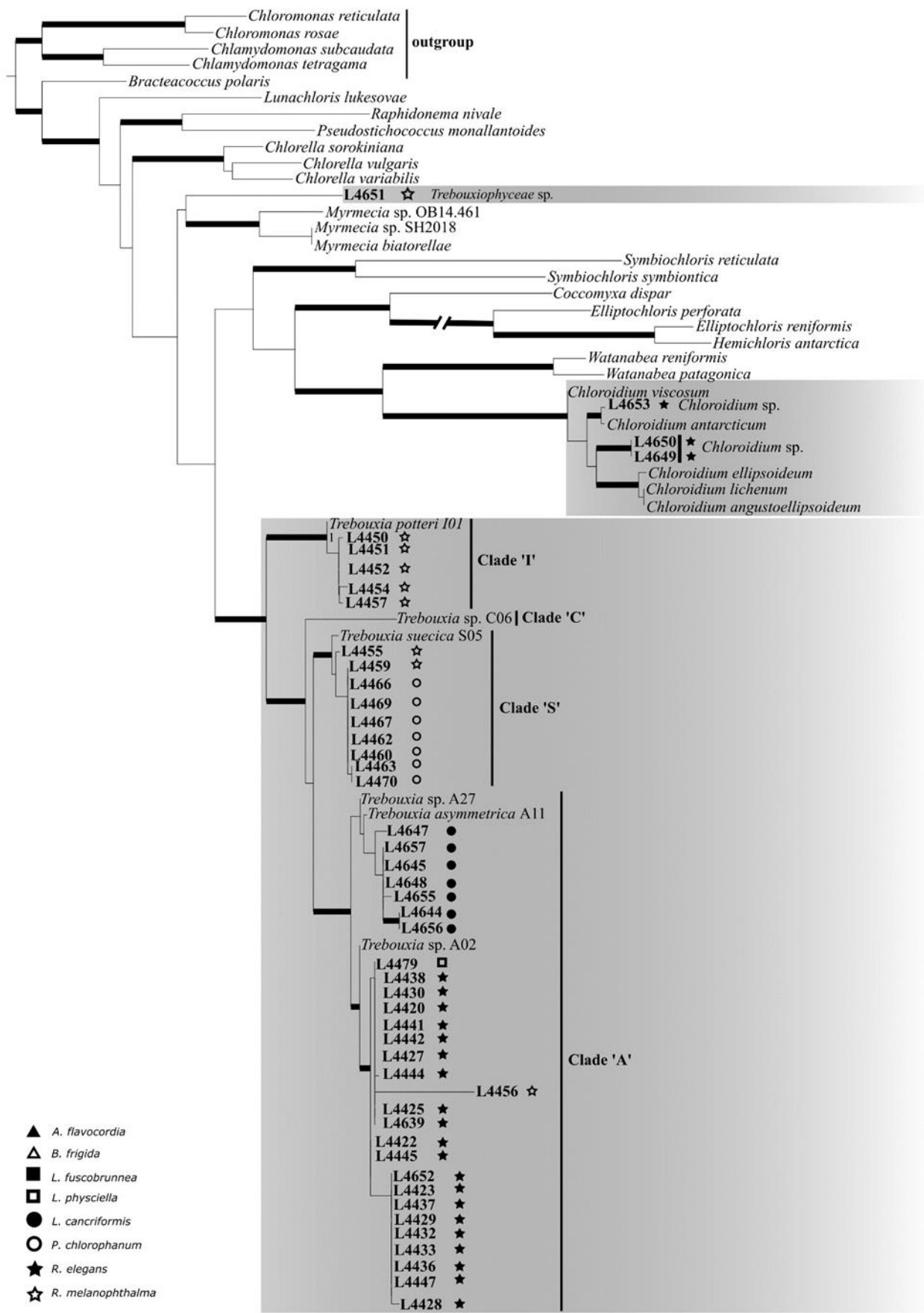


Figure 4.9. Phylogenetic inference of algal strains genus based on RbcL dataset. branches in bold denote RAxML bootstrap support $>75\%$ and Bayesian posterior probabilities ≥ 0.9 . Newly obtained sequences are in bold and enclosed in a gray box. *Trebouxia* clades are named according to Muggia et al. (2020a). Symbols next to the strain number correspond to the lichen species from which they were isolated (see legend).

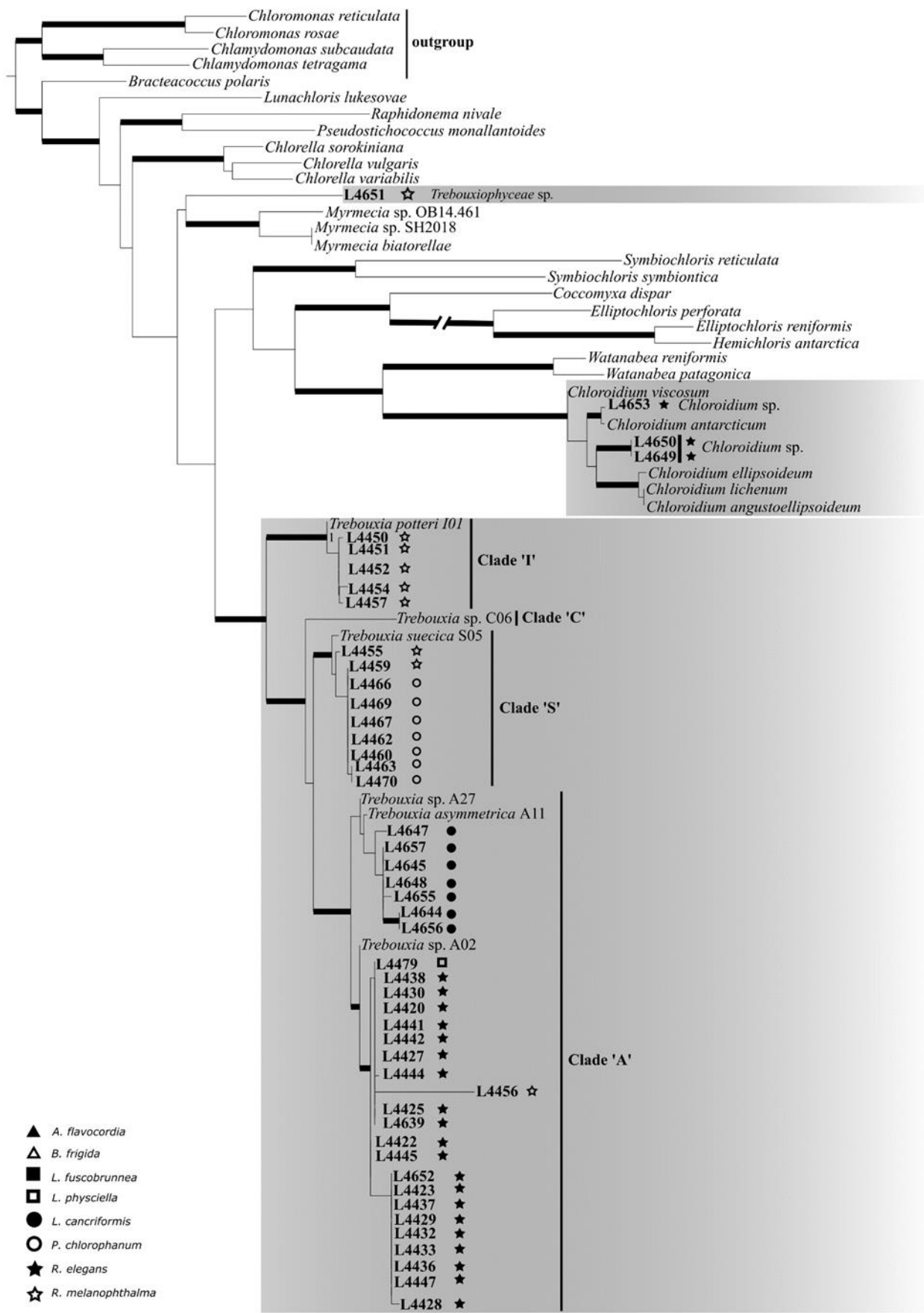


Figure 4.10. Phylogenetic inference of algal strains genus based on nuclear ITS dataset. branches in bold denote RAxML bootstrap support >75% and Bayesian posterior probabilities ≥ 0.9 . Newly obtained sequences are in bold and enclosed in a gray box. *Trebouxia* clades are named according to Muggia et al. (2020a). Symbols next to the strain number correspond to the lichen species from which they were isolated (see legend).

4.3.3 Taxonomy

The definitive formal description of new species will be included in the final manuscript after publication of this thesis.

Type species: ***Petrophila complexa***, (preliminary figure table is shown in Figure 14)

MycoBank: MBXXX

Holotype: MUT XXX = MNA-CCFEE 6751 cultured strain, isolated from lichen thallus of *Lecidea cancriformis* collected in Campo Icaro, Antarctica. The culture is preserved in a metabolically inactive state at -150 °C.

Petrophila complexa MUT XXX is the unique identifier of the holotype sheet in the Mycotheca Universitatis Taurinensis (MUT), Department of Life Sciences and Systems Biology, University of Turin.

Etymology: the species is named after the unusual multiple structures that the fungus may display, despite being included in the group of Black Fungi, known for their very poor morphology.

Lichen-associated, asexual fungus. Strictly psychrophilic, optimal growth temperature 15°C, unable to grow over 20°C. Colonies black obverse and reverse, clumpy, slightly velvety, irregular, not flat margin. Very slowly growing *in vitro*, attaining 3-5 mm diameter in 1 month at optimal 15°C (Fig. 1A). Filamentous hyphae, septate, smooth, 3–3.5 µm wide, pale brown pigmented. Occasionally more or less spherical, smooth- and thick-walled, dark-brown terminal clamidospores up to 6-7 µm in diam with a basal septum were observed (Fig. 1B). Torulose hyphae with thick cell wall 4.5 µm wide, from brown to dark brown (Fig. 1C), showing polar growth by enteroblastic elongation with lateral branching (Fig. 1D). Intercalar or terminal swollen cells 7.5 µm wide, eventually resulting in endoconidia formation, 3.2 µm wide (Fig. 1D-F), occasionally seceding rexolithically or evolving in resistant structures, globose or sub-globose (7-8 µm wide), with very thick cell wall (Fig. 1I,J). Endoconidia globose 4.5-10 µm once liberated (Fig. G,H). Meristematic growth present (Fig. 1K). Conidiophores erect, micronematous, with intercalary or terminal conidiogenous cells producing 1 celled conidia (Fig. 1L).

Distribution: Continental Antarctica, isolated from *Lecidea cancriformis*, Campo Icaro and Prior Island, Northern Victoria Land.

Notes: as reported for *P. incerta*, *P. complexa* displays intercalary conidiogenous cells procuding 1 celled conidia. Unlike *P. incerta*, *P. complexa* displays endoconidiation and meristematic growth. *P. complexa* is also peculiar for its ecology and distribution, differently from *P. incerta*, occurring on rocks in Spain, it occurs associated with lichen thallus in Antarctica.

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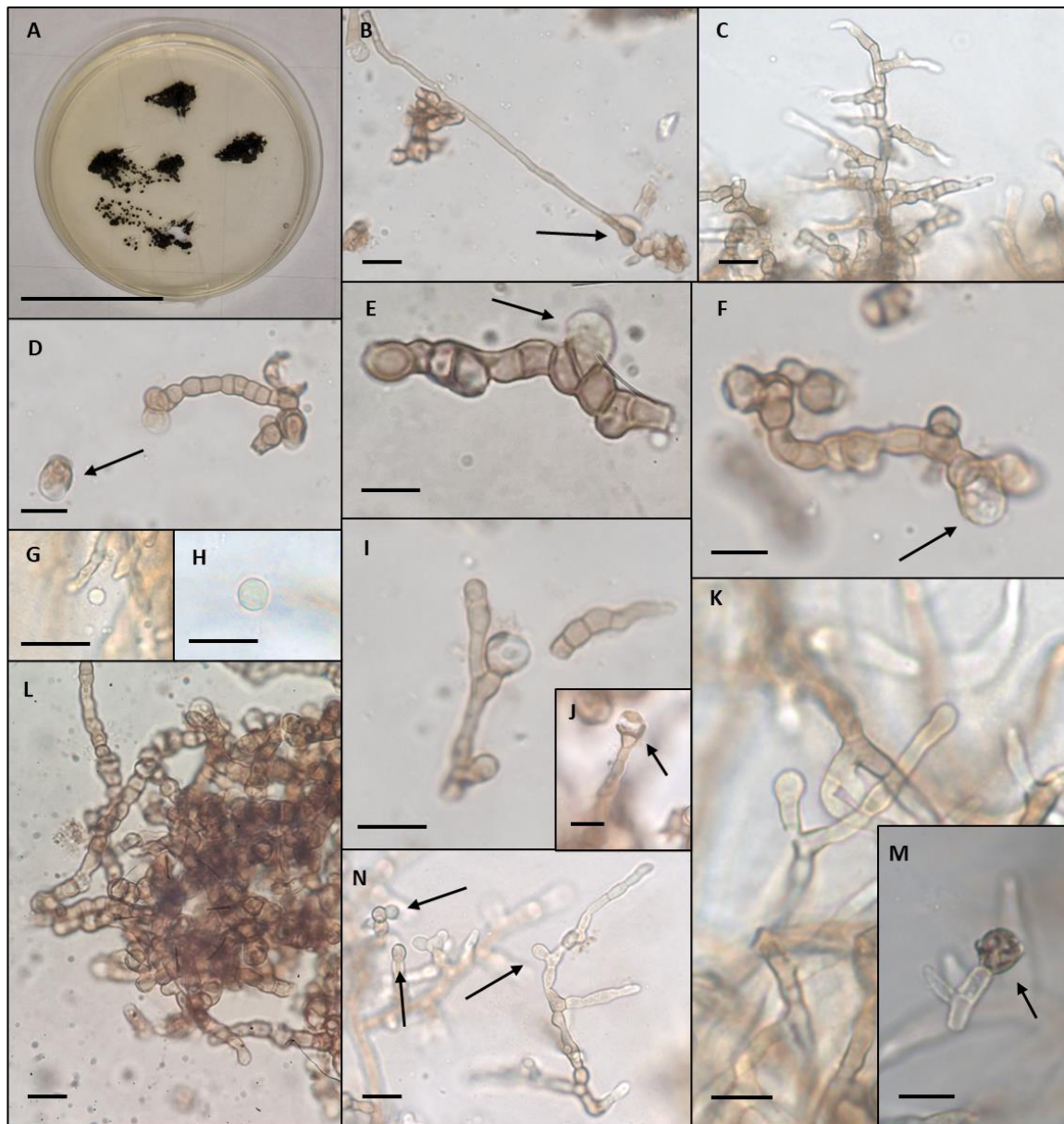


Figure 4.11. Morphological characters of *Petrophila complexa* analyzed in the isolated strains MNA-CCFEE 6751, MUT XXXX; *Petrophila complexa* colonies cultured onto Malt Extract Agar medium **A**. Filamentous hyphae with terminal clamidospore (arrow) **B**. Enteroblastic proliferation and branching by lateral enteroblastic elongation **C**. Torulose hyphae with thicker cell wall 4.5 µm wide and lateral or terminal swollen cells occasionally seceding rexolithically or evolving in resistant structures, globose or sub-globose (arrows) **I,J,K,N** (arrows) **D-F**. Endoconidia globose 4.5-10 µm once liberated **G,H**. Meristematic growth present **L**. Conidiophores erect, micronematous, with intercalary or terminal conidiogenous cells producing 1 celled conidia (arrows) **N**. Scale bars: **(A)** 5 cm; **(D,E,F,I,J,K,M)** 10 µm; **(B,C,G,H,L,N)** 20 µm.

Dactylospora, sp. nov. (preliminary figure table is shown in Figure 14)

Mycobank: MBXXX

Etymology:

Holotype: L4632 cultured strain, preserved in metabolically inactive state in MY medium (September 2022, date at which it were first identified in culture isolation), isolated

from the thallus of *R. elegans* L4428; TSBXXX).

Description: endolichenic (i.e., cryptically present in lichen thalli) fungus derived likely from hyphae fragments or resting spores entrapped in the thalline matrix of the lichen hosts, growing in vitro rather fast. The mycelium is composed by a dense aggregate of hyaline hyphae that builds a whitish-creamy colony with irregular margin, sometimes plaques are present. Most of the hyphae are composed by cylindrical cells ($2 \times 15\text{--}20\text{ }\mu\text{m}$) from which branches generate (Figure 12). Pachybasium-like conidiophores (conidia-laden) and slightly oval amenospora-type spores (3-4 µm long) were observed.

Distribution: reported for the first time as isolated from lichens growing on siliceous-granitic, and sandstone rocks in Victoria Land, Antarctica (sites: Random Hills, Star Nunnatak, Edmonson Point). Isolated so far from the following lichen species: *A. flavocordia*, *R. elegans*. Material examined: ANTARCTICA, Victoria Land, Random Hills on granitic rocks, endolichenic fungi isolated from *A. flavocordia* lichen thalli, strain numbers: S51, S53, S54, S55, S56 and S57. Isolated from *R. elegans* lichen thalli, strain numbers L4632. ANTARCTICA, Victoria Land, Edmonson Point, on granitic rocks, endolichenic fungi isolated from *R. elegans* lichen thalli, strain numbers: L4529 and L4532. ANTARCTICA, Victoria Land, Star Nunnatak, on granitic rocks, fungi isolated from *R. elegans* lichen thallus, strain number: L4593.

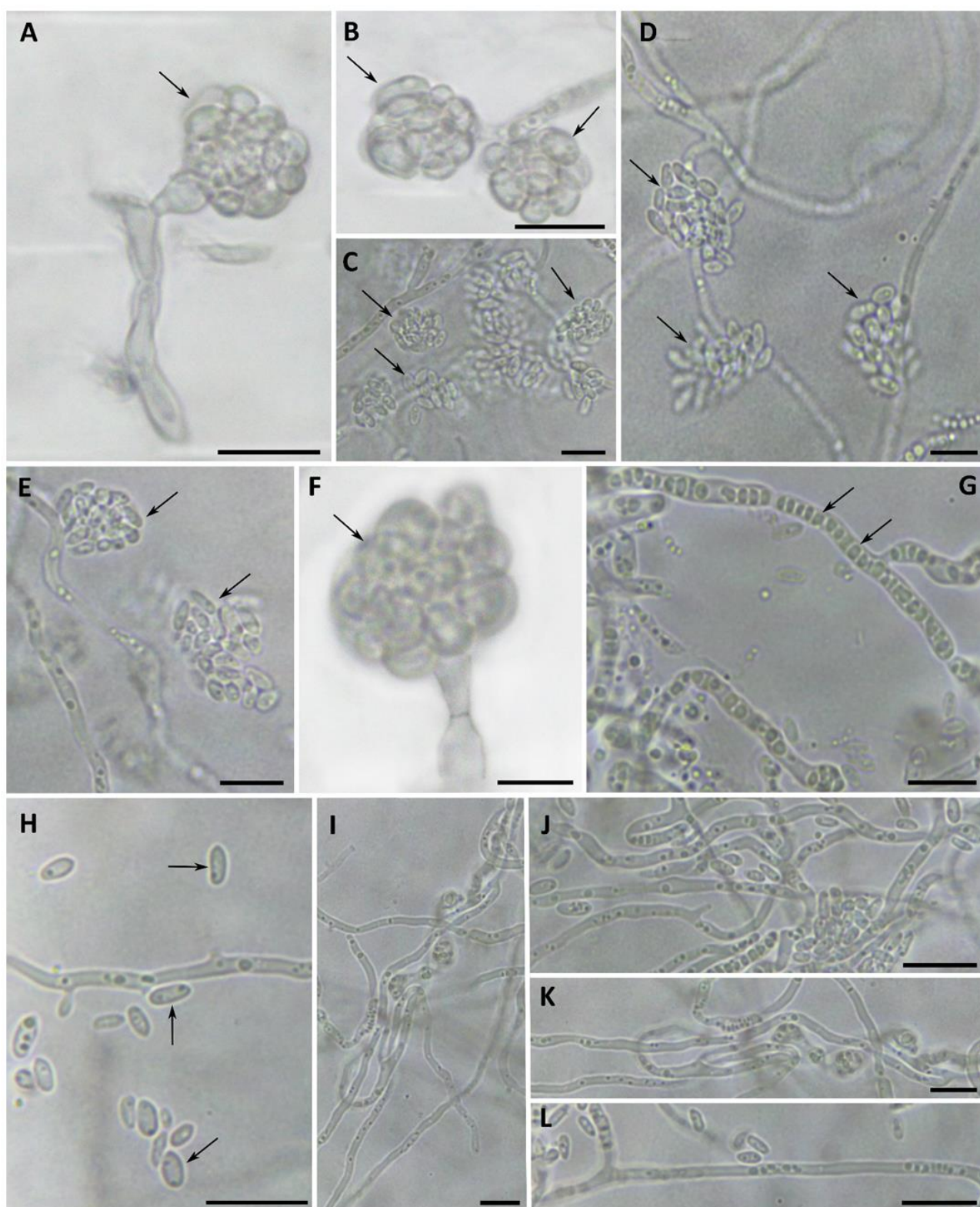


Figure 4.12. Morphology of new clade of *Dactylospora* sp. nov. The studied strains are L4532 (A-D), L4632 (E, F), S38 (G, H), and S51 (I-L). Black arrows (in A-E, H) indicate conidiophores and conidiospores. Scale bars: A-H, J-L) 5 µm; I) 10 µm.

Knufia, sp. nov. (preliminary figure table is shown in Figure)

Mycobank: MBXXXX

Etymology:

Holotype: L4489 cultured strain, preserved in metabolically inactive state in MY medium (September 2022, date at which they were first identified in culture isolation), isolated from the thallus of *R. elegans* (L4420; TSBXXX)

Description: endolichenic (i.e., cryptically present in lichen thalli) fungus derived likely from hyphae fragments or resting spores entrapped in the thalline matrix of the lichen hosts, growing in vitro slowly. The mycelium is composed by a dense aggregate of strong melanized hyphae that builds a black granulose colony with irregular margin. Most of the hyphae are composed by cylindrical or rectangular cells of variable size ($3\text{--}4 \times 5\text{--}10\ \mu\text{m}$) from which branches generate (Figure 13). Budding and ramification were observed.

Distribution: reported for the first time as isolate from lichens growing on siliceous-granitic rocks in Victoria Land, Antarctica (sites: Random Hills). Isolated so far from the following lichen species: *R. elegans*.

Material examined: ANTARCTICA, Victoria Land, Random Hills on granitic rocks, endolichenic fungi isolated from *R. elegans* lichen thalli, strain number: L4489. ANTARCTICA, Victoria Land,

Star Nunnatak, on granitic rocks, fungi isolated from *R. elegans* lichen thallus, strain numbers: L4521. L4543 and L4544.

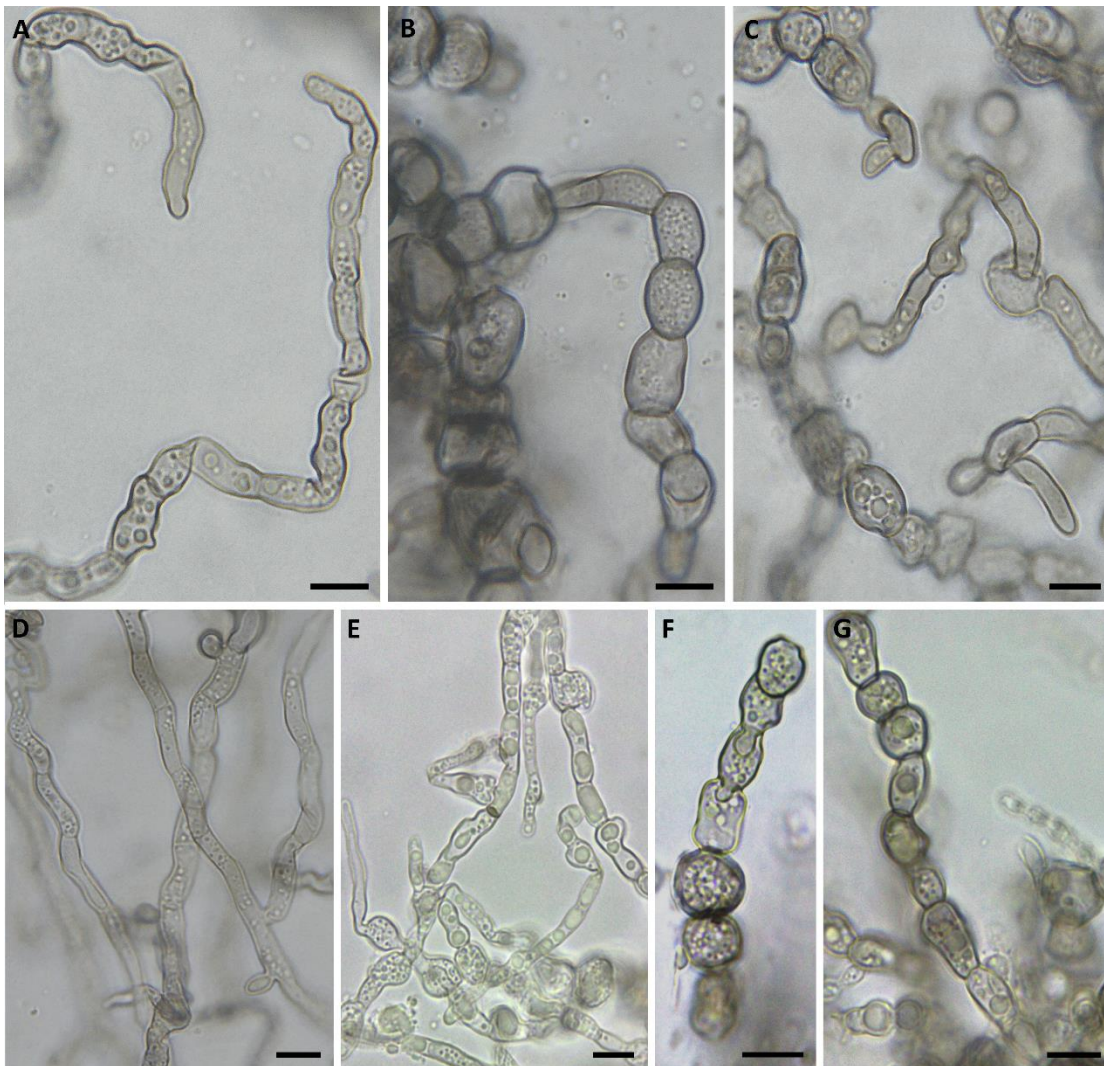


Figure 4.13. Morphology of three strains of new *Knufia* sp. nov., included in the phylogenetic analysis of figure 2. Strains L4489 (A-D), L4543 (E), and L4544 (F-G) are reported. Filamentous, septate, and melanized hyphae (A, D). Branching hyphae signed with white arrows (E, D). Cytoplasmic inclusion white arrows in (E). scale bar 10μm.

Pseudeurotium, sp. nov. (preliminary figure table is shown in Figure 14)

Mycobank: MBXXXX

Etymology:

Holotype: L4625 cultured strain, preserved in metabolically inactive state in MY medium (September 2022, date at which they were first identified in culture isolation), isolated from the thallus of *R. melanophthalma* (L4456; TSBXXX).

Description: endolichenic (i.e., cryptically present in lichen thalli) fungus derived likely from hyphae fragments entrapped in the thalline matrix of the lichen hosts, growing in vitro fast. The mycelium is composed by a dense aggregate of light melanized hyphae that builds a brownish colony with regular margin . Most of the hyphae are composed of cylindrical or rectangular cells of variable size ($3\text{--}4 \times 10\text{--}20 \mu\text{m}$) from which branches generate (Figure 14). Intracellular inclusions were often observed.

Distribution: reported for the first time as isolated from lichens growing on siliceous-granitic rocks in Victoria Land, Antarctica (sites: Random Hills). Isolated so far from the following lichen species: *R. melanophthalma*.

Material examined: ANTARCTICA, Victoria Land, Random Hills on granitic rocks, endolichenic fungi isolated from *R. melanophthalma* lichen thalli, strain numbers: L4523, L4524, L4602, L4603, L4604, L4605, L4606, L4608, L4609, L4610, L4611, L4612, L4614, L4616, L4622, L4624, L4625, L4626, L4636.

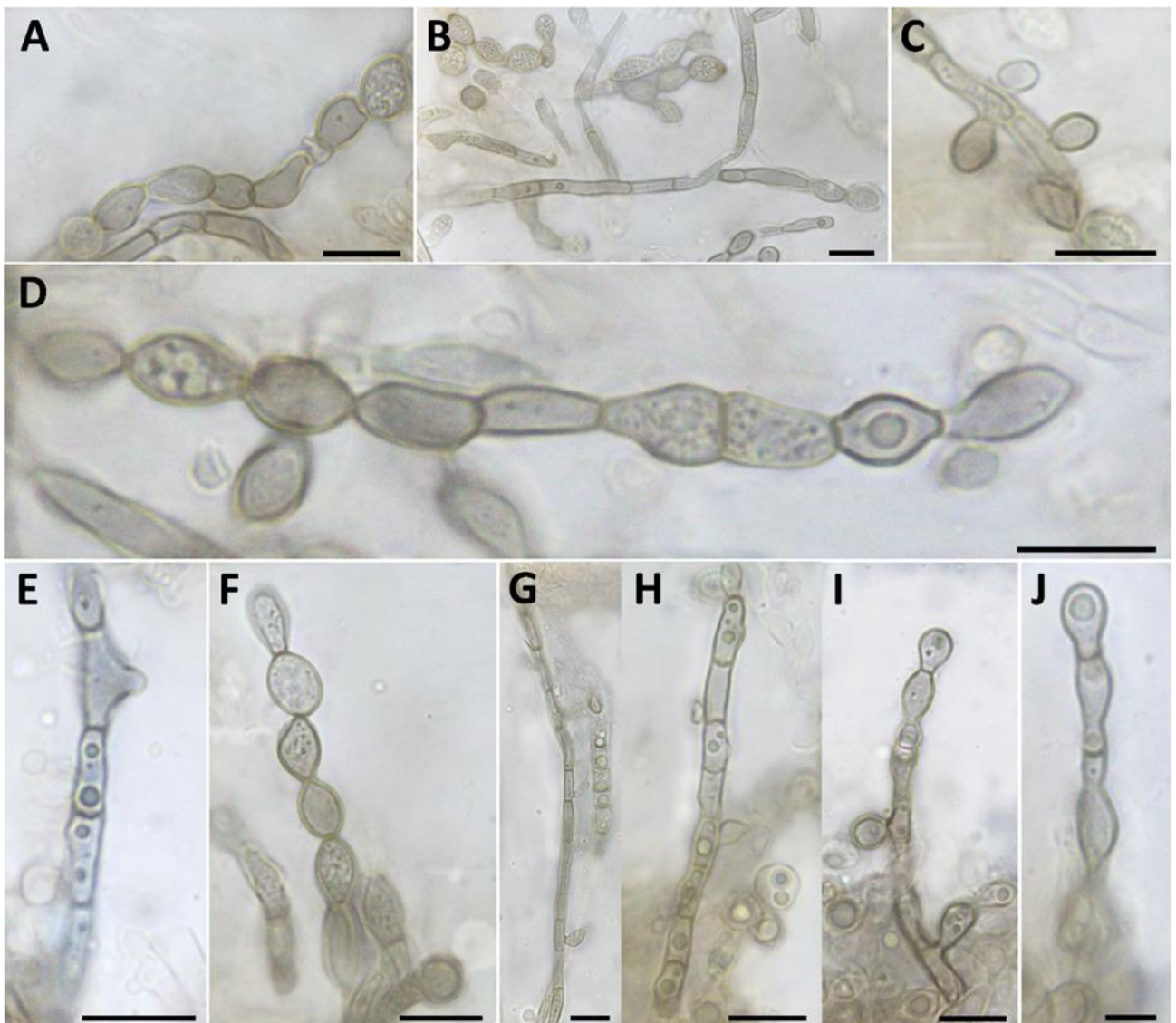


Figure 14. Morphology of two strains of *Pseudeurotium* sp. nov. included in phylogeny figure 3. Strains photographed are L4525 (A-D) and L4625 (E-J). Scale bars: A-J) 5μm.

4.4 Discussion

High throughput sequencing has greatly improved the study of the lichen mycobiome by making available a large amount of data regarding the diversity of lichen-associated fungi, i.e. the mycobiome. Still, these ‘new generation’ molecular techniques are affected by amplification biases and primer mismatches that may let some taxa undetected, but will likely be overcome by future metagenomic studies. Thus, an integrative taxonomic approach, in which molecular data are complemented by culture-dependent methods still allows to reach a more comprehensive understanding of the hidden biodiversity. For that it concerns fungi of the lichen mycobiome, the isolation of environmental strains in axenic culture is already a well-established application, that has proven to be fundamental for a reliable description and characterization of those new species cryptically inhabiting the lichen thalli (Girlanda et al., 1997; Arnold et al., 2009; Muggia et al., 2016; Smith et al., 2020; Cometto et al., 2022), but not always detected by the amplicon sequencing method. Here we were able to culture more than 300 fungal and algal inocula from thalli of five Antarctic endemic lichen species (i.e., *A. flavocordia*, *B. frigida*, *L. fuscobrunnea*, *L. physciella*, *L. cancriformis*), and three cosmopolitan species (i.e. *P. chlorophanum*, *R. melanophthalma* and *R. elegans*) collected in the Victoria Land in Antarctica. Among the isolated fungi we recognized species already described in previous studies (Selbmann et al., 2008, 2013; Egidi et al., 2014; Cometto et al., 2022, 2023), as well as five taxa that are here firstly recognized at the species level. Most sequences belonged to the phylum Ascomycota, particularly to the big fungal classes Dothideomycetes and Eurotiomycetes, in which already most of the fungi isolated from epilithic lichens were recognized so far (Selbmann et al., 2013b; Muggia et al., 2016, 2020b; Fernandez-Mendoza et al., 2017; Cometto et al., 2023). The metabarcoding analyses performed on the same lichen samples (see Stoppiello et al., submitted) showed that Basidiomycetes were present in less amounts than ascomycetes (in line with other studies, e.g., Smith et al., 2020; Lendemer et al., 2019; Cometto et al., 2022), and indeed a corresponding lower number of Basidiomycetes isolates was obtained. However, this time a substantial amount of Basidiomycetes yeasts grew in culture. This may be hypothesized because of a lower inter-species competition of fewer fungi within the thalli due to the extreme and selective environmental conditions. Yeasts are known to adopt strategies to survive and thrive in extreme environments, such as production of cold-active enzymes, ‘anti-freeze’ compounds and extracellular polymers (Siddiqui and Cavicchioli 2006; Turk et al., 2011; Buzzini et al., 2012; Gunde-Cimerman et al., 2014), and this would facilitate their permanence within the lichen thalli in Antarctica. A key role could be also played by their production of exopolysaccharides that would make them also more resistant toward the lichen secondary metabolites (Spribille et al., 2016).

4.4.1 Ascomycota diversity

The phylum Ascomycota is the most represented within the lichen mycobiomes in general (Zhang et al., 2015, 2016; Fernández-Mendoza et al., 2017; U'Ren et al., 2010, 2012; Banchi et al., 2018). So far, the classes found most frequently associated to epilithic lichens were Eurotiomycetes, Dothideomycetes and Leotiomycetes, with a great majority of taxa representatives of the polyextremotolerant black fungi (especially from the order Capnodiales and Chaetothyriales; Selbmann et al., 2013b, Santiago et al., 2015, Muggia et al., 2021; Cometto et al., 2023). Black fungi have evolved adaptations that allow them to survive in environments such as Antarctica or high mountain altitudes (Onofri et al., 2007; Selbmann et al., 2015; Coleine et al., 2021; De Los Ríos et al., 2014, Cometto et al. 2022). Here we report four new species of ascomycetes inhabiting the lichen thalli, highlighting that there is a still unknown diversity to be uncovered. The species isolated from our samples belonged to the classes Dothideomycetes, Eurotiomycetes and Leotiomycetes. Within Eurotiomycetes in particular, the new taxa were recognized as *Dactylospora* sp. nov. (Dactylosporaceae) and *Knufia* sp. nov. (Chaetothyriales). *Dactylospora* is a genus of fungi comprising saprobic organisms that inhabit wood and liverworts but are also found in lichen thalli (Pang et al. 2014, Jaklitsch et al. 2016). The genus was reported for Antarctica from the South Shetland Islands as a lichenicolous fungus by Alstrup et al. (2018). Here we found it for the first time as lichenicolous fungus on lichen collected in the continental region. The phylogeny of this taxon was resolved by Ekanayaka et al. (2019), with the recognition of the order Dactylosporales (Eurotiomycetes). Our sequences formed a clade fully supported and closely related to *Sclerococcum*, which has been hypothesized to be the asexual form of *Dactylospora* (Ekanayaka et al. 2019). For the first time we were able to isolate *Dactylospora* strains in culture and we were able to analyze the morphology. These strains present hyaline conidiospores, that substantially differ from those described previously for other *Dactylospora* species, i.e. uniseptate dark brown spores (Olech et al. 1996, Pang et al. 2014, Alstrup et al. 2018, Ekanayaka et al. 2019). Though, in Dobbeler et al. (2017) atypical not-septate spores were reported in the species *Dactylospora inopina*, and it might be that in the isolated strains we observed spores in an immature stage.

During the analysis of the Chaetothyriales, it also emerged the presence of the two species *Paracladophialophora lichenicola* and *Cladophialophora lichenicola* recently isolated and described from lichens growing in extreme environments (Cometto et al. 2023). These fungi turned out to be strictly lichenicolous and were described only as isolates from lichen thalli coming from different sites in Europe and South America. Their presence among the isolates from Antarctica, and furthermore their isolation from lichen species endemic of Antarctica, i.e. *Acarasopora flavocordia*

and *Buellia frigida* further strengthens their association to lichen thalli, and highlights how they have differentiated genetically from the already reported strains. Indeed, as it emerges in the phylogenetic analyses, the strains of both *P. lichenicola* and *C. lichenicola* from Antarctica have their own nucleotide polymorphisms in their ITS sequences that segregate them in their own clades. This result further suggests that lichen thalli are pivotal systems in extreme environments, where they act as niches for the evolution and preservation of biodiversity .

The genus *Knufia* (Chaethotyriales), includes endolithic and saprotrophic species in general (Tsuneda et al., 2011; He et al., 2013; Edmane et al., 2022; Mehrabe et al., 2018) and its presence within the lichen-dominated endolithic communities in Antarctica was already known and confirmed by culture isolation (Selbmann et al. 2013a, Coleine et al. 2018); however, here *Knufia* was found to be one of the most abundant genera among the isolated endolichenic fungi. *Knufia* was abundantly found also using an amplicon sequencing approach by Stoppiello et al. (here chapter 2), and it is already reported multiple time as lichen isolate (Selbmann et al., 2013b; Reblova et al., 2013; Cometto et al., 2023). Altogether these results confirm its ability to inhabit lichen thalli. Most of the newly isolated strains were recognized to be the endolithic species *Knufia separata*, recently described by Sun et al. (2020) as rocks inhabiting fungi in China. Interestingly, this species has been isolated from lichen thalli from Antarctica and from thalli of *R. melanophthalma* from South and North America collected at high elevation above 3.000 m (Cometto et al., 2022). The ecology of *Knufia separata* confirms its nature as an extremophilic fungus (Sun et al., 2020), but the new species *Knufia* sp. nov. isolated from thalli of *R. elegans* is a major result and hints to a further differentiation of this genus into new endolichenic taxa, likely exploring new niches for their evolution.

We recognized a new clade also within Leotiomycetes, representing the new species *Pseudeurotium* sp. nov (Thelebolaceae). The presence of this genus and its family Thelebolaceae in the Antarctic continent was reported for the mycobiota of permafrost and maritime Antarctic region especially in glaciers and aqueous environments, such as lake sediments, glacier layers or peninsula region (Santiago et al., 2015, Ogaki et al., 2020; Santos et al., 2020; da Silva et al., 2020; Quijada et al., 2022). Our phylogenetic analyses reveal that this new clade of *Pseudeurotium* is closely related to *Antarctomyces* (de Menezes et al., 2017) and *Thelebolus*, thus evidencing the diversification of this lineage in Antarctica. The phylogenetic placement of this new lineage inside Thelebolaceae might be better solved in upcoming studies, as the phylogeny of the family is so far unresolved and needs further analyses (Quijada et al., 2022). The presence of this taxon inside the lichen thalli could let assume its parasitic lifestyle towards the lichen photobionts, as species of *Pseudeurotium* outside the Antarctic regions have been reported as parasites of algae and plants (Manzotti et al., 2020, Quijada

et al., 2022). Whether *Pseudeurotium* develops a parasitic lifestyle toward the lichen photobiont(s) could be tested with co-culture experiments using the available strains in the future.

Our phylogenetic inference has highlighted a well supported and separated clade in the genus *Petrophila*, order Capnodiales, here described as the new species *Petrophila complexa*. The genus *Petrophila* Hoog and Quaedvlieg gen. nov., with the sole new species *P. incerta* de Hoog and Quaedvlieg sp. nov., were introduced for the first time in 2014 (Egidi et al., 2014). The strains were isolated from rocks collected in the Mediterranean area, Mallorca, Spain. In this study, the genus *Petrophila* was found for the first time in Antarctica; the new species here described shows, in addition to a different geographic distribution, also a different ecology respect to *P. incerta*, being associated exclusively to lichen thalli of the endemic lichen species *L. cancriformis*.

4.4.2 Basidiomycota diversity

Basidiomycetes yeasts are an important component of the lichen mycobiota (Spribille et al., 2016, 2018; Cometto et al., 2022); so far, some taxa could be isolated in cultures and described from boreal lichens and lichens from harsh, high altitude mountain environments (Černajová and Skaloud, 2019, Cometto et al., 2022). The culturable fraction of the Basidiomycetes mycobiota of Antarctic lichens could be studied here for the first time and interestingly we found again a few taxa previously isolated (Cometto et al., 2022). Culturable Basidiomycetes yeasts from Antarctic lichen thalli are majorly represented by Cystobasidiomycetes, Tremellomycetes, Agaricostilbomycetes, and Filobasidiales. Most of the newly isolated strains were identified as *Cystobasidium laryngis* (Cystobasidiales) and were closely related to other two species, i.e. *Cystobasidium ritchiei* and *C. pinicola*, that are known to be psychrophilic (Turchetti et al., 2018). The new strains of *C. laryngis* were isolated from three Antarctic endemic (*A. flavocordia*, *B. frigida*, *L. cancriformis*) and two cosmopolitan species (*R. elegans* and *R. melanophthalma*), suggesting that the occurrence of this yeast species is more dependent on the cold environmental conditions than on the ecology and distribution of the lichen hosts, as it was not isolated before from thalli of *R. melanophthalma* collected outside Antarctica (Cometto et al., 2022).

Within Agaricostilbomycetes the new species of *Kurtzmanomyces* sp. nov. was represented by nine strains that formed an own lineage with other four strains isolated previously by Cometto et al. (2022) from the lichens *Tephromela atra* and *R. melanophthalma* from the Italian Alps and North America, respectively. This result strengthens the assumption that there are certain strains particularly associated to the lichen thalli and distributed globally, and that deserve species description and

taxonomical recognitions as cryptic lichenicolous fungus, in the same way as already done for some cryptically occurring ascomycetes (Muggia et al., 2021a, Cometto et al., 2023)

The genus *Naganisha* is also confirmed to occur in Antarctic lichens with the species *N. friedmannii* and *N. albidosimilis*. Both species are psychrophilic as their relatives, were originally described from Antarctica, and were already found in Antarctic soil and in lichen thalli of *Usnea antarctica*, *U. aurantiacoater* and *Ramalina terebrata* (Bab'eva and Golubev, 1969; Vishniac, 1985; Montes et al., 1999; Pavlova et al., 2002; Thomas-Hall et al., 2002; Thomas-Hall et al., 2010; Duarte et al., 2013, 2016).

Species of the genus *Tremella* are known to be important lichen parasite distinguishable on the lichen thalli by their galls, in which the reproductive structures (basidia with basidiospores) are formed (Millanes et al., 2011, 2012, 2014, 2015). However, Cometto et al. (2022) already reported the cryptic presence of *Tremella* in lichens and here we also confirm it, as *Tremella macrobasidiata* was isolated from thalli of *R. elegans* devoid of any symptom of infection. Interestingly, so far *T. macrobasidiata* was found strictly associated with *Lecanora* lichen species (Zamora et al., 2011, 2016; Tuovinen et al., 2021). Its cryptic presence in thalli of species other than *Lecanora*, could be explained by its dimorphic state, as it would be present as yeast cells among the mycobiont hyphae, instead forming a filamentous mycelium and developing the typical galls. It may be hypothesized that the yeast growth is preferred by this fungus under harsh climatic/environmental conditions and thus it could occur cryptically and unspecific in different lichens.

4.4.3 Photobiont diversity

Lichens in Antarctica are subjected to several factors that limit the photosynthesis of their photobiont, such as low temperatures, lack of water, and high levels of UV radiation. Choosing a partner that can somehow mitigate these dangers is crucial for the survival of the lichen itself (Determeyer-Wiedmann et al., 2019). Studies on the diversity of photobionts in Antarctic lichens have previously shown that the genus *Trebouxia* Puymaly is the most frequently found as lichen photobiont in these environments (Ruprecht et al. 2012, 2020; Wagner et al., 2020; Pérez-Ortega et al., 2012, 2023). Also in the analysed samples a clear predominance of *Trebouxia* was found. This result is supported by the identification of the photobionts by both ITS and RbcL markers. Most of the ITS sequences obtained (48%) belong to the *Trebouxia* A02 lineage, which has already been found as the main photobiont in Antarctic lichens (Pérez-Ortega et al. 2012; Ruprecht et al., 2012, 2020; Wagner et al., 2020 and De Carolis et al., 2022). *Trebouxia* A02 associates as photobiont to many mycobiont species worldwide,

such as *Austrolecia*, *Buellia*, *Carbonea*, *Huea*, *Lecanora*, *Lecidea*, *Lecidella*, *Rhizoplaca* (Wagner et al., 2020); *Acarospora*, *Caloplaca*, *Polysporina*, *Sarcogyne*, and *Umbilicaria* (Pérez-Ortega et al., 2012); *R. melanophthalma* and *Tephromela* (De Carolis et al., 2022), and in this study we report it as the main photobiont of *R. elegans* and *L. Physciella*. However, due to the lack of axenic cultured strains needed for the characterization of morphological and ultrastructural traits, this lineage still miss a formal species description.

The lineage *Trebouxia* S02 and *T. impressa* were the second most frequently recovered photobiont in the studied thalli. *Trebouxia* S02 has been already known for its occurrence in alpine environments, likely for its capacity to cope well with drought conditions, and thus able to thrive in the Antarctic environments (Blaha et al., 2006; Garrido-Benavent et al., 2020; Ruprecht et al., 2012, 2020). This lineage has been previously reported for *T. atra* and *R. melanophthalma* as well (Muggia et al., 2010, 2014b; De Carolis et al., 2022), while here we report it for the first time for *P. cholorophanum*. It is worth noting that the diversity of *Trebouxia* species found in the here selected lichens from Antarctica is very similar to that reported by De Carolis et al. (2022) for lichens from very high elevation. Indeed the three lineages *Trebouxia* A02, *Trebouxia* S02 and *T. impressa*, seem to be the best adapted and most present in harsh conditions, suggesting them as the best competitive lineages of *Trebouxia* in such extreme environments.

A new *Trebouxia* lineage was isolated from the thalli of *Lecidea cancriformis* and likely represents a new species. However, the strains grow relatively slow in culture and only a future morphological and ultrastructural characterization could support and confirm its monophyly. Certainly a description of a further *Trebouxia* species would highlight the extraordinary diversity and the evolutionary success of this genus as lichen photobiont.

Only four RbcL sequences revealed the presence of *Myrmecia* (L4651) and *Chloroidium* (L4653, L4650, L4649). The *Myrmecia* (L4651) strain was isolated from a thallus (*R. melanophthalma* L4452) of which the thallus sequence corresponded to *Trebouxia Impressa*. On the other way, the genus *Chloroidium* is known from Antarctic soils (Dariencko et al., 2018; Rybalka et al., 2023) and it is likely an epithalline algae unspecifically associated to the epilithic lichens.

4.5 Conclusions

The diversity of the mycobiota of Antarctic lichen species and the associated algae/photobionts is still far from being completed. The present study revealed an additional fraction of that hidden diversity

of fungal and algal species in lichens, this time with a special focus on some crustose and sub-foliose/sub-fruticose species of Antarctic lichens. We presented further evidence that the lichen mycobiota is majorly composed by ascomycetous taxa and that Basidiomycetes are represented by yeast species. Both asco- and Basidiomycetes species seem to select lichens as their preferred niche in Antarctica and worldwide. Indeed, the major representatives of the lichen mycobiota are fungi that do not show a specificity toward a particular lichen host species, rather toward the lichen thallus in general as a structure in which spores, yeast cells and mycelia fragments thrive or rest. All the investigated species have *Trebouxia* as principal photobiont and this highlights the extraordinary diversity and the evolutionary success of this genus as symbiotic partner also under extreme environmental conditions. In Antarctica, where living conditions on rock surfaces are far more selective than elsewhere, lichen thalli would be even more exploited as suitable niches by other fungi and their evolution. The role of lichens as hotspots for microbial diversity and niche for diversification will become fundamental for the study of conservative strategies in Antarctic regions.

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CHAPTER 5. Synthesis and conclusions

In Chapter 1 - we introduced Antarctica, and particularly the Victoria Land. How this area is characterised by extreme climatic events that apply selective pressure on the organisms dwelling in these areas. Here, due to the harsh environmental conditions, vascular plants do not settle and are replaced by cryptogamic communities composed of mosses and, above all, lichens. Moreover, we discussed lichen symbiosis in depth and several species we considered in this work of thesis as: *Buellia frigida*, *Lecidea cancriformis*, *Lecanora fuscobrunnea*, *Acarospora flavocordia*, *Rauvskia elegans*, *Rhizoplaca melanophthalma* and *Lecacora physciella*. Furthermore, these organisms are not only associations between algae and fungi but can also host a wide variety of biodiversity in their thallus, representing a unique microcosm for diversity.

In Chapter 2 - In this study we performed an amplicon-sequencing analysis approach to investigate the lichen associated with bacterial assemblages and, for the first time, with mycobiota. We found that the lichen mycobiota is predominantly composed of *Ascomycota* belonging to the classes *Chaetothyriomycetes* and *Dothideomycetes*, while a few key representative taxa were recognized as basidiomycetous yeasts. On the other hand, bacteria associated with lichens were represented by *Pseudomonadota*, *Cyanobacteria* and *Bacteroidota* in which psychrophilic genera were identified. The microbiota was diverse among the lichen species and their variation is driven by the lichen forming-fungus, i.e. the mycobiont, and/or by their endemic or cosmopolitan distribution. There was, in fact, a strong association of the microbial communities linked to the lichen itself, rather than to the specific characteristics of the collecting site. The lichen thallus, thus, plays an important role in microbial diversification and may potentially act as a selective biodiversity filter in which different fungal and bacterial communities thrive in it.

In Chapter 3 - In this study, we investigated the intricate microbial communities thriving within the endolithic niches of Antarctic granite and epilithic habitats. Using amplicon sequencing, we analyzed 90 samples, including 24 from granite substrates and 66 from 6 different lichen species. Our findings revealed distinct patterns of diversity and composition between these two ecological niches. Endolithic communities exhibited higher microbial diversity, particularly among bacteria, compared to epilithic habitats. The prevalence of lichens in both habitats suggests their pivotal role in shaping these communities. Furthermore, specific fungal and bacterial taxa were identified, highlighting their adaptation to extreme environmental conditions. This study enhances our understanding of microbial ecology in polar regions and emphasises the need for further research to elucidate the mechanisms driving microbial diversity and adaptation in extreme environments.

In Chapter 4 - In this study we investigated antarctic epilithic thalli of five endemic and three cosmopolitan lichen species for their potential role as fungal species niches of diversification. The culturable fraction of the Antarctic lichen-associated fungi and algae was uncovered with the aim to isolate as many culturable species as possible and find whether taxa are strictly connected to the Antarctic environment or are cosmopolitan species that particularly associate to lichens in harsh, extreme environments. Over 300 fungal and algal inocula grew in culture, among which we recognized species previously described as well as five new fungal species of ascomycetes and basidiomycetes in the genera *Dactylospora*, *Knufia*, *Petrophila*, *Pseudeurotium*, and *Kurtzmanomyces*. These species seem to select lichens as their preferred niche in Antarctica and worldwide. Indeed, the major representatives of the lichen mycobiota are fungi that do not show any specificity toward a particular lichen host species, rather toward the lichen thallus in general as a structure in which spores, yeast cells and mycelia fragments thrive or rest. In Antarctica, where living conditions on rock surfaces are far more selective than elsewhere, lichen thalli would be even more exploited as suitable niches by other fungi for their evolution and diversification.

The main findings of this work are: *i)* having established the fungal and bacterial diversity associated to the lichen thalli; *ii)* thallus, more than the site, is determinant for shaping the associated fungal and bacterial assemblages; *iii)* we determined the presence of a potential core of taxa that are uniquely associated with endemic lichen represented by endemic species; *iv)* we have determined that endolithic communities in granite substrates exhibit greater biodiversity compared to the lichens inhabiting the same areas, possibly due to their composition of numerous lichen species beyond the six we examined; *v)* we isolated new taxa and described 5 new species of fungi of the genera of *Dactylospora*, *Knufia*, *Petrophila*, *Pseudeurotium*, and *Kurtzmanomyces*.