UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXXV CICLO DEL DOTTORATO DI RICERCA IN AMBIENTE E VITA

STABILITY AND VARIATION OF LICHEN MYCOBIOME

Settore scientifico-disciplinare: BIO/01

DOTTORANDA
AGNESE COMETTO

COORDINATORE
PROF. GIORGIO ALBERTI

SUPERVISORE DI TESI
PROF.SSA LUCIA MUGGIA

CO-SUPERVISORE DI TESI
PROF. ALBERTO PALLAVICINI

ANNO ACCADEMICO 2021/2022

INDEX

THESIS ABSTRACT

Lichens – as self-sustaining, mutualistic and symbiotic systems – house a multiplicity of microorganisms (prokaryotes, microalgae and microfungi), whose biodiversity and roles in the symbioses are still largely unknown. Until recently, only a few studies focused on the overall diversity of the lichen-associated fungi which constitute the lichen mycobiome. In this research, I characterized the diversity of the lichen mycobiome in two cosmopolitan, epilithic lichens, i.e. *Rhizoplaca melanophthalma* (DC.) Leuckert & Poelt and *Tephromela atra* (Huds.) Hafellner var. *atra,* that were chosen as study organisms. Their mycobiomes were investigated for the first time at a global scale, as lichen samples were collected across their entire geographic range and both a culture-dependent and environmental DNA (eDNA) metabarcoding approaches were performed. I aimed at *i)* characterizing if any stable 'core mycobiome' can be detected for each lichen species and if a shared mycobiome exists; *ii)* understanding the role of the environmental conditions in determining the lichen mycobiome composition.

By culture-dependent approach, I isolated and identified, using an integrated approach based on morphological and phylogenetic data, 76 basidiomycete yeasts (belonging to the five classes Agaricostilbomycetes, Cystobasidiomycetes, Microbotryomycetes, Tremellomycetes and Ustilaginomycetes) and 131 ascomycetes fungi (belonging to the three classes Eurotiomycetes, Dothideomycetes and Sordariomycetes). Furthermore, within the ascomycetes fungi I identified two new lineages in Chaetothyriales. Most of the isolated fungi, either the ascomycetes and basidiomycetes, have been previously found in lichens, or resulted to be taxa closely related to species which usually occupy other ecological niches (i.e. plants, rocks and soil) and which adopt different lifestyles (i.e. plant pathogens, opportunists and saprotrophs).

By DNA metabarcoding, I first tried to design and test a set of blocking primers, which were specifically designed to reduce the amplification of *R. melanophthalma* and *T. atra* mycobionts and to enhance the amplification of all the other fungi present within the lichen thalli. However, these primers did not prevent the amplification of the mycobionts and I carried out the metabarcoding approach without using them. The comparison of the fungal communities highlighted low diversity within single thalli with the presence of few ascomycetes consistently present in both lichen species, mostly belonging to Chaetothyriales, Capnodiales and Helotiales. Basidiomycetes account only for 7-14% of the sequences, mostly from Tremellales. The environmental conditions, especially when harsh (e.g. extreme elevation) and the geographic isolation possibly played the main role in shaping the mycobiome composition, however also the lichen host seemed to influence the mycobiome community, though in a lesser extent. A stable species-specific core mycobiome

was hardly identifiable both in *R. melanophthalma* and *T. atra* as most of the mycobiome taxa were present with low frequency of occurrence and no taxon was ubiquitously present in neither lichen. I also compared the fungal diversity detected by DNA metabarcoding with the one obtained by the culture-dependent approach, to understand which fraction of the mycobiome was only detected either by culture isolation or by eDNA analyses. Even if DNA metabarcoding described most of the diversity of the lichen mycobiome, and many of the metabarcoded taxa were also isolated in culture, surprisingly there were some fungal species which grew in culture but were not detected by the metabarcoding analysis.

In conclusion, both the culture-dependent and the DNA metabarcoding approaches well complemented each other and allowed to obtain a fairly complete estimation of the global lichen mycobiome diversity in *R. melanophthalma* and *T. atra*. My results confirm that lichens are cradles of microfungi, either yeasts or filamentous, that can be isolated in culture, while they reside in the thalli likely exploiting thallus resources but without being specific to any lichen symbiosis.

RIASSUNTO DELLA TESI

I licheni – essendo sistemi autosufficienti, mutualistici e simbiotici – ospitano una molteplicità di microrganismi (procarioti, microalghe e microfunghi), la cui biodiversità e ruolo nella simbiosi è ancora ampiamente sconosciuta. Fino ad oggi, pochi studi si sono concentrati sulla diversità complessiva dei funghi associati ai licheni che costituisce il micobioma lichenico. In questa ricerca, ho caratterizzato la diversità del micobioma di due licheni epilitici e cosmopoliti, i.e. *Rhizoplaca melanophthalma* (DC.) Leuckert & Poelt e *Tephromela atra* (Huds.) Hafellner var. *atra*, che sono stati scelti come organismi studio. Avendo campioni di licheni provenienti da tutto il loro areale geografico è stato possibile per la prima volta analizzare il micobioma su scala globale. Per fare questo è stato eseguito sia l'approccio di coltura-dipendente che quello di metabarcoding del DNA ambientale (eDNA). L'obiettivi erano *i)* caratterizzare possibili comunità fungine che si mantengono stabili per una data specie di lichene e verificare se una parte di questa comunità fosse condivisa tra i due organismi studio; *ii)* capire se le condizioni ambientali influenzano la composizione e specificità del micobioma dei licheni.

Utilizzando l'approccio di coltura-dipendente, ho isolato e identificato sulla base di dati morfologici e genetici, 76 lieviti basidiomiceti (appartenenti alle classi Agaricostilbomycetes, Cystobasidiomycetes, Microbotryomycetes, Tremellomycetes and Ustilaginomycetes) e 131 funghi ascomiceti (appartenenti alle classi Eurotiomycetes, Dothideomycetes e Sordariomycetes). Inoltre, per i funghi ascomiceti ho descritto due nuove specie nell'ordine Chaetothyriales. La maggior parte dei funghi isolati, sia ascomiceti che basidiomiceti, erano già stati precedentemente descritti in licheni, o risultavano filogeneticamente vicini a specie che solitamente occupano altre nicchie ecologiche (i.e. piante, rocce e suolo) e che hanno evoluto stili di vita diversi (patogeni di piante, opportunisti o saprotrofi).

Tramite il metabarcoding del DNA, prima di tutto ho disegnato e testato un set di cosiddetti 'blocking primers' i quali riducono specificatamente l'amplificazione dei micobionti di *R. melanophthalma* e *T. atra* e di conseguenza permettono l'identificazione di tutti gli altri funghi presenti nei licheni. Tuttavia, questo esperimento non ha funzionato e ho eseguito l'approccio di metabarcoding senza utilizzarli. Il confronto della comunità fungine ha mostrato una bassa diversità per i singoli talli con la comparsa di pochi ascomiceti costantemente presenti in entrambe le due specie di licheni, la maggior parte appartenenti all'ordine Chaetothyriales, Capnodiales and Helotiales. I basidiomiceti rappresentano solo il 7-14% delle sequenze, e la maggioranza appartiene all'ordine Tremellales. Le condizioni ambientali, soprattutto le più rigide (e.g. elevate altitudini) e isolamento geografico sono i principali fattori che influiscono sulla composizione del micobioma

dei licheni, mentre il micobionte sembra avere un ruolo minore. In *R. melanophthalma* e *T. atra* non è stata identificata una comunità fungina stabile e specifica poiché soli pochi taxa sono presenti nei licheni con bassa frequenza e nessuno di questi è presente in entrambe le specie studio.

Infine, ho confrontato la diversità fungina ottenuta applicando l'approccio di metabarcoding con quella ottenuta con metodo di coltura-dipendente, per capire quale frazione del micobioma è identificabile utilizzando diversi approcci. Anche se il DNA metabarcoding permette di catturare una maggiore diversità, è interessante notare come alcune specie di funghi che sono cresciuti in coltura non è stata identificata dalle analisi di metabarcoding.

Per concludere, l'approccio di coltura-dipendente e il DNA metabarcoding integrandosi l'uno con l'altro hanno permesso di ottenere una visione completa della diversità del micobioma in *R. melanophthalma* e *T. atra*. I miei risultati confermano che i licheni sono nicchie per microfunghi, sia filamentosi che lieviti, i quali probabilmente sfruttano le risorse dei talli senza avere un ruolo rilevante nella simbiosi lichenica.

.

GENERAL INTRODUCTION

Lichens are symbiotic organisms, traditionally recognized as mutualistic associations between a main fungal partner, the mycobiont, and one or more populations of phototrophic organisms (i.e. unicellular green algae and/or cyanobacteria), the photobionts (Nash 2008; Hawksworth and Honegger 1994). The interdependent relationship between these two major symbionts is the basis of the lichenization and it is essential for the partners to receive mutual advantages and protection (Ahmadjian 1993; Wang et al. 2014). Indeed, the photobionts biosynthesize carbohydrates (e.g. acyclic sugar alcohols like ribitol and sorbitol) which are used by the fungus as energy source (Richardson et al. 1968; Honegger 1997). On the other hand, the mycobiont provides to the photobionts protection from environmental stresses (e.g. solar radiation, drought and mechanical damages) and guarantees within the thallus water, moisture and minerals (Nash 2008; Wang et al. 2014). As the mycobionts create the "greenhouse" for the photobionts, lichens were proposed to be "fungi that discovered agriculture", also because mycobionts choose, with a diverse degree of selectivity and specificity, the photosynthetic partners to "farm" (Hyvärinen et al. 2002; Lücking et al. 2009a,b,c, 2020; Lücking and Lumbsch 2014). The resulting lichens usually are the symbiotic phenotypes of the mycobionts and only in a few documented cases the thallus phenotype is determined by the biologically relevant structure of the photobiont cells (e.g. Sanders and Lücking 2002).

The view of lichens as two-partner symbiotic relationships has been challenged by many recent discoveries that reappraised lichens as open houses able to host many additional microorganisms (Hawksworth and Grube 2020). Indeed, besides the principal symbionts, also bacteria, virus, additional microalgae and microfungi live in association with the lichen thalli, potentially contributing to the symbiotic outcome on its whole (e.g., Grube et al. 2009; Wilkinson et al. 2015; Aschenbrenner et al. 2017; Moya et al. 2017; Muggia and Grube 2018; Petrzik et al. 2019; Hawksworth and Grube 2020 and references therein). Thereby, the symbiotic concept of lichen should consider the entire diversity of the associated microorganisms. In that way lichens could be defined as true "holobiont", in which the microbial fractions would be represented by their microbiome (bacteria), mycobiome (fungi) and phycobiome (green microalgae; Fig. 1; Simon et al. 2019). Still functions and taxonomic diversity of the all lichen-associated microorganisms are largely unknown and inestimable (Spribille 2018; Hawksworth and Grube 2020).
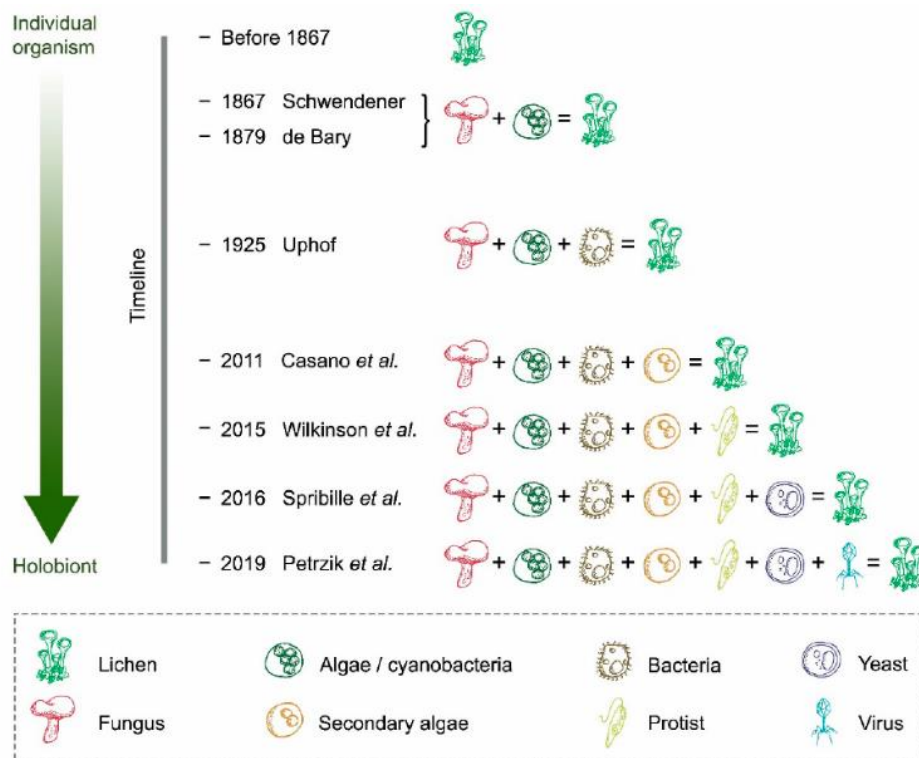
Figure 1. Overview of the lichen system from considering them as a single organism to holobiont [figure retrieved by Morillas et al. (2022)].

Lichen-associated fungi, also known as lichenicolous fungi, have been the focus of uncountable studies since the mid-19ᵗʰ century (Lindsay 1869; Olivier 1907; Vouaux 1912). Many of them are represented by highly specialized and successful group of fungi that live exclusively on lichens often establishing an obligate parasitic relationship with a certain mycobiont species or genus (Lawrey and Diederich 2003, 2018). Most of the known lichenicolous fungi cause weak damages in their host lichen thallus but can also induce the formation of galls, discolorations and lesions on it (Lawrey and Diederich 2003). Only in few cases the lichenicolous fungi are also responsible of the death of their lichen host (Hawksworth 1982). Still it is unclear whether lichenicolous fungi behave as parasite of the mycobionts or of the photobionts exploiting their photosynthates. Until recently, these fungi have mainly been classified on the bases of their reproductive characters and symptoms detectable on the lichen thalli. The degree of "lichenicolous fungus-lichen host specificity" was argued in terms of coevolution, as the high level of host-specificity of many lichenicolous fungi could derive by co-evolutionary speciation (Page 2003). On the other way, Millanes et al. (2014) suggested that the lichen host specificity shown by certain lichenicolous fungi could be driven by the ecological condition – e.g. different ecological niches or the geographical barriers.Lichens can also harbour many symptomless fungi which reside cryptically in their thalli (Suryanarayanan et al. 2005; Li et al. 2007; Harutyunyan et al. 2008; Fleischhacker et al. 2015; Zhang et al. 2015; Muggia

et al. 2016; Spribille et al. 2016; Wang et al. 2016; Fernández-Mendoza et al. 2017; Banchi et al. 2018; Smith et al. 2020; Yang et al. 2022). These fungi were firstly detected in the 1990s by the application of culture-dependent approaches (Petrini et al. 1990; Girlanda et al. 1997). More recently also *ad hoc* fluorescence in situ hybridization (FISH) coupled with confocal laser scanning microscopy (CLSM) and DNA metabarcoding enable their detection (U'Ren et al. 2010; Spribille et al. 2016; Fernández-Mendoza et al. 2017; Banchi et al. 2018; Touvinen et al. 2019). In particular the culture-independent approaches – DNA-metabarcoding techniques and High Throughput Sequencing (HTS) – have been employed to catch the highest possible diversity of lichenicolous fungi from lichen thalli (Zhang et al. 2015; Wang et al. 2016; Fernández-Mendoza et al. 2017; Banchi et al. 2018; Smith et al. 2020; Yang et al. 2022). The results obtained by the DNA metabarcoding highlighted that lichen mycobiomes comprise both symptom producing, well-known lichenicolous fungi as well as cryptically occurring lichenicolous fungi. Many of these are facultative opportunistic fungi in other lichen species, while they do not develop any recognizable infections structures on different hosts (Harutyunyan et al. 2008; Fleischhacker et al. 2015; Muggia et al. 2016). Furthermore, many other fungi cryptically occurring in lichens are closely related to fungi known from different niches (e.g. plant, rocks and soil) and having a different lifestyle (e.g. opportunistic and saprotrophic). Hofstetter et al. (2007) suggested for the first time that these non-lichen fungal taxa may represent a previously unrecognized source of fungal diversity in lichens. Two years later, Arnold et al. (2009) defined these symptomless and cryptically occurring fungi as "endolichenic" for their analogy with the plant-associated endophytes. In fact, the endolichenic fungi are phylogenetically related to the primarily non-lichenized lineages of euascomycetes (Sordariomycetes, Dothideomycetes, Leotiomycetes and Pezizomycetes) and do not share evolutionary origins with the lichen-forming fungi (Lecanoromycetes, Arthoniomycetes and Lichinomycetes).

All lichenicolous fungi could occur in lichens as trapped resisting spores, remaining unrecognized yeast or hyphal stages, while the formation of distinct asexual and sexual reproductive structures could depend on certain (still not investigated) abiotic and biotic conditions. Giving that, all fungi living in the lichen thalli, with the exclusion of the mycobiont, are generally referred as "lichen-associated fungi". A few years ago, Hafellner (2018) proposed a new definition of lichenicolous fungi which clarified the distinction between those occurring symptomatically and cryptically on lichen, as "all lichen-inhabiting fungi, both non-lichenized and lichenized, either obligate or facultative, with a colonization inducing symptoms on the host or not". In particular, Hafellner (2018) recognized three major subgroups of the lichen-associated fungi, such as: *i)* lichenicolous fungi s.str, *ii)* endolichenic fungi and *iii)* lichen epiphytes. Lichenicolous fungi s.str

are all the fungi that live exclusively in the lichen thalli and most of them are non-lichenized fungi. Endolichenic fungi, as defined by Arnold et al. (2009), are those fungi that occur symptomless in lichen and are often facultative opportunistic or saprotrophic. Lichen epiphytes are usually lichenized fungi that grow or overgrow in other (macro)lichens. All these lichen-inhabiting fungi build the lichen mycobiome in which to date a total of 2319 lichenicolous species are recognised: 2000 are obligately lichenicolous species, 257 are lichenicolous lichens and 62 are facultatively lichenicolous species (Diederich et al. 2018). Most of them are filamentous ascomycetes belonging to the subphylum Pezizomycotina (Dothideomycetes, Eurotiomycetes, Leotiomycetes, Sordariomycetes and Lecanoromycetes) which represent about the 96% of the known lichen-associated fungi (Diederich et al. 2018; Muggia and Grube 2018). Basidiomycetes represent instead the minor fraction of lichenicolous fungi and they have been rarely isolated in culture and they were often neglected or overlooked as considered possible contaminants (Ekman 1999). However, recent works identified a relevant fraction of basidiomycetes yeasts in lichens, most of them belong to the classes of Agaricomycetes (Lawrey et al. 2007; Millanes et al. 2021), Tremellomycetes (Zamorra et al. 2011; Millanes et al. 2011; Tuovinen et al. 2019) and Cystobasidiomycetes (Spribille et al. 2016; Millanes et al. 2016; Černajová and Škaloud 2019, 2020).

In general, to date little is known about the overall diversity of lichen mycobiomes and how it is shaped by the main environmental drivers and/or by the lichen host. So far, it is known that the lichen mycobiome composition could be influenced by the environmental conditions, i.e. climate and substrate of the original lichen habitats (Zhang et al. 2015; Wang et al. 2016). Indeed, Eurotiomycetes and Dothideomycetes are frequently detected and isolated from rock-inhabiting lichens and are closely related to melanized rock-inhabiting fungi (RIF, Sterflinger and Krumbein 1995; Wollenzien et al. 1995), plant pathogens and opportunistic fungi (Harutyunyan et al. 2008; Muggia et al. 2016, 2019, 2021; Muggia and Grube 2018; Quan et al. 2020). Leotiomycetes and Sordariomycetes taxa, instead, have been identified from soil- and bark-inhabiting lichens collected in temperate, humid, Antarctic and boreal environments and they are more closely related to lineages of plant endophytes (Arnold et al. 2009; U'Ren et al. 2010, 2012; Muggia et al. 2016). Most Tremellomycetes and Cystobasidiomycetes were isolated from lichens collected in alpine, subalpine and boreal habitats (Fernández-Mendoza et al. 2017; Banchi et al. 2018). Moreover, also the growth form of the lichens could have a role in shaping the mycobiome composition (Smith et al. 2020). Diverse studies did not identify a stable lichen-associated fungal community, referred as "core lichen mycobiome", which would be maintained stable across the geographic and ecological distances of certain species (Fleischhacker et al. 2015; Fernández-Mendoza et al. 2017; Yu et al. 2018) and a general low specificity of lichen mycobiome towards their lichen hosts has been

observed. In this regard, Spribille et al. (2016) specifically identified a Cystobasidiomycetes yeasts (Basidiomycota, Pucciniomycotina) in the thallus cortex of *Bryoria* specimens and speculated that this fungus could be a "third symbiotic partners" in the lichen symbiosis. The presence of this peculiar yeast in lichens stimulated the search of further yeast taxa in other lichen symbioses, so that further basidiomycetes yeasts were discovered in lichens. Millanes et al. (2016) described in species of *Hypogymnia* and *Usnea* (Parmeliaceae) the new genus *Cyphobasidium*, placed in Cystobasidiomycetes, able to develop the sexual stage on specific host, as typical for lichenicolous fungi. Later on, Černajová and Škaloud (2019) found in 95% of the *Cladonia* specimens collected across Europe other Cystobasidiomycetes yeasts, which were both present in corticated and ecorticate thalli of the *Cladonia* species. These results in a way supported the speculation of Spribille et al. (2016) but none could prove which kind of association would "third partner" yeasts establish with the two major lichen symbionts. In contrast, it was already well known that lichens were parasitized by different basidiomycetes representatives of the class Tremellomycetes (e.g., Diederich 1996; Fernández-Mendoza et al. 2017; Banchi et al. 2018), and that these fungi were characterized by a dimorphic life cycle. The haploid yeast phase was known to be the initial stage of their ontogeny, less selective in choosing the host as substrate to live, and a second filamentous stage which develop more specifically in the host. Because Spribille et al. (2016) evidenced only the yeast unicellular stage and did not detect any further hyphal features and developmental stages into meiosporangia, it was argued that the yeasts in lichens were only the vegetative propagules of mycoparasites without any functional relationship with the lichen symbioses (Oberwinkler 2017). The random presence of basidiomycete yeasts in lichens was also strengthen by the results of a few metagenomic analysis on lichen thalli from diverse origins and growth forms (Lendemer et al. 2019; Fernández-Mendoza et al. 2017; Banchi et al. 2018; Smith et al. 2020). Furthermore, Mark et al. (2020) used interaction networks and multivariate analyses to demonstrate that, even if Cystobasidiomycetes were found to be frequent in the studied lichen species, these yeasts were not associated with the symbiosis as the algal partner does.

So far only a very few works have reported on the lichen mycobiome diversity, and these were majorly focused on lichen communities at local scale (Fernández-Mendoza et al. 2017; Banchi et al. 2018). Furthermore, it is not totally known if lichens host a stable fungal community, such as a "core mycobiome", that would be specific for a certain lichen species and could play a functional role in the symbiosis.

In this context, the present doctoral thesis aims at uncovering the diversity and specificity of the lichen mycobiome to demonstrate if *i)* lichen thalli growing in different ecologies are characterized by a distinct, ecology-related mycobiome, which is transient among diverse habitats,

ii) while there would be a core species-specific mycobiome that would be peculiar for the lichen species, independently from the ecological conditions. The leading hypothesis is that multiple ecological guilds of fungi are associated with lichens, but that only a specific fraction of these mycobiomes, i.e. the core mycobiome, is always stable, whereas the ecology-related mycobiome, is transient but homogeneous in its composition only under the same ecological conditions. To achieve these aims two lichen species were selected as models, i.e. *Rhizoplaca melanophthalma* (DC.) Leuckert & Poelt and *Tephromela atra* (Huds.) Hafellner var. *atra*, and were collected across their whole geographic range. *R. melanophthalma* and *T. atra* were chosen because they both *i)* have a worldwide distribution under diverse ecological conditions and occur at different elevations, *ii)* form large populations of conspicuous thalli on rock substrates, *iii)* have been comprehensively studied for their speciation patterns, chemical, morphological and genetic diversity at both individual and population level, and for the specificity/selectivity of their mycobiont-photobiont (genus *Trebouxia*) relationships (Leavitt et al. 2011, 2016a,b; Muggia et al. 2008, 2010, 2014a,b). Also, the mycobionts and several photobionts of both lichens have been already isolated in culture and morphologically characterized.

The research was carried out by applying both culture-dependent experiments and DNA metabarcoding analyses. The culture-dependent approach was performed to isolate fungal strains that strictly reside inside the thalli (Petrini et al. 1990; Girlanda et al. 1997; Prillinger et al. 1997; Arnold et al. 2009; Peršoh and Rambold 2012) and would represent potentially still uncultured taxa. Indeed this method is essential for retrieving physical isolates that can be morphological characterized and potentially described as new species, beside that they can serve for future functional studies or preparation of synthetic communities. Because many fungi do not grow under axenic culture conditions (U'Ren et al. 2014; Muggia and Grube 2018; Wijayawardene et al. 2021), this approach may lead to an underestimation of the whole fungal diversity present in environmental samples. It is thus successfully complemented by the eDNA metabarcoding approach, in which most of the diversity present in a sample is caught by High Throughput Sequencing (HTS, Bates et al. 2012; Zhang et al. 2015; Fernández-Mendoza et al. 2017; Banchi et al. 2018; Smith et al. 2020; Yang et al. 2022).

I have organized the research work into four main parts which here correspond to individual chapters that either have been already published (chapter 1), or in preparation for soon submission (chapter 2), or report on a methodological approach (chapter 3), or is a contribution presently under review (chapter 4). Some chapters comprise also a part of supplementary materials, which is presented and the end. Literature used as reference for the whole thesis is reported at the end. The research overarches cultures-based and molecular approaches, including morphological (light

microscopy) and phylogenetic analyses. The general structure and the topics dealt with in each chapter are outlined here below. The funding for this study was provided by the Italian Ministry of University and Research (MIUR, Ministero dell'Università e della Ricerca) project PRIN2017 (project code 20177559A5) assigned to my supervisor.

Chapter 1 is entitled "The yeast lichenosphere: high diversity of basidiomycetes from the lichens *Tephromela atra* and *Rhizoplaca melanophthalma*". It corresponds to a paper published in July 2022 in the journal *Fungal Biology*. In this work I characterized the diversity of cultivable basidiomycetes yeasts and investigated if it was related to *i)* the lichen species or *ii)* to the geographic (ecological) origin in which the lichen samples were collected. Furthermore, I also investigated whether the isolated and well-known lichen-associated fungi Cystobasidiomycetes and *Tremella macrobasidiata* yeasts (for which species-specific primers were already available; Millanes et al. 2011; Spribille et al. 2016; Tuovinen et al. 2021) were detectable in the lichen thalli by PCR amplification.

Chapter 2 is entitled "Tackling fungal diversity in lichen symbioses: molecular and morphological data recognize new lineages in Chaetothyriales (Eurotiomycetes, Ascomycota)". It corresponds to a manuscript in its almost final form to be submitted by the end of the year (2022) to the journal *Mycological Progress*. In this work I aimed at describing the culturable fraction of the cryptically occurring ascomycetes fungi from thalli of *R. melanophthalma* and *T. atra*. I identified fungi that were already known to occur in lichens and others that have not been found so far. For these I proposed the recognition of two new lineages in Chaetothyriales and species descriptions were prepared.

Chapter 3 is about the experimental procedure carried out to implement the use of blocking primers in metabarcoding analysis of fungi. Here I explain the setup of the experiment and comment on its suitability as an application on lichen samples.

Chapter 4 is entitled "Largely transient mycobiomes shape fungal diversity in two globally distributed lichens". It corresponds to a paper under review in the journal *FEMS Microbiology Ecology*. In this work I aimed at studying the mycobiome diversity of *R. melanophthalma* and *T. atra* applying a metabarcoding approach in order to *i)* understand if the two target species host significantly different mycobiomes according to their geographic origin, *ii)* characterize if any "core mycobiome" can be detected and if it is stable and specific to either lichen species, *iii)* identify the mycobiome fraction which can be isolated in culture (chapters 1 and 2) and is also detected by metabarcoding analyses, and which fraction is instead only detected by one approach.

During the doctoral period I also have been involved in other projects that are reported in the Appendix section. Appendix 1 is about the morphological and genetic characterization of

ascomycetes and algae isolated from rocks collected over 6000 meters a.s.l. at the top of the mountains Muztagh Ata (China) and Cerro Mercendario (Argentina). This work entitles "Life on top: cryptoendolithic ascomycetes and microalgae isolated from over 6000 m altitude" and was published in July 2022 in the journal *Plant and Fungal Systematics*. Appendix 2 is the abstract of a submitted manuscript entitled "The origin of human pathogenicity and biological interactions in Chaetothyriales", under review in the journal *Fungal Diversity*, in which I contributed in generating sequence data for the phylogenetic analysis and in the preparation of the chapter dealing with the lichen-associated fungi.

CHAPTER 1

The yeast lichenosphere: high diversity of basidiomycetes from the lichens *Tephromela atra* and *Rhizoplaca melanophthalma*

Abstract

Lichens are well-known examples of complex symbiotic associations between organisms from different kingdoms. Microfungi in particular, establish diverse associations with the hosting lichen thallus, as species-specific parasites or transient co-inhabitants. The whole community of lichen-associated fungi constitute the "lichen mycobiome" and comprises both ascomycetes and basidiomycetes, including filamentous and yeast taxa. Metabarcoding results and microscopy analyses show that in some thalli, basidiomycetes can be frequent lichen-associated fungi but so far only a few species could be axenically isolated and morphologically characterized. Within a broad project aiming at characterizing the mycobiome diversity by culture-dependent and independent approaches in two lichen species selected as reference models – *Rhizoplaca melanophthalma* Leuckert & Poelt and *Tephromela atra* (Huds.) Hafellner var. *atra* – we succeed in isolating and culturing 76 new strains of basidiomycetous yeasts. The lichen thalli were collected in different mountain regions worldwide and at relatively high elevation. The yeast strains were isolated on different growth media and were studied for their morphological and genetic diversity. Nuclear internal transcribed spacer (nucITS) and nuclear ribosomal large subunit (nucLSU) sequence analyses identified them to belong to ten families within the orders Agaricostilbomycetes, Cystobasidiomycetes, Microbotryomycetes, Tremellomycetes and Ustilaginomycetes. The yeasts here detected only showed patterns of host-preference in a few cases and they are potentially related to the ecological conditions.

Keywords

Introduction

Recent discoveries have promoted the reconsideration of lichens, expanding our understanding beyond a simple "two-partner-symbiosis". These symbioses are now considered self-sustaining ecosystems derived from mutualistic association of a biotrophic fungus (mycobiont) and phototrophic microorganisms (photobionts, e.g. chlorophytes and/or cyanobacteria), along with an indeterminate number of other microscopic organisms (Hawksworth and Grube 2020). The "lichen"

resulting from these interactions can be considered as the symbiotic phenotype of the lichen-forming fungus, i.e., the mycobiont (Honegger 2012), although in a few documented cases the thallus phenotype may be determined by the biologically relevant photobiont (e.g. Sanders and Lücking 2002). The multiplicity of microorganisms associated with the lichen thalli spans from prokaryotes, microalgae to microfungi (e.g., Grube et al. 2009; Aschenbrenner et al. 2017; Moya et al. 2017; Muggia and Grube 2018 and references therein). Some of these microbes may grow independently of lichen systems under certain conditions in nature and in axenic cultures (Arnold et al. 2009; Muggia et al. 2016, 2017). However, knowledge on their diversity and potential role(s) that they can play in the lichen symbioses is still incomplete (Spribille 2018; Muggia and Grube 2018; Tagirdzhanova et al. 2021).

Among lichen-associated microorganisms, fungi in particular engage in diverse associations with the hosting lichen thallus, often as species-specific parasites or transient generalist co-inhabitants within the thalli (Fernández-Mendoza et al. 2017; Muggia and Grube 2018). The presence of accessory fungi in lichen thalli has attracted interest for many years. Fungi recognized to be specific parasites of different lichen species have been well studied and are formally recognized as "lichenicolous fungi" (Crittenden et al. 1995; Lawrey and Diederich 2003; Diederich et al. 2018). Lichenicolous fungi have traditionally been considered to develop symptomatically on the lichens and build their reproductive structures on the surface of the thalli, or semi-immersed within (Lawrey and Diederich 2003, 2011; Hawksworth 1982; Muggia et al. 2015; Rambold and Triebel 1992). Most of the lichenicolous fungi are very slow growing in axenic culture, and the majority of the taxa have been studied only from the environmental lichen samples (Ertz et al. 2014; Muggia et al. 2015, 2019). In contrast, fungi which develop cryptically within lichen thalli and which mycelia are hardly detectable by standard microscopy techniques have been known since 1990 by the application of culture-dependent approaches (Petrini et al. 1990; Girlanda et al. 1997). Because their lifestyle in lichens resembles that of endophytic fungi in plants, these taxa have been commonly termed "endolichenic fungi" (Arnold et al. 2009; U'Ren et al. 2012, 2014; Muggia et al. 2016, 2017; Fernández-Mendoza et al. 2017; Banchi et al. 2018; Muggia and Grube 2018). Endolichenic fungi grow relatively quickly in vitro, especially when their culture isolation is performed from thallus fragments (Muggia et al. 2016, 2017; Muggia and Grube 2018). However, High Throughput Sequencing (HTS) and metabarcoding analyses demonstrated that also the lichenicolous fungi can be cryptically present in thalli that do not correspond to their specific lichen host on which they produce symptoms, behaving as endolichenic fungi (Fernández-Mendoza et al. 2017; Banchi et al. 2018; Tuovinen et al. 2021). Thus, it is often difficult to make a clear distinction between the two fungal groups. Both lichenicolous and endolichenic fungi are now regarded as

"lichen-associated fungi" and constitute the "lichen mycobiome" (Fernández-Mendoza et al. 2017; Banchi et al. 2018; Muggia and Grube 2018). Similar mycobiomes (in terms of species composition) may be present in thalli of closely related mycobionts (Fernández-Mendoza et al. 2017; Smith et al. 2020).

Lichen mycobiomes comprise both ascomycetes and basidiomycetes, either filamentous (mycelium consisting of hyphae) or yeast (unicellular) taxa (Diederich 1996; Millanes et al. 2011, Fernández-Mendoza et al. 2017; Diederich et al. 2018). Spribille et al. (2016) suggested that basidiomycetous yeasts in the Cyphobasidiales (Cystobasidiomycetes) could be a potential third, biologically relevant partner in the lichen symbioses. Since then, the presence of yeasts in lichens – belonging to Cystobasidiomycetes and Tremellomycetes – has been documented very specifically by fluorescent in situ hybridization (FISH), coupled with confocal laser scanning microscopy (CLSM; Spribille et al. 2016; Tuovinen et al. 2019, 2021). Early metabarcoding molecular data showed that up to 18% of endolichenic taxa are representatives of Basidiomycota (Zhang et al. 2015, 2016) and in some thalli basidiomycetes can even be the dominant lichen-associated fungi (Fernández-Mendoza et al. 2017). On the contrary, some subsequent analyses hardly detected Cystobasidiomycetes yeasts using metabarcode sequencing (Lendemer et al. 2019; Smith et al. 2020).

Dimorphism, i.e., the alternating formation of both a haploid unicellular yeast phase and a dikaryotic filamentous mycelium during their life cycle, is common in basidiomycetes (Oberwinkler 1987; Bandoni 1995; Sampaio 2004; Boekhout et al. 2011; Millanes et al. 2021). In his revision about Pucciniomycotina yeasts, Oberwinkler (2017) suggested that, as is the case for other basidiomycete yeasts, the Cyphobasidiales yeasts in lichens are a part of the lifecycle of these basidiomycetes growing and forming large colonies inside the lichen thalli. It is also generally assumed that lichenicolous species in the Tremellomycetes also have a yeast stage. Although less frequently detected by light microscopy, basidiospores germinating by budding have been observed and documented in the basidiomata of lichenicolous *Tremella* and in isolated and cultivated Fellomyces yeasts from lichens (Diederich 1996; Prillinger et al. 1997; Zamora et al. 2011, 2016). Tuovinen et al. (2019, 2021) demonstrated that the yeast stage of several lichen-associated *Tremella* is common and widespread within the lichen thalli. Dimorphism has been also confirmed for other lichenicolous species of mycelial basidiomycetes, such as *Cyphobasidium* species (Spribille et al. 2016).

Despite the potential importance of basiomycetous yeasts in lichens, host-yeast association are still incompletely understood and the yeast diversity in these symbiotic systems remains largely unknown. Extreme habitats – as the cold Arctic and Antarctica – where lichens dominate (Bridge

and Spooner 2012; Santiago et al. 2015; Duarte et al. 2016; Pankratov et al. 2017) would be particularly interesting to study the diversity of lichen-inhabiting yeasts. Psychrophilic yeasts in lichens were detected in subfossils of glacier-preserved thalli and were identified to belong to Cystofilobasidiales (DePriest et al. 2000). More recent studies highlighted the presence of new basidiomycetous species in the genera *Fellomyces*, *Mrakia*, *Naganishia*, *Piskurozyma* and *Vishniacozyma* exclusively from lichens (Pankratov et al. 2017). Also, a geographically widespread association between *Cladonia* lichen species and the recently discovered Cystobasidiomycetes yeast *Lichenozyma pisutiana* has been reported (Černajová and Škaloud 2019). Although some previous research investigated the presence of basidiomycetous yeasts in lichen species of the family Parmeliaceae and in the genera *Lecanora* and *Cladonia* (Spribille et al. 2016; Tuovinen et al. 2019, 2021; Černajová and Škaloud 2019), and strengthened the perception of a great diversity of lichen-associated yeasts, other lichens have been comparatively less studied.

Thus, in the frame of a wider project investigating the mycobiomes of two cosmopolitan lichens selected as study models – *Rhizoplaca melanophthalma* Leuckert & Poelt and *Tephromela atra* – we applied a targeted culture-dependent approach to better understand the range of basidiomycetous yeasts associating with lichens. We aimed at investigating if the diversity of cultivable yeasts is related to *i)* the lichen species or *ii)* to their respective geographic origin (the two species were sometimes collected in the same locality side by side). We also investigated whether the isolated Cystobasidiomycetes and *Tremella macrobasidiata* yeasts (for which species-specific primers were already available; Millanes et al. 2011; Spribille et al. 2016; Tuovinen et al. 2021) were detectable in the lichen thalli by PCR amplification. The isolated strains were studied in their morphological and phylogenetic diversity, and were recognized to belong to ten families among Agaricostilbomycetes, Cystobasidiomycetes, Microbotryomycetes, Tremellomycetes and Ustilaginomycetes.

Material and methods

Sampling

The two lichen species *Rhizoplaca melanophthalma* and *Tephromela atra* were chosen as study systems because of their worldwide distribution under diverse ecological conditions and because their symbioses have been abundantly investigated in the past (Leavitt et al. 2011, 2016a,b; Muggia et al. 2008, 2010, 2014a,b). *R. melanophthalma* is characterized by an umbilicate thallus (attached at a single point), whereas *T. atra* builds a crustose thallus composed of adjacent areoles (Figs. 1a, b). Lichen thalli of both species were collected in different localities trying to cover as much as possible their ecological (type of substrates and climate) and geographical distributional ranges

worldwide (Table 1, Fig. 1e). The sampling was performed in boreal, alpine, temperate, humid and arid habitats in Europe (Alps and Spain), North America (Rocky Mountains), South America (Andes) and Tasmania (Three Thumbs) and covered diverse elevations from 500 m up to 5100 meters above sea levels (m a.s.l.; Figs. 1a-e). Lichen thalli growing on different rock types, such as quartzite, granitic, schist- sandstone and siliceous rocks, were collected (Table 1). In total, 136 thalli of *R. melanophthalma* coming from 34 populations, and 84 thalli of *T. atra* from 21 distinct populations were used for fungal isolation. All the lichen samples were deposited at the herbarium of the University of Trieste (TSB).
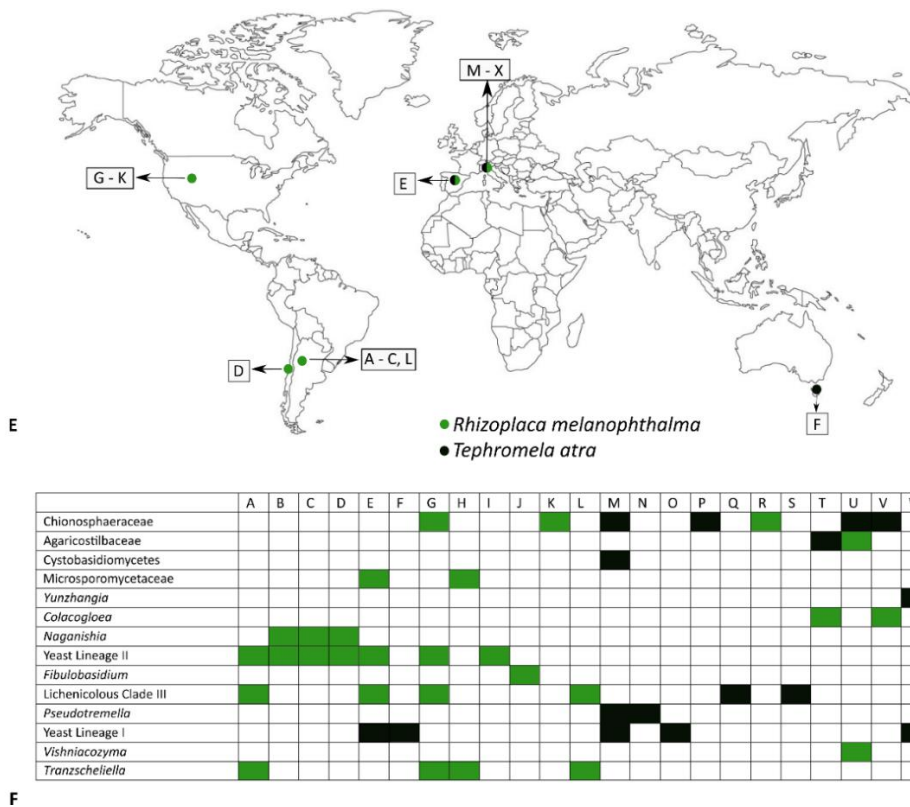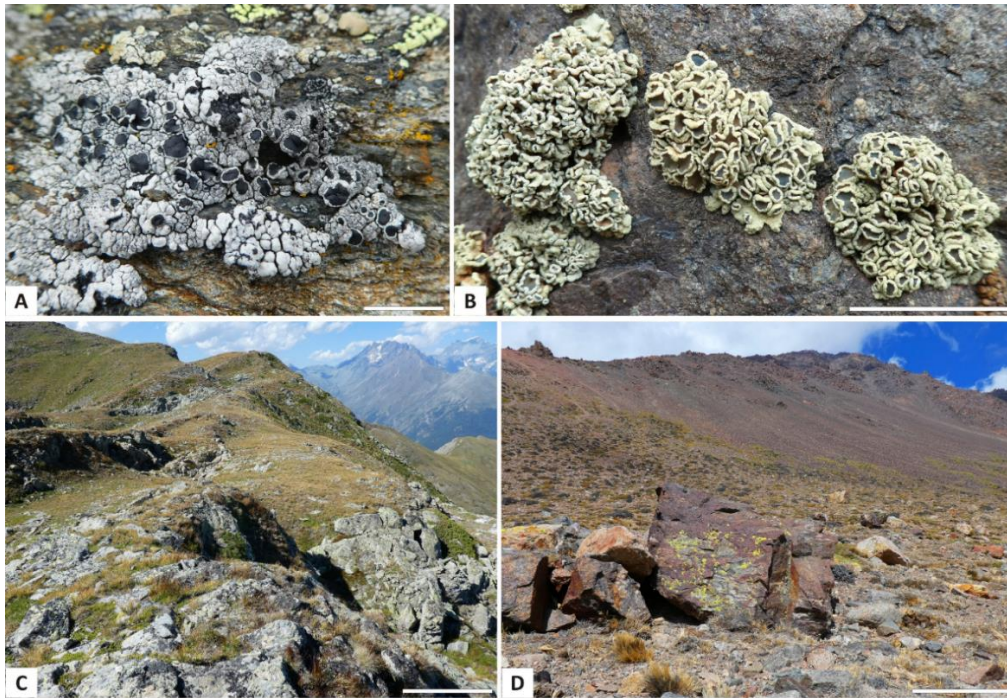
Figure 1. Lichen species (A,B) and the environments (C,D) in which basidiomycetes diversity was investigated: A) *Tephromela atra* (collected in the Alps, Italy); B) *Rhizoplaca melanophthalma* (collected in the Cordillera de los Andes, Argentina); C) Italian Alps (locality P, Table 1); D) Cordillera de los Andes (locality B, Table 1); E) Map of the localities (letters as in Table 1) from which we successfully isolated yeast strains from *R. melanophthalma* (green) and *T. atra* (black); F) Presence-absence matrix of isolated yeast strains (indicated according to their phylogenetic placement; Table 2) from *R. melanophthalma* (green) and *T. atra* (black), localities are identified by letters (Table 1). Scale bars: 1 cm (A), 2 cm (B), 1 m (C, D).

Table 1. Samples of *Rhizoplaca melanophthalma* and *Tephromela atra* are reported with their thallus ID and the geographic origins; localities are identified by alphabetic letters (A-X).

Lichen host	Thallus ID	Altitude (m a.s.l.)	Substrate	Geographic origin	Locality ID
R. melanophthalma	(L2383-L2391)	1450	basaltic boulders	Argentina, prov. Mendoza, dep. Malargue, Laguna de Llancanelo, RP186, 20 km after the crossroad with RN40; S/SW exposed, scattered in dry pampa vegetation, ca. 35˚42'50" S/ 69˚27'18" W (*L. Muggia*).	A
R. melanophthalma	L2436-L2455)	3550	granitic boulders	Argentina, prov. Mendoza, Tunuyan, Cordillera del los Andes (E side), road 94 towards portillo Argentino, camp 'Yareta', 3550 m a.s.l., on acid big boulder, E-S exposed (*L. Muggia*).	B
R. melanophthalma	(L2457-L2470)	3330	acid rocks	Argentina, prov. Mendoza, Cordillera de los Andes (E side), Las Cuevas, lowest border of Mt. Tolosa glacier, S-W exposed (*L. Muggia*).	C
R. melanophthalma	(L2526-L2544)	3300	acid rock	Chile, prov. Santiago de Chile, Valle (valley) del Yeso, Cordillera del Los Andes (W side), on the path going from the Bano del Plomo to the Laguna de los Patos, S-exposed (*J. Orlando & D. Leiva*).	D
T. atra *R. melanophthalma*	(L2583-L2584) (L2585-L2594)	1900	siliceous-granitic boulders	Europe, Spain, prov. Madrid, Miraflores de la Sierra, Puerto de la Morquera, towards Pico Najarra, about 150m above Puerto de la Morquera, ca. 40˚49'22" N/3˚49'49" W (*L. Muggia & S. Perez-Ortega*).	E
T. atra	(L2595-L2603)	545	dolorite boulders	Australia, Tasmania, three Thumbs, summit area, 42˚36'S/147˚52'E, Grid; 570752828/ Grid. Sq.: 5728; in dry sclerophyll forest (*G. Kantvilas*).	F
R. melanophthalma	(L2635-L2667)	1700	quartzite	USA, Utah, Utah Co., Rock Canyon, ca. 2 km from trailhead, on exposed quartzite outcrop on north-facing side of canyon; 40.2649, -111.6179 (*S.D. Leavitt 19-303*).	G
R. melanophthalma	(L2668-L2685)	1665	sandstone boulders	USA, Utah, Emery County, vic. of Horse Canyon Rest Area along US Highway 6, on sandstone in Pinyon/Juiper woodland; 39.4123, -110.4320 (*S.D. Leavitt 19-235*).	H
R. melanophthalma	(L2686-L2703)	2020	wasatch formation	USA, Utah, Rich Co., southeast of Bear Lake along Highway 30 and west of Sage Creek Junction, on rock in sage-steppe habitat; 41.7905, -111.2129 (*S.D. Leavitt 19-157*).	I
R. melanophthalma	(L2704-L2721)	2490	sandstone boulder	USA, Utah, Duchesne Co., Ashley National Forest, South Unit, on Nutter's Ridge, on sandstone outcrup north-east of exclusure site (*S.D. Leavitt*).	J
R. melanophthalma	(L2722-L2731)	1845	basalt/volcanic rocks	USA, Idaho, Owyhee Co. Along Mud Flat Rd, 27.7 miles from Highway 78; 42.704228-166.3832 (*S.D. Leavitt 19.233*).	K
R. melanophthalma	(L2786-L2799)	2700	acidic rocks	Argentina, prov. Mendoza, road RP52, near to Paramillo, ca. 30 m above the road, ca. 32°30´13" S/ 69°03´18" W (*L. Muggia*).	L
T. atra	(L3274-L3286)	2150	siliceous rocks/cliffs	Italy, Trentino Alto Adige, prov. Trento, Pergine Valsugana, Val dei Mocheni, Passo La Portella, S-exposed, ca. 46°05'38" N/ 11°21'57" E (*L. Muggia & A. Cometto*).	M
R. melanophthalma *T. atra*	(L3333-L3350) (L3351-L3361)	2100	siliceous rocks	Italy, Trentino Alto Adige, prov. Bolzano, Mazia Valley (Matschertal), path to Tartscher Kreuz, on boulders in open meadow, S-exposed, ca. 46°41´33" N/ 10°35´49" E (*L. Muggia & A. Cometto*).	N

T. atra	(L3396-L3403)	1650	siliceous/shists tiles	Italy, Piemonte, prov. Verbania-Cusio-Ossola, Val Vigezzo, Alpe Villasco, on roof tile, N-exposed (*L. Muggia & A. Cometto*).	O
T. atra *R. melanophthalma*	(L3404-L3418) (L3419-L3437)	2300	granitic boulders	Italy, Aosta Valley, saddle below Mt. Chaligne S/E side, alpine vegetation, ca. 45°46′08" N/ 7°14′52" E (*L. Muggia A. Cometto*).	P
T. atra	(L3472-L3480)	1950	silecous bricks/rocks	Italy, Aosta Valley, prov. Aosta, Gressoney Valley, path to Colle Pinter, Alta Via n. 1, about 100 height meter above Alm Alpenzu, N/W/S-exposed, ca. 45°48′13" N/ 7°48′50" E (*L. Muggia & A. Cometto*).	Q
R. melanophthalma	(L3481-L3495)	2800	granitic-siliceous cliff	Italy, Aosta Valley, prov. Aosta, Gressoney Valley, Colle Pinter, Alta Via n. 1 (AV1, path n. 6), big cliffs right above the pass, S/W-exposed, 45°49′12" N/ 7°47′14" E (*L. Muggia & A. Cometto*).	R
T. atra	(L3520-L3527)	1550	siliceous rocks/cliffs	Italy, Aosta Valley, prov. Aosta, Gressoney Valley, Alta Via n. 1 (AV1, path n. 6), path from Gressoney to Alpe Alpenzu, S/E-exposed, ca. 45°48′263" N/ 7°48′11" E (*L. Muggia & A. Cometto*).	S
T. atra *R. melanophthalma*	(L3528-L3536) (L3537-L3553)	1750	granitic boulders	Italy, Piemonte, prov. Turin, Valley D' Ala (Lanzo Valley), Ala di Stura, loc. Balme, path n. 228 to Lago Ru, open Larix vegetation on broad bankings, S-exposed (*L. Muggia & A. Cometto*).	T
T. atra *R. melanophthalma*	(L3554-L3562) (L3563-L3575)	1500	granitic rocks	Italy, Piemonte, prov. Turin, Valley D' Ala (Lanzo Valley), Ala di Stura, loc. Balme, path n. 228 to Lago Ru, at bifurcation with the path to climbing crag "Le Ginevre", 100 height m above Balme, shadowed, 45°18'11" N/ 7°12'56" E (*L. Muggia & A. Cometto*).	U
R. melanophthalma *T. atra*	(L3616-L3639) (L3640-L3651)	2250	siliceous rocks/boulders	Italy, Piemonte, prov. Cuneo (Alpi Cozie), Val Varaita-Val Maira, Colle di Sampeyre, W of the pass, 44°33'06" N/ 7°07'05" E (*L. Muggia & A. Cometto*).	V
T. atra	(L3694-L3707)	2100	schist-sandstone rocks	Italy, Piemonte, prov. Cuneo (Alpi Marittime), Mt. Ventoso, below the summit, W-exposed, ca. 44°04'56" N/ 7°42'58" E (*L. Muggia & A. Cometto*).	W
T. atra *R. melanophthalma*	(L3720-L3722) (L3723-L3729)	2150	schist-sandstone rocks	Italy, Piemonte, prov. Cuneo (Alpi Marittime), Mt. Saccarello, few meters S/E of the summit, S-exposed, ca. 43°03'40" N/ 7°42'46" E (*L. Muggia & A. Cometto*).	X

Culture isolation

Fungal isolation from *Rhizoplaca melanophthalma* and *Tephromela atra* thalli followed the protocol of Yamamoto et al. (2002). Approximately 2 mm^2 fragments of lichen thalli were dissected with a sterile razor blade. For *R. melanophthalma*, one marginal lobe and one apothecium were taken, while for *T. atra*, one marginal areole and one apothecium. The fragments were washed three times for 15 minutes with sterile water, followed by 30 minutes of washing with 500 µl of Tween80 diluted 1:10. A final washing step was performed rinsing the thallus fragments three times for 15 minutes 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. Six

different media were used to promote the growth of as many different fungi as possible: Trebouxia medium (TM, Ahmadjian 1987), Lilly and Barnett (LB, Lilly and Barnett 1951), Sabouraud (SAB, Pagano et al. 1958), Potato Dextrose agar (PDA, ApplChem A5828), Dichloran/Glycerol agar (DG18, Hocking and Pitt 1980) and Malt Yeast-extract (MY Lilly and Barnett 1951). We inoculated two tubes of the same medium for each sample for a total of 12 inocula from each lichen individual. The tubes were incubated in growing chamber under the following conditions: 17 °C, 20 µmol × photons m^{-2} × s $^{-1}$, with a light/dark cycle of 14/10 h. After three to six months, the inocula had reached a diameter of about 1-3 mm and were checked for the yeast morphology using light microscopy to exclude any – similarly looking – contamination by bacteria. The confirmed yeast strains were then further processed to set subcultures on Petri plates using the same growth medium where the inocula were isolated successfully. Three subcultures were prepared for each strain.

Once the strains were taxonomically identified (see below), accumulation curves for each lichen species were built to assess if the sampling effort was sufficient. Moreover, a Venn diagram was generated to compare the yeast taxa shared between *R. melanophthalma* and *T. atra*.

Morphological analysis

Cell morphological traits of the cultured yeast strains were analysed using light microscopy. Part of the colony was removed with a sterile loop, diluted in a drop of water and the cells were mounted in water or were additionally stained with 1% Phloxin B after pre-treatment with 5% KOH (Diederich 1996). Digital photos were taken with a Zeiss AXIO Imager A2 coupled to a Thorlabs digital camera and were slightly improved for colour saturation and sharpness with Adobe Photoshop 7.0 (Adobe System Incorporated, San Jose, CA, USA) and photo-tables were assembled using CorelDRAW X7 (Corel Corporation, Ottawa, Canada).

Molecular analyses: DNA extraction, PCR amplification and sequencing

Small parts of the cultured yeast colonies were taken with a sterile inoculation loop and transferred into 1.5 ml reaction tubes, containing three sterile tungsten beads for homogenization, frozen and ground using a TissueLyserII (Retsch). The DNA extractions were performed following the C-TAB protocol of Cubero et al. (1999), with minor adjustments. The identity of the cultured strains was checked with sequences of the nuclear internal transcribed spacers (nucITS) and 5.8S rDNA ribosomal gene and the nuclear ribosomal large subunit (nucLSU). The nucITS fragment was amplified with the primers ITS1F (Bruns and Gardes 1993) and ITS4 (White et al. 1990), while the nucLSU was amplified with the primers LR0R and LR5 (Vilgalys and Hester 1990; http://www.biology.duke.edu/fungi/mycolab/ primers.htm). All strains were sequenced for their ITS locus; if the ITS sequences were identical (99%-100% identity) for strains sharing the same origin –

i.e. isolated from the same lichen host thallus, or from thalli coming from the same population – the LSU locus was further sequenced only for a subset of the strains. Polymerase chain reactions (PCR) were prepared for a 25 μl final volume containing 5 μl DNA, 12.5 μl of AccuStart II PCR ToughMix, 0.4 μl for each of the 10 μM primers. PCR amplifications were performed under the following conditions: one initial heating step of 3 minutes at 94 °C linked to 35 cycles of 45 seconds at 94 °C, 45 seconds at 55 °C, 1 minute at 72 °C, and one final extension step of 5 minutes at 72°C after which the samples were kept at 4 °C. A negative control was used to verify the absence of non-specific amplification products.

PCR amplifications were also performed on the DNA extracts of those lichen thalli from which yeast strains corresponding to Cystobasidiomycetes and *Tremella macrobasidiata* were isolated in culture. Doing so we aimed at inferring the presence of the yeasts inside the thalli. This additional PCR analysis was possible for Cystobasidiomycetes and *Tremella macrobasidiata* because specific primers for only these two taxa have proven to work well to amplify the DNA of asymptomatic yeasts isolated from lichen thalli (Spribille et al. 2016; Tuovinen et al. 2021). DNA extractions from the thalli were performed using approximately 2 mm^2 fragments of lobes and areolas of *R. melanophthalma* and *T. atra*, respectively, of the same lichen thalli used for culture isolation. The thallus fragments were previously washed (as described for culture isolations) and DNA extraction followed the C-TAB protocol (Cubero et al. 1999). Thalli of *R. melanophthalma* L2590 and L2668 and *T. atra* L3276 were checked for Cystobasidiomycetes using the Cystobasidiomycetes-specific primers ITS_symrho_2F and LR0_symrho_R (Spribille et al. 2016). PCR amplifications were performed under the following conditions: one initial heating step of 3 minutes at 94 °C linked to 30 cycles of 30 seconds at 94 °C, 1 minute at 48 °C, 1 minute at 72 °C, and one final extension step of 2 minutes at 72 °C after which the samples were kept at 4 °C. Thalli of *R. melanophthalma* L2589, L2636, L2637 and L2786 and *T. atra* L3472 and L3523 were checked for *T. macrobasidiata* using *Tremella* specific primers TmM_ITS_970F (Tuovinen et al. 2021) and Basid-LSU3-3 (Millanes et al. 2011). PCR amplifications were performed with touch down PCR conditions, i.e., one initial heating step of 3 minutes at 94 °C linked to 4 cycles of 40 seconds at 94 °C, 40 seconds at 64 °C, 90 seconds at 72 °C, 4 cycles of 30 seconds at 94 °C, 30 seconds at 62 °C, 90 seconds at 72 °C, 32 cycles of 30 seconds at 94 °C, 30 seconds at 60 °C, 90 seconds at 72 °C, and one final extension step of 7 minutes at 72°C after which the samples were kept at 4 °C. A positive (derived from the culture yeast DNA extraction) and a negative control were used.

All the amplicons were checked for their quality and size by 1% agarose gel electrophoresis stained with Green Safe Gel and purified using Mag-Bind® Normalizer Kit (Omega bio-tek). Clean amplicons were sent for Sanger sequencing to Macrogen Europe (The Netherlands).

Phylogenetic analysis

A first approximation of the identity of the newly generated nucITS and nucLSU sequences was checked with blast similarity search (Altschul 1990) using sequences available in Genbank database. As our sequences showed high similarity with representatives of the classes Agaricostilbomycetes, Cystobasidiomycetes, Microbotryomycetes, Tremellomycetes and Ustilaginomycetes, we prepared individual multiple sequence alignments (MSA) for each of these major basidiomycetous classes and for each sequenced locus. The taxon sampling for each analysis was constructed based on the blast similarity sequence results and on the groups and sequences retrieved from previous phylogenetic studies; sequences of type materials were included when available (Supplementary Tables S1-S6). The taxon sampling of Agaricostilbomycetes was based on Millanes et al. (2021) and Diederich et al. (2022), that of Cystobasidiomycetes on Černajová and Škaloud (2019) and Millanes et al. (2016), that of Microbotryomycetes on Kachalkin et al. (2019) and Yurkov et al. (2016), and that of Ustilaginomycetes on Li et al. (2017) and Wang et al. (2015a). For the class Tremellomycetes, two separate MSAs were prepared, one for the order Tremellales and the other for Filobasidiales. Representative taxa were selected from the phylogenetic studies of Duarte et al. (2016), Millanes et al. (2011), Scorzetti et al. (2002) and Zamora et al. (2016). A further MSAs was specifically prepared for a reduced group in the Tremellales, using *Phaeotremella* as outgroup (Supplementary Table S7).

The MSAs were prepared in Bioedit v7.2.5 (Hall, 1999) and initially aligned in MAFFT v.7 (Katoh et al. 2013) using the g-ins-I substitution model. We manually removed ambiguous single nucleotide polymorphisms (SNPs) and introns from the alignment. We analyzed single locus datasets using Maximum Likelihood (ML) and Bayesian Inference (BI) approaches running the analyses on CIPRES Science Gateway v.3.3 web portal (Miller et al. 2011). RAxML v.8.2 (Stamatakis 2014) was used for the ML analysis applying GTRGAMMA substitution model and 1000 bootstrap pseudoreplicates. The BI analysis was performed with the program MrBayes v.3.2 (Ronquist et al. 2012) running 5 million generations with 6 chains starting from a random tree. Every 100th tree was sampled, and the first 25% of data were discarded as burn-in. The distribution of log-likelihood scores was examined using the program Tracer v1.5 (Rambaut and Drummond 2007) to determine that stationary phase for each search was reached and chains had achieved convergence. The first 25% of the sampled topologies were discarded as part of a burn-in procedure, while the remaining trees were used for calculating the posterior probabilities in the

majority rule consensus tree. The convergence of the chains was also confirmed by the Potential Scale Reduction Factor (PSRF), which approached 1 (Ronquist et al. 2011). After checking the phylogenetic concordance between the nucITS and the nucLSU datasets for each of the six taxonomic groups (Agaricostilbomycetes, Cystobasidiomycetes, Microbotryomycetes, Filobasidiales, Tremellales and Ustilaginomycetes) we concatenated the two loci, using the program SequenceMatrix v.1.9 (Vaidya et al. 2011), for the final class level analyses. The combined datasets were analysed with both RAxML and MrBayes programs following the same conditions previously described. The phylogenetic trees were visualized in TreeView v.1.6.6 (Page, 1996).

Results

Culture isolation

Starting from 136 thalli belonging to 34 populations of *Rhizoplaca melanophthalma* and 84 thalli belonging to 21 population of *Tephromela atra*, a total 76 basidiomycetous yeast strains grew from 29 thalli belonging to 18 populations of *R. melanophthalma* and 18 thalli belonging to 13 populations of *T. atra*, collected in 24 different localities (Table 1). In particular, we isolated 51 basidiomycetes yeast strains from *R. melanophthalma* and 25 from *T. atra*. Because of the relatively low success rate of the isolation, it was not possible to homogeneously isolate a certain number of species from different lichen samples. The Venn diagram (Supplementary Fig. S1) shows that there are only three taxa found in both lichen species (Chionosphaeraceae, Lichenicolous clade III of Tremellales, and Agaricostilbaceae; Supplementary Fig. S1a). While the accumulation curves show that there is an extremely low increase of yeast species diversity among the analyzed thalli (Supplementary Fig. S1b). The pattern of isolated yeasts was very uneven among localities, as a single isolate could be obtained for six localities (i.e., J, K, P, Q, R and S; Supplementary Table S8). The 76 basidiomycetous yeast strains belonged to five different classes (Table 2) as follow: ten strains belonged to the class Agaricostilbomycetes, isolated from thalli of both lichen species collected between 2500 and 1500 m a.s.l. of North and South America and in the Alps; five strains belonged to the class Cystobasidiomycetes, isolated from thalli of both lichen species collected between 2150 and 1600 m a.s.l. in North America, Spain and the Alps; three strains belonged to the class Microbotryomycetes, isolated from thalli of both lichen species collected between 2250 and 1740 m a.s.l. in the Alps; 54 strains belonged to the class Tremellomycetes, isolated from the thalli of both lichen species collected in wide range of habitats below 3500 m a.s.l., worldwide; four strains belonged to the class Ustilaginomycetes, isolated only from thalli of *R. melanophthalma* between 2700 and 1540 m a.s.l. in North and South America. Sites G (Utah, USA) and M (Italy) are the sites from which the highest diversity of yeast taxa was isolated from thalli of *R.*

melanophthalma and *T. atra*, respectively. Here from either lichen species up to four different yeast species could be identified (Fig. 1f; Supplementary Table S8), while from the other sites only one or up to three different yeast species could be isolated (Supplementary Table S8). Furthermore, from site G three different yeast species could be isolated from the lichen sample *R. melanophthalma* L2637, while in site M two yeast species could be isolated from the sample *T. atra* L3276.

Calacogloea sp., *Naganishia* sp*., Tranzscheliella* sp., *Vishniacozyma* sp., the Yeast lineage II in Tremellales, *Fibulobasidium* sp. and a Microsporomycetaceae sp. were isolated only from *R. melanophthalma* thalli coming from different localities and growing mainly on granitic boulders (Table 1; Fig. 1f; Supplementary Table S8, Fig. S1a). Yeast Lineage I in Tremellales, *Pseudotremella* sp*., Yunzhangia* sp. and an unknown Cystobasidiomycetes were isolated only from *T. atra* thalli growing on schistous-siliceous rocks (Table1; Supplementary Table S8, Fig. S1a). Instead, unknown Chionosphaeraceae, Lichenicolous Clade III in Tremellales and unknown Agaricostilbaceae were isolated from both lichen species collected in different localities (Supplementary Fig. S1a). Moreover, in only four cases yeast strains were successfully isolated from both *R. melanophthalma* and *T. atra* collected in the same locality: i.e. from the locality E (Spain) *Tremella macrobasidiata,* Yeast lineage II, and *Lichenozyma pisutiana* were isolated from *R. melanophthalma* while Yeast Lineage I from *T. atra*; from the locality T (Italy) *Colacogloea* sp. was isolated from *R. melanophthalma* while unknown Agaricostilbaceae from *T. atra*; from the locality U (Italy) unknown Agaricostilbaceae and *Vishniacozyma* sp. were isolated from *R. melanophthalma* while unknown Chionosphaeraceae from *T. atra*; from the locality V (Italy) *Colacogloea* sp. was isolated from *R. melanophthalma* while unknown Chionosphaeraceae from *T. atra* (Fig. 1f).

Many other fungal strains belonging to the classes of Eurotiomycetes, Dothideomycetes, Sordariomycetes, Lecanoromycetes and Leotiomycetes were isolated and identified by ITS sequences during the screening of the yeast strains, but they will be analyzed in detail in another study.

Table 2. Origin data and sequence accession numbers of Basidiomycetes strains newly isolated in culture: culture ID, the original lichen host (thalli of *Rhizoplaca melanophthalma* and *Tephromela atra* and their ID), the phylogenetic placement, the geographic origin of the original lichen samples (letter-code as in Table 1), and the new corresponding NCBI accession numbers are reported.

ID culture	Lichen host	Phylogenetic placement	ID localities	nucITS	nucLSU
L3034	*R. melanophthalma* L2637	Agaricostilbomycetes / Agaricostilbales / Chionosphaeraceae	G	OP045981	OP045800
L3045	*R. melanophthalma* L2638	Agaricostilbomycetes / Agaricostilbales / Chionosphaeraceae	G	OP045982	-

L3044	*R. melanophthalma* L2725	Agaricostilbomycetes / Agaricostilbales / Chionosphaeraceae	K	OP045983	OP045801
L4046	*T. atra* L3275	Agaricostilbomycetes / Agaricostilbales / Chionosphaeraceae	M	OP045974	OP045802
L4051	*T. atra* L3407	Agaricostilbomycetes / Agaricostilbales / Chionosphaeraceae	P	OP045975	OP045803
L4089	*R. melanophthalma* L3483	Agaricostilbomycetes / Agaricostilbales / Chionosphaeraceae	R	OP045976	-
L4101	*T. atra* L3556	Agaricostilbomycetes / Agaricostilbales / Chionosphaeraceae	U	OP045978	-
L4100	*T. atra* L3643	Agaricostilbomycetes / Agaricostilbales / Chionosphaeraceae	V	OP045977	OP045804
L4105	*T. atra* L3530	Agaricostilbomycetes / Agaricostilbales / Agaricostilbaceae	T	OP045980	-
L4069	*R. melanophthalma* L3565	Agaricostilbomycetes / Agaricostilbales / Agaricostilbaceae	U	OP045979	OP045836
L4045	*T. atra* L3276	Cystobasidiomycetes	M	OP045984	OP045807
L4050	*T. atra* L3276	Cystobasidiomycetes	M	OP045985	-
L3243	*R. melanophthalma* L2668	Cystobasidiomycetes / Cystobasidiales / Microsporomycetaceae	H	OP046047	OP045806
L3244	*R. melanophthalma* L2668	Cystobasidiomycetes / Cystobasidiales / Microsporomycetaceae	H	OP046048	-
L3041	*R. melanophthalma* L2590	Cystobasidiomycetes / Cystobasidiales / Microsporomycetaceae	E	OP045986	OP045805
L4063	*T. atra* L3695	Microbotryomycetes / *Yunzhangia*	W	OP045987	OP045808
L4070	*R. melanophthalma* L3541	Microbotryomycetes / *Colacogloea*	T	OP045988	OP045809
L4072	*R. melanophthalma* L3617	Microbotryomycetes / *Colacogloea*	V	OP045989	OP045810
L2767	*R. melanophthalma* L2454	Tremellomycetes / Filobasidiales / *Naganishia*	B	OP045990	OP045811
L2770	*R. melanophthalma* L2468	Tremellomycetes / Filobasidiales / *Naganishia*	C	OP045991	OP045812
L2774	*R. melanophthalma* L2469	Tremellomycetes / Filobasidiales / *Naganishia*	C	OP045992	OP045813
L2781	*R. melanophthalma* L2543	Tremellomycetes / Filobasidiales / *Naganishia*	D	OP045993	OP045814
L2615	*R. melanophthalma* L2390	Tremellomycetes / Tremellales / Yeast Lineage II	A	OP045995	-
L2766	*R. melanophthalma* L2454	Tremellomycetes / Tremellales / Yeast Lineage II	B	OP045996	-
L2776B	*R. melanophthalma* L2469	Tremellomycetes / Tremellales / Yeast Lineage II	C	OP045997	-
L2867	*R. melanophthalma* L2460	Tremellomycetes / Tremellales / Yeast Lineage II	C	OP045994	-
L3738	*R. melanophthalma* L2460	Tremellomycetes / Tremellales / Yeast Lineage II	C	OP046000	OP045818
L2779	*R. melanophthalma* L2543	Tremellomycetes / Tremellales / Yeast Lineage II	D	OP045999	-
L2885	*R. melanophthalma* L2528	Tremellomycetes / Tremellales / Yeast Lineage II	D	OP045998	-
L2887	*R. melanophthalma* L2528	Tremellomycetes / Tremellales / Yeast Lineage II	D	OP046004	OP045815
L2860	*R. melanophthalma* L2590	Tremellomycetes / Tremellales / Yeast Lineage II	E	OP046001	-
L2895	*R. melanophthalma* L2637	Tremellomycetes / Tremellales / Yeast Lineage II	G	OP046005	OP045817
L2894	*R. melanophthalma* L2686	Tremellomycetes / Tremellales / Yeast Lineage II	I	OP046002	OP045816
L3022	*R. melanophthalma* L2686	Tremellomycetes / Tremellales / Yeast Lineage II	I	OP046003	-
L3038	*R. melanophthalma* L2686	Tremellomycetes / Tremellales / Yeast Lineage II	I	OP046007	-
L3039	*R. melanophthalma* L2686	Tremellomycetes / Tremellales / Yeast Lineage II	I	OP046006	-
L3046	*R. melanophthalma* L2686	Tremellomycetes / Tremellales / Yeast Lineage II	I	OP046009	-
L3743	*R. melanophthalma* L2686	Tremellomycetes / Tremellales / Yeast Lineage II	I	OP046015	-
L3051	*R. melanophthalma* L2687	Tremellomycetes / Tremellales / Yeast Lineage II	I	OP046010	OP045835
L3080	*R. melanophthalma* L2687	Tremellomycetes / Tremellales / Yeast Lineage II	I	OP046013	-
L3740	*R. melanophthalma* L2687	Tremellomycetes / Tremellales / Yeast Lineage II	I	OP046014	-
L3744	*R. melanophthalma* L2687	Tremellomycetes / Tremellales / Yeast Lineage II	I	OP046016	-
L3026	*R. melanophthalma* L2688	Tremellomycetes / Tremellales / Yeast Lineage II	I	OP046012	-
L3052	*R. melanophthalma* L2688	Tremellomycetes / Tremellales / Yeast Lineage II	I	OP046011	-
L3742	*R. melanophthalma* L2688	Tremellomycetes / Tremellales / Yeast Lineage II	I	OP046017	-
L3029	*R. melanophthalma* L2689	Tremellomycetes / Tremellales / Yeast Lineage II	I	OP046008	-

L3025	*R. melanophthalma* L2704	Tremellomycetes / Tremellales / *Fibulobasidium*	J	OP046025	OP045819
L3024	*R. melanophthalma* L2387	Tremellomycetes / Tremellales / Lichenicolous Clade III	A	OP046022	-
L2892	*R. melanophthalma* L2589	Tremellomycetes / Tremellales / Lichenicolous Clade III	E	OP046021	-
L3023	*R. melanophthalma* L2636	Tremellomycetes / Tremellales / Lichenicolous Clade III	G	OP046019	-
L3027	*R. melanophthalma* L2636	Tremellomycetes / Tremellales / Lichenicolous Clade III	G	OP046020	OP045820
L3741	*R. melanophthalma* L2637	Tremellomycetes / Tremellales / Lichenicolous Clade III	G	OP046023	-
L2898	*R. melanophthalma* L2638	Tremellomycetes / Tremellales / Lichenicolous Clade III	G	OP046018	-
L3785	*R. melanophthalma* L2786	Tremellomycetes / Tremellales / Lichenicolous Clade III	L	OP046024	OP045821
L4075	*T. atra* L3472	Tremellomycetes / Tremellales / Lichenicolous Clade III	Q	OP046026	-
L4085	*T. atra* L3523	Tremellomycetes / Tremellales / Lichenicolous Clade III	S	OP046027	OP045822
L4044	*T. atra* L3274	Tremellomycetes / Tremellales / *Pseudotremella*	M	OP046028	OP045823
L4066	*T. atra* L3353	Tremellomycetes / Tremellales / *Pseudotremella*	N	OP046029	-
L4067	*T. atra* L3353	Tremellomycetes / Tremellales / *Pseudotremella*	N	OP046030	OP045824
L4074	*T. atra* L3354	Tremellomycetes / Tremellales / *Pseudotremella*	N	OP046031	-
L4077	*T. atra* L3354	Tremellomycetes / Tremellales / *Pseudotremella*	N	OP046033	-
L4076	*T. atra* L3721	Tremellomycetes / Tremellales / *Pseudotremella*	X	OP046032	-
L4080	*T. atra* L3721	Tremellomycetes / Tremellales / *Pseudotremella*	X	OP046034	OP045825
L4091	*T. atra* L3720	Tremellomycetes / Tremellales / *Pseudotremella*	X	OP046035	-
L2889	*T. atra* L2583	Tremellomycetes / Tremellales / Yeast Lineage I	E	OP046037	OP045827
L2878	*T. atra* L2596	Tremellomycetes / Tremellales / Yeast Lineage I	F	OP046036	OP045826
L4049	*T. atra* L3276	Tremellomycetes / Tremellales / Yeast Lineage I	M	OP046038	OP045828
L4087	*T. atra* L3276	Tremellomycetes / Tremellales / Yeast Lineage I	M	OP046040	-
L4093	*T. atra* L3396	Tremellomycetes / Tremellales / Yeast Lineage I	O	OP046041	-
L4065	*T. atra* L3398	Tremellomycetes / Tremellales / Yeast Lineage I	O	OP046039	OP045829
L4102	*T. atra* L3695	Tremellomycetes / Tremellales / Yeast Lineage I	W	OP046042	OP045830
L4103	*R. melanophthalma* L3566	Tremellomycetes / Tremellales / *Vishniacozyma*	U	OP046043	OP045831
L2609	*R. melanophthalma* L2390	Ustilaginomycetes / Ustilaginales / *Tranzscheliella*	A	OP046044	OP045832
L3062	*R. melanophthalma* L2635	Ustilaginomycetes / Ustilaginales / *Tranzscheliella*	G	OP046046	-
L2891	*R. melanophthalma* L2669	Ustilaginomycetes / Ustilaginales / *Tranzscheliella*	H	OP046045	OP045833
L2900	*R. melanophthalma* L2788	Ustilaginomycetes / Ustilaginales / *Tranzscheliella*	L	OP046049	OP045834

Phylogenetic analysis

A total of 76 new nucITS and 37 new nucLSU sequences were obtained for the cultured yeasts (Table 2). The combined nucITS-nucLSU phylogenetic trees are presented in Figs. 2-7. We performed six separate phylogenetic analyses corresponding to Agaricostilbomycetes, Cystobasidiomycetes, Microbotryomycetes, Tremellales and Filobasidiales (Tremellomycetes) and Ustilaginomycetes. In general, our phylogenetic reconstructions were well-supported and topologically congruent with the phylogenies we used as references, i.e., the studies of Liu et al. (2015a,b), Li et al. (2017, 2020), Wang et al. (2015a,b) and Zamora et al. (2016), Černajová and Škaloud (2019), Millanes et al. (2011, 2016). Also, the phylogenetic trees inferred by ML and

Bayesian analyses did not report topological incongruence, neither between the two single loci nucITS and nucLSU individually, nor in the combined nucITS-nucLSU analyses.

Agaricostilbomycetes (Fig. 2) – Two strains, L4069 and L4105, isolated from thalli of *R. melanophthalma* and *T. atra*, respectively, sampled on granitic rocks from the Alps (in two closely located sites), belonged to the family Agaricostilbaceae, in which they were closely related to *Sterigmatomyces* spp. and *Pseudobensingtonia* spp. (Wang et al. 2015b). We refer to them as still unknown Agaricostilbaceae. Eight other strains, four isolated from thalli of *T. atra* collected in four different localities of the Alps (L4046, L4051, L4100, L4101), and four strains isolated from thalli of *R. melanophthalma* collected in two different localities of the Alps and two in North America (L3034, L3044, L3045, L4089), were grouped into the family of Chionosphaeraceae and were very closely related to *Kurtzmanomyces nectairei*; we refer to them as still unnamed *Kurtzmanomyces* sp.

Cystobasidiomycetes (Fig. 3) – Although the backbone phylogeny was not supported, all the major family and order-level lineages were fully supported. Here, two strains isolated from one thallus of *T. atra* from the Alps (L4045 and L4050), grouped into a still unnamed lineage (likely of still *incertae sedis* in Cystobasidiomycetes) with three samples of uncultured Cystobasidiomycetes detected previously by Černajová and Škaloud (2019) and Mark et al. (2020). Two other strains, isolated from thalli of *R. melanophthalma* collected in North America (L3243 and L3244), were nested within Microsporomycetaceae. A fifth strain isolated from a *R. melanophthalma* collected in Spain (L3041), also in Microsporomycetaceae, was closely related to the recently described species *Lichenozyma pisutiana* isolated from *Cladonia* lichen thalli by Černajová and Škaloud (2019).
The detection of Cystobasidiomycetes within thalli of *R. melanophthalma* L2590 and L2668 and *T. atra* L3276 performing PCR amplifications using the Cystobasidiomycetes specific primers failed, as no PCR products were obtained.

Microbotryomycetes (Fig. 4) – Two strains isolated from the thalli of *R. melanophthalma* collected in two localities of the Alps (L4070 and L4072) were found in the lineage of *Colacogloea* spp., a yeast genus isolated from soil (Yurkov et al. 2016; Kachalkin et al. 2019). A third strain isolated from a thallus of *T. atra* sampled on the Alps (L4063), formed a small clade with *Yunzhangia auriculariae*; however, this relationship received no support.
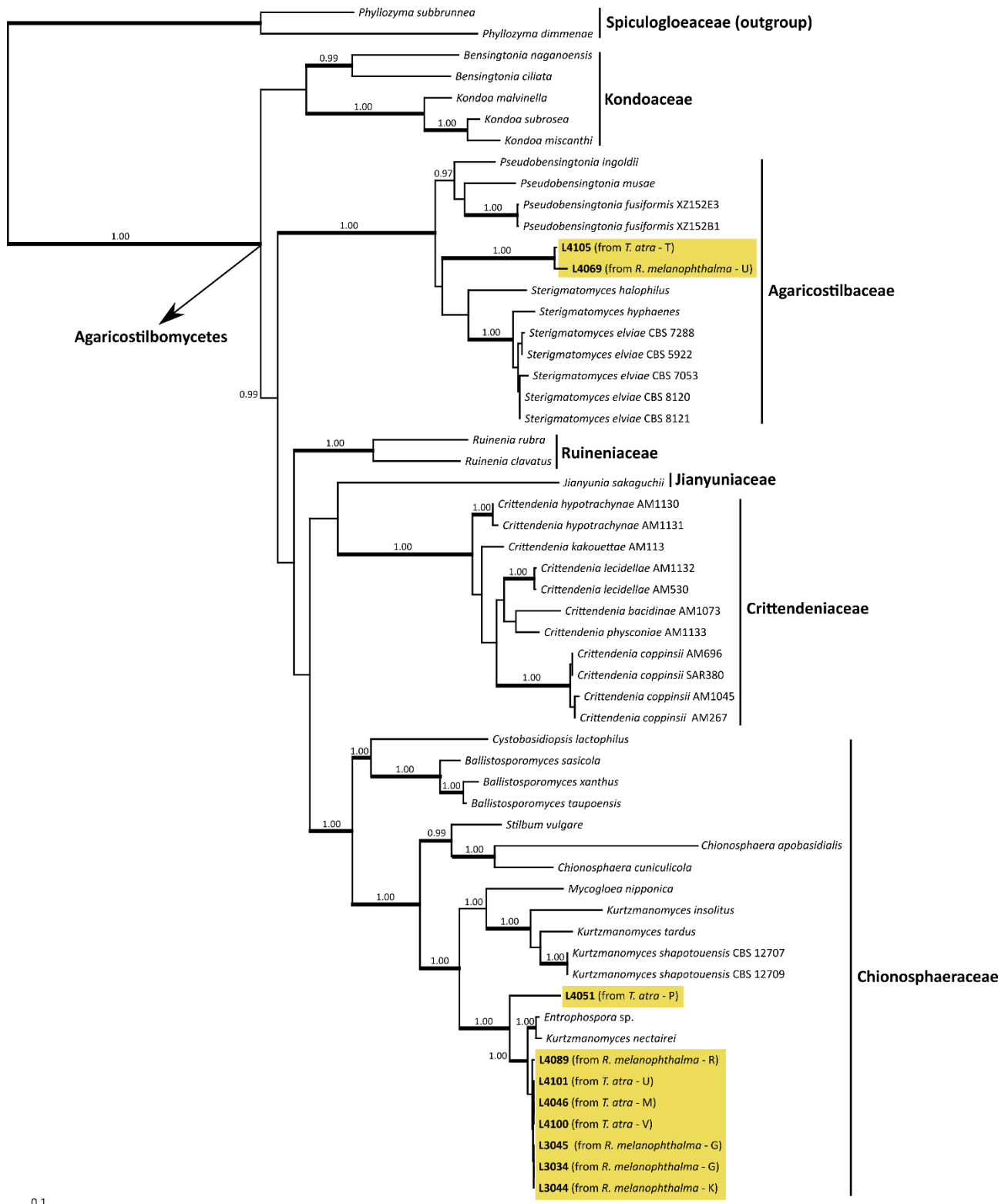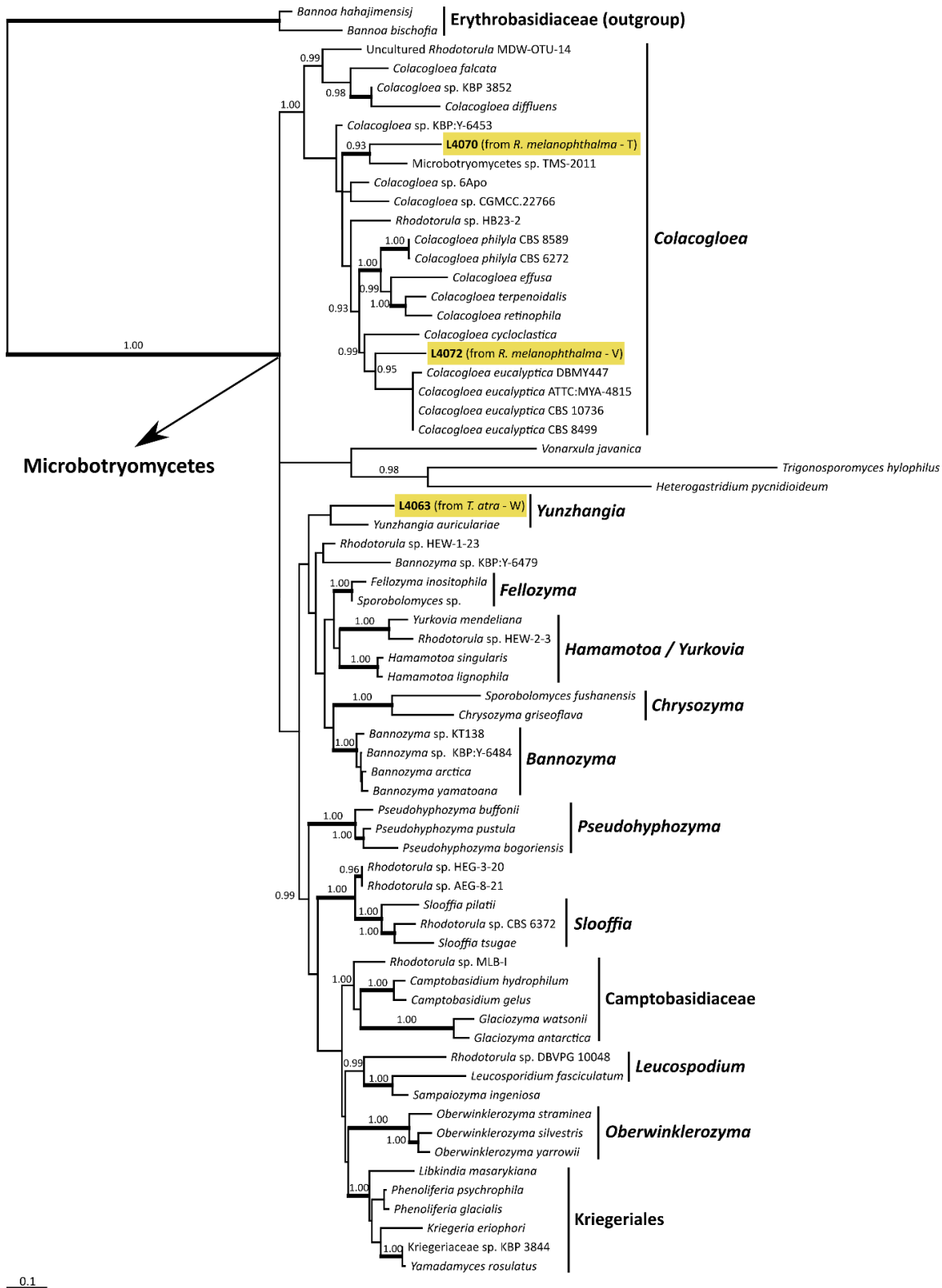
Figure 2. Phylogenetic inference of Agaricostilbomycetes: Bayesian analysis based on the concatenated nuclear ITS-LSU dataset. Bayesian posterior probabilities ≥0,8 are reported above branches; branches in bold denote RAxML bootstrap support >75%. Newly obtained sequences are in bold and highlighted in yellow; in parenthesis the original lichen species and the sampling locality (according to Table 1) are reported. Agaricostilbomycetes clades are named according to the phylogenetic study of Millanes et al. (2021).
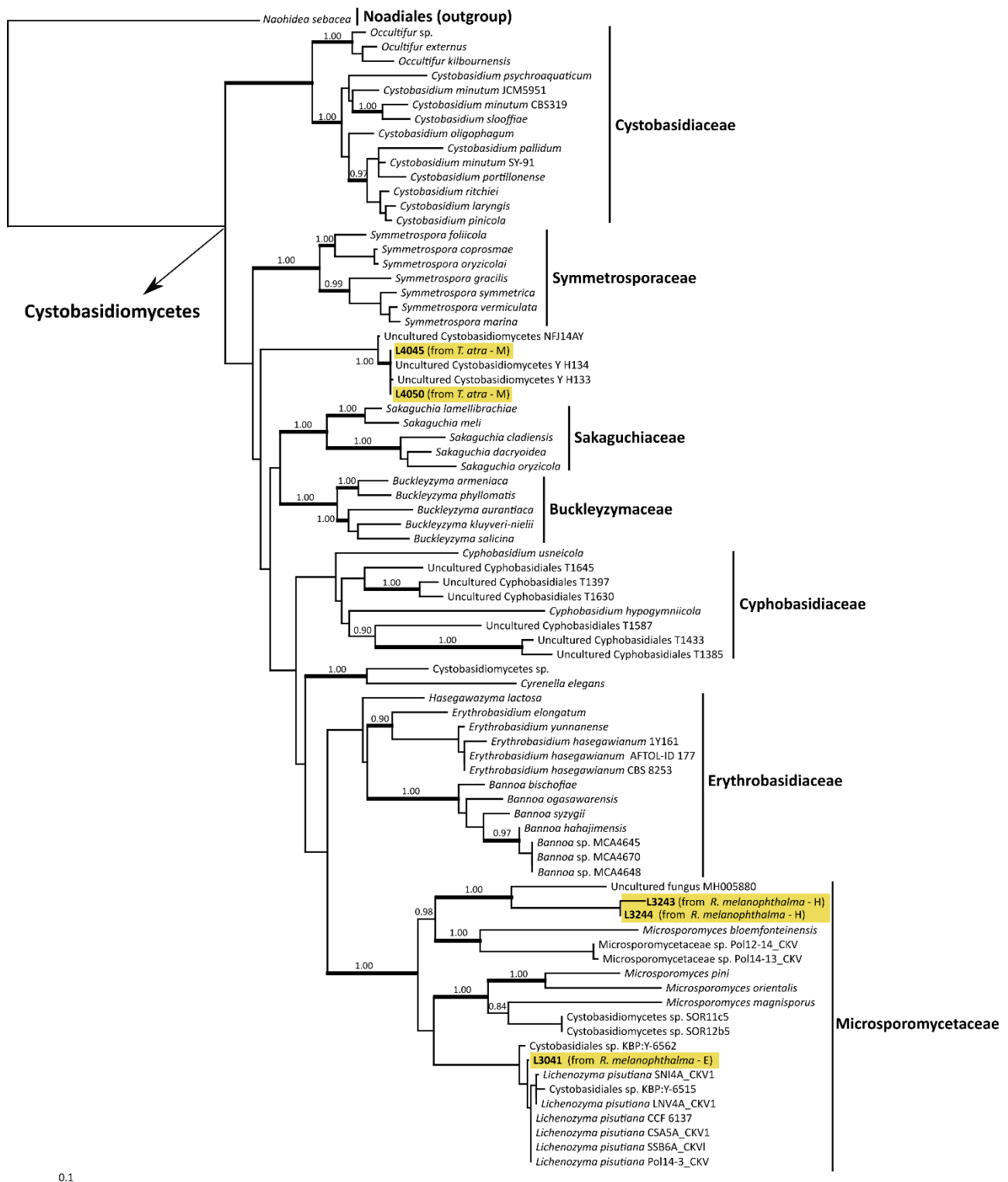
Figure 3. Phylogenetic inference of Cystobasidiomycetes: Bayesian analysis based on the concatenated nuclear ITS-LSU dataset. Bayesian posterior probabilities ≥0,8 are reported above branches; branches in bold denote RAxML bootstrap support >75%. Newly obtained sequences are in bold and highlighted in yellow; in parenthesis the original lichen species and the sampling locality (according to Table 1) are reported. Cystobasidiomycetes clades are named according to the phylogenetic studies of Černajová and Škaloud (2019) and Millanes et al. (2016).
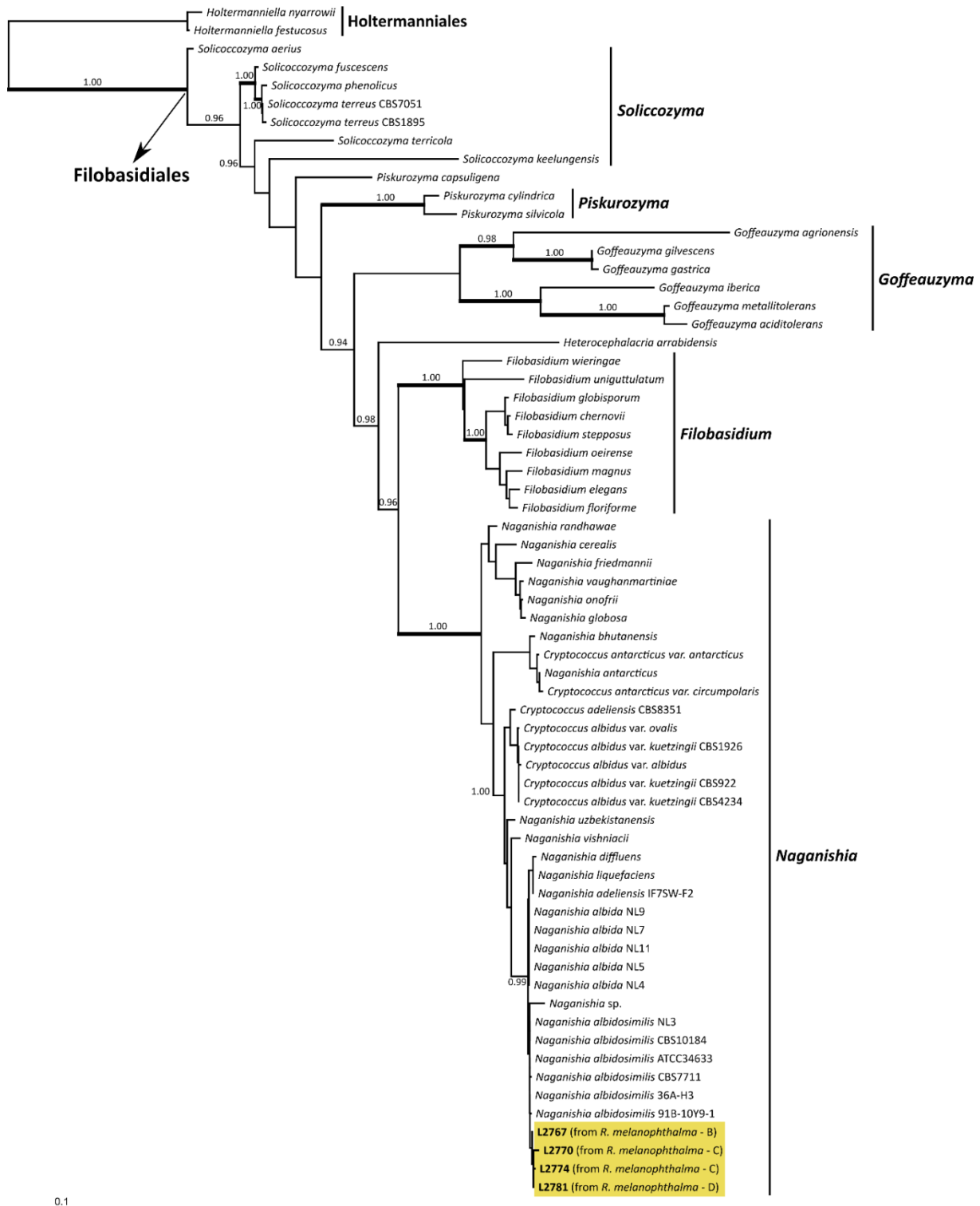
Figure 4. Phylogenetic inference of Microbotryomycetes: Bayesian analysis based on the concatenated nuclear ITS-LSU dataset. Bayesian posterior probabilities ≥0,8 are reported above branches; branches in bold denote RAxML bootstrap support >75%. Newly obtained sequences are in bold and highlighted in yellow; in parenthesis the original lichen species and the sampling locality (according to Table 1) are reported. Microbotryomycetes clades are named according to the previous phylogenetic studies of Yurkov et al. (2016) and Kachalkin et al. (2019).

Tremellomycetes (Figs. 5, 6) – The phylogenetic inference of Filobasidiales (Fig. 5) recovered five well supported clades – "aerius" (*Solicoccozyma*), "cylindricus" (*Piskurozyma*), "gastricus" (*Goffeauzyma*), *Filobasidium* and "albidus" (*Naganishia*) in accordance with Liu et al. (2015a,b) and Boekhout et al. (2011). Four strains isolated from thalli of *R. melanophthalma* collected in three localities of South America (L2767, L2770, L2774 and L2781) were placed in Filobasidiales, all of them were nested in the genus *Naganisha* (Fig. 5).

Within the order Tremellales (Fig. 6), the newly sequenced strains belonged to six different lineages. Seven strains isolated from thalli of *T. atra* sampled in five localities of the Alps, Spain and Tasmania (L2878, L2889, L4049, L4065, L4087, L4093 and L4102) grouped within a clade named "Yeast Lineage I" and were closely related to another yeast (GB accession number KBPY6612) isolated for the first time from the lichen *Cladonia rangiferina* by Kachalkin and Pankratov (unpublished work). The strain L4103 isolated from *R. melanophthalma* from the Alps was nested within *Vishniacozyma*. The single strain L3025 was within Fibulobasidium clade, closely related to *Fibulobasidium* spp. and *Sirobasidium* spp.. Twenty-three strains isolated from thalli of *R. melanophthalma* of seven populations sampled in North and South America, Spain and the Alps (L2615, L2776B, L2779, L2860, L2867, L2885, L2887, L2894, L2895, L3022, L3026, L3029, L3038, L3039, L3046, L3051, L3052, L3738, L3740, L3742, L3743 and L3744) formed a distinct lineage (named 'Yeast Lineage II', Fig. 6) along with four samples of uncertain position. Closely related to the clade named "Lichenicolous Clade III" by Millanes et al. (2011) we found two isolates from thalli of *T. atra* collected in two localities in the Alps (L4075 and L4085) and seven isolates coming from *R. melanophthalma* collected in four different localities of North and South America and Spain (L2892, L2898, L3023, L3024 L3027, L3741 and L3785).
The phylogenetic placement of these strains was further confirmed by the analyses performed only with species belonging to Tremellales and including the sequences successfully obtained from the thallus PCR amplification with the *Tremella* specific primers (Supplementary Fig. S2). Indeed, the sequences obtained from thalli of *R. melanophthalma* L2636, L2637, L2589 and L2786 and *T. atra* L3472 and L3523 correspond to *T. macrobasidiata* and were grouped together with the respective sequences derived from the isolated strains L2892, L2898, L3023, L3024 L3027, L3741, L3785 L4075 and L4085. Eight strains isolated from thalli of *T. atra* of three populations collected in the Alps (L4044, L4066, L4067, L4074, L4076, L4077, L4080 and L4091 and L4085) corresponded to *Pseudotremella.*

Ustilaginomycetes (Fig. 7) – Four strains isolated from thalli of *R. melanophthalma* collected in three localities in North and South America (L2609, L2891, L2900 and L3062) were nested within a large lineage including *Tranzscheliella* spp.

Figure 5. Phylogenetic inference of Filobasidiales: Bayesian analysis based on the concatenated nuclear ITS-LSU dataset. Bayesian posterior probabilities ≥0,8 are reported above branches; branches in bold denote RAxML bootstrap support >75%. Newly obtained sequences are in bold and highlighted in yellow; in parenthesis the original lichen species and the sampling locality (according to Table 1) are reported. Filobasidiales clades are named according to the previous phylogenetic studies of Boekhout et al. (2011) and Liu et al. (2015a,b).
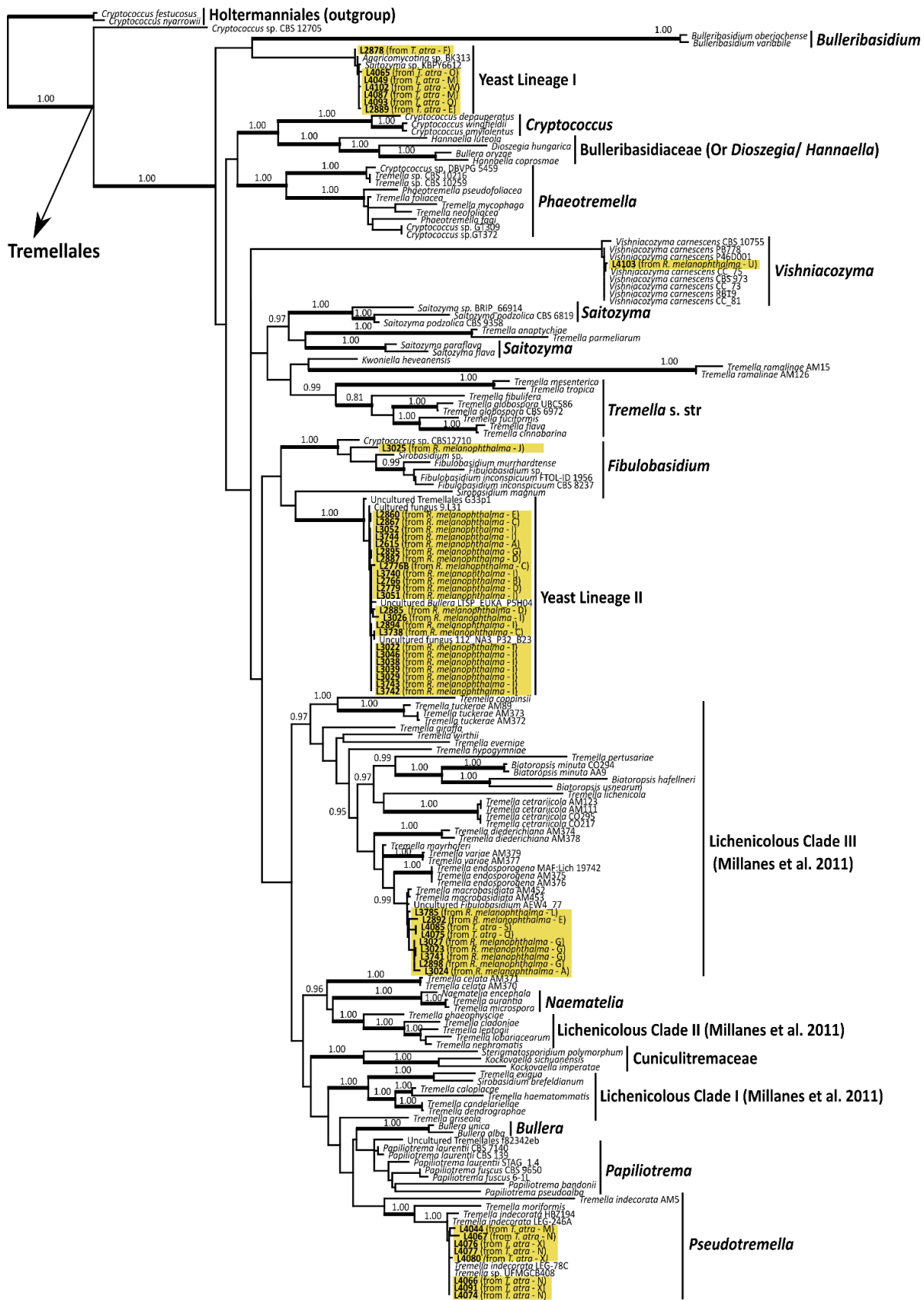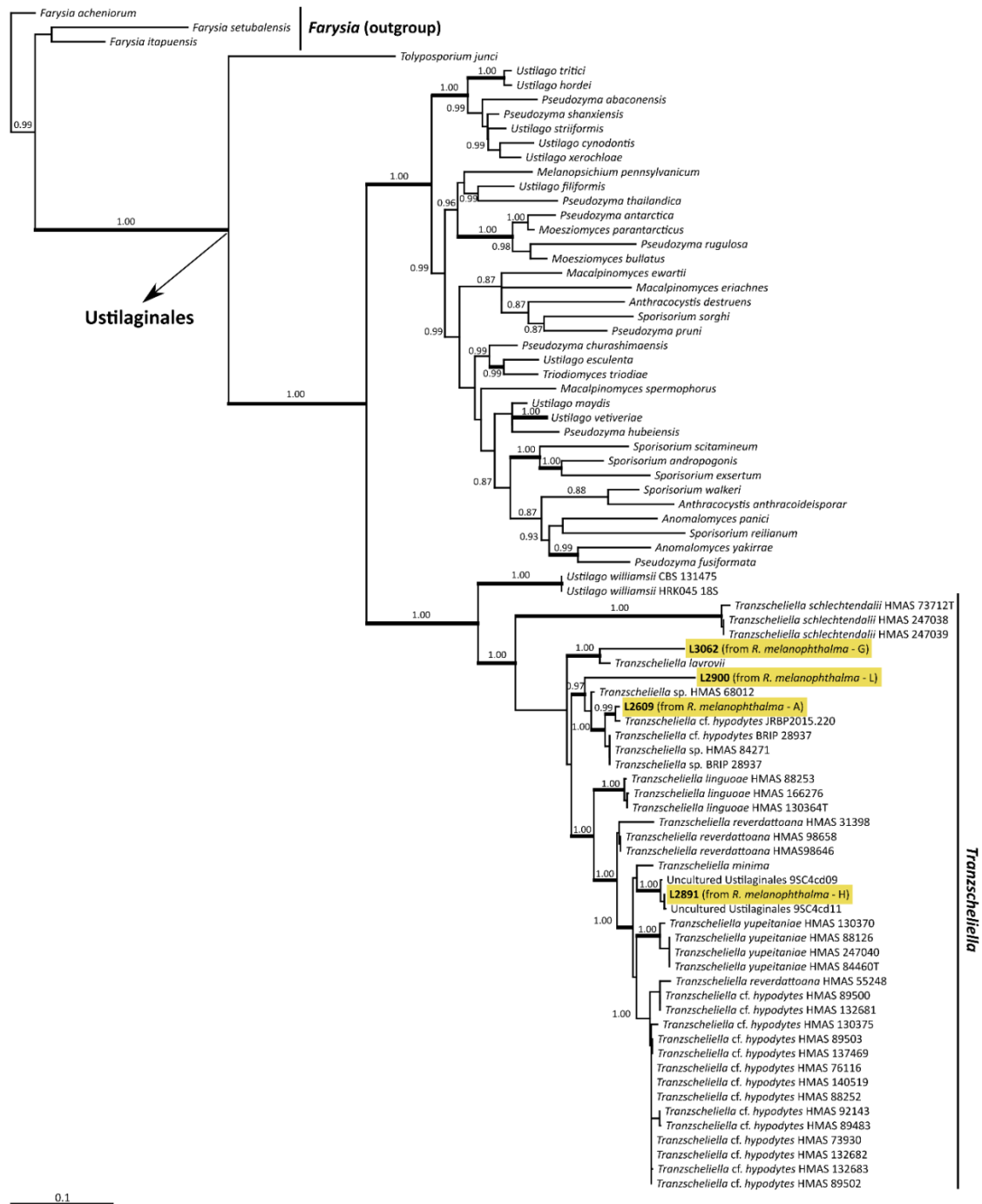
Figure 6. Phylogenetic inference of Tremellales: Bayesian analysis based on the concatenated nuclear ITS-LSU dataset. Bayesian posterior probabilities ≥0,8 are reported above branches; branches in bold denote RAxML bootstrap support >75%. Newly obtained sequences are in bold and highlighted in yellow; in parenthesis the original lichen species and

the sampling locality (according to Table 1) are reported. Tremellales clades are named according to the previous phylogenetic studies of Duarte et al. (2016), Millanes et al. (2011) and Scorzetti et al. (2000), Zamora et al. (2017).

Figure 7. Phylogenetic inference of Ustilaginomycetes: Bayesian analysis based on the concatenated nuclear ITS-LSU dataset. Bayesian posterior probabilities ≥0,8 are reported above branches; branches in bold denote RAxML bootstrap support >75%. Newly obtained sequences are in bold and highlighted in yellow; in parenthesis the original lichen species and the sampling locality (according to Table 1) are reported. Ustilaginomycetes clades are named according to the previous phylogenetic studies of Li et al. (2017) and Wang et al. (2015a).

Morphological analysis

 Agaricostilbomycetes (Fig. 8) – The strains belonging Chionosphaeraceae (L3034, L3044, L3045, L4046, L4051, L4089, L4100 and L4101) were characterized by colonies of 1.5 cm in diameter and pinkish coloured (Figs. 8a-e). Most of the cells were isodiametric to ellipsoid (5 × 4 μm; Figs. 8f, h-k); budding polar cell were present (Figs. 8f, h-k). Germination cells were observed only in a single strain (L4046; Figs. 8i, j). The strains recovered within the family Agaricostilbaceae (L4069 and L4105) were characterized by colonies of 1.5 cm in diameter and orange coloured. Cells were usually ellipsoidal (7 × 4 μm) and budding cells were present (Figs. 8g, l).
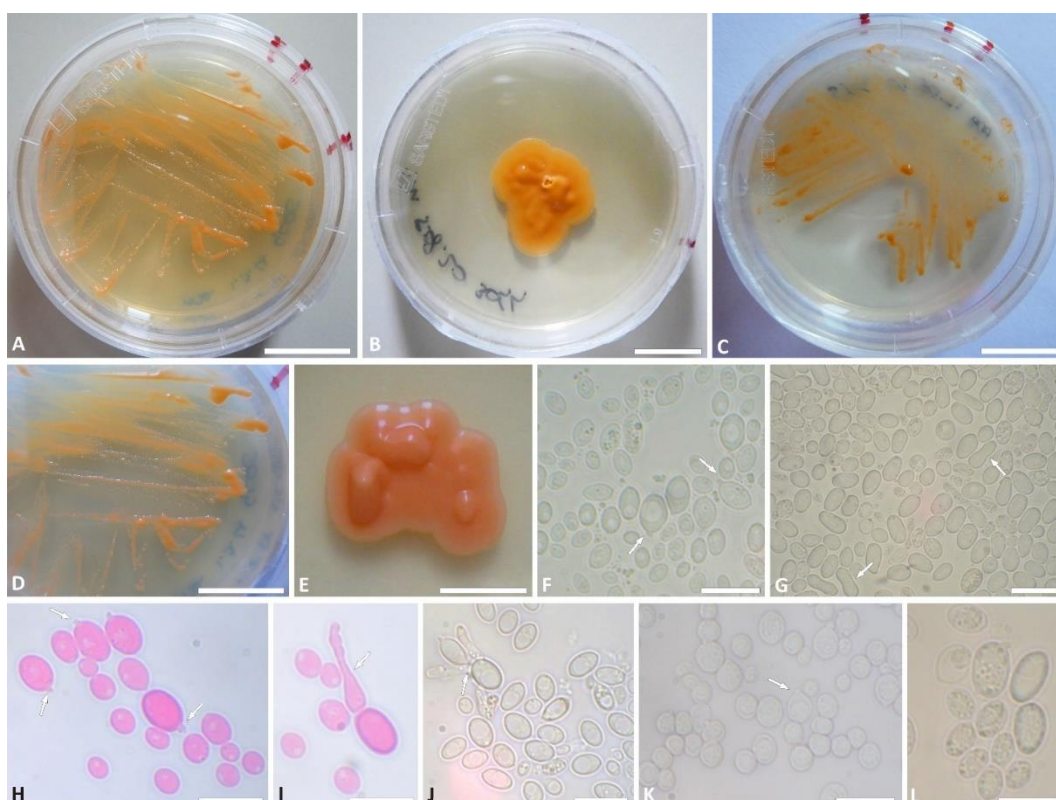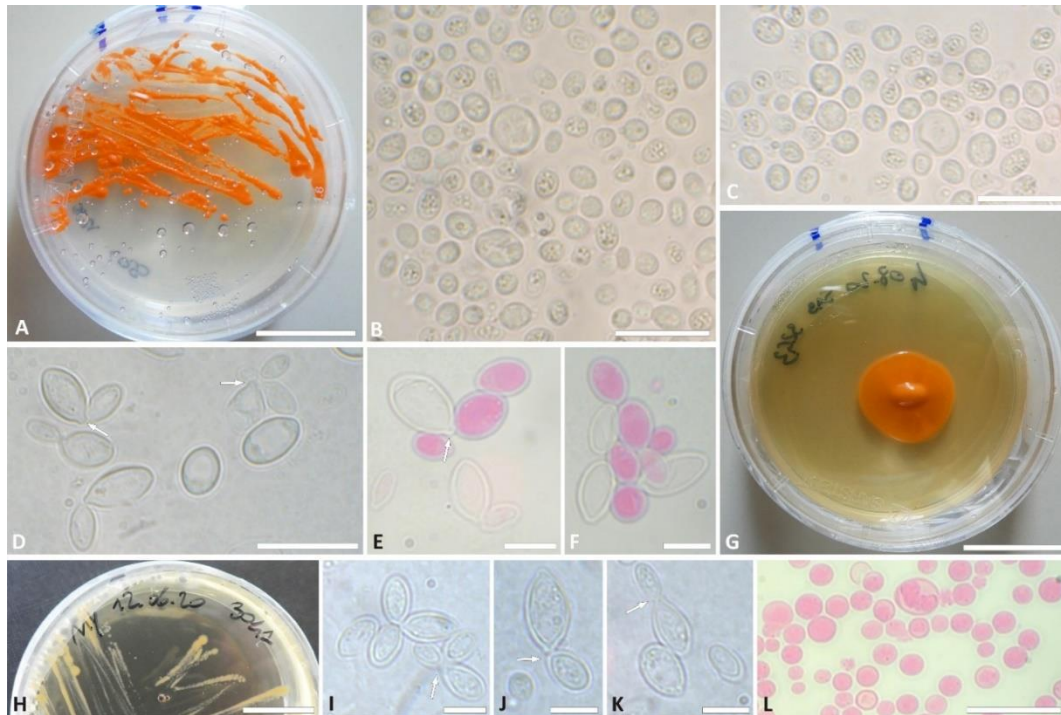

Figure 8. Morphology of six-month old representative cultured fungal strains belonging to Agaricostilbomycetes and included in the phylogenetic analysis of Fig. 2. Colony shape of *Kurtzmanomyces* sp. L4046 (A), L3044 (B), L3045 (C), L4046 (D) and L3034 (E); mature cells and polar budding cells of *Kurtzmanomyces* sp. L4046 (F), of Agaricostilbaceae sp. L4069 (G, L); mature cells and polar budding cells strained with Phloxin B of *Kurtzmanomyces* sp. L4046 (H, I), germination cells are visible in (I, J), cells and polar budding cells of *Kurtzmanomyces* sp. L3044 (K). Scale bars: 1,5 cm (A-E), 10 μm (F-L).

 Cystobasidiomycetes (Fig. 9) – The two strains L4045 and L4050, forming a still unnamed lineage with other three uncultured Cystobasidiomycetes, were characterized by orange colonies (Fig. 9a). Their cells were isodiametric (5 μm) to ellipsoid (8 × 4 μm; Figs. 9b-f); budding polar cell were present (Figs. 9c-e). The two strains (L3243 and L3244) belonging to *Microsporomyces* spp.,

grew as orange colonies of about 1.5 cm diameter (Fig. 9g). Their cells were usually isodiametric (5-8 µm diameter; Fig. 9l); budding cells were not observed. The strain L3041, nested within *Lichenozyma pisutiana,* was characterized by ochraceous to pale orange colony (Fig. 9h) and ellipsoid cells (7 × 4 µm) often presenting budding polar cells (Figs. 9i, j).

Figure 9. Morphology of six-month old representative cultured fungal strains belonging to Cystobasidiomycetes and included in the phylogenetic analysis of Fig. 3. Colony shape of uncultured Cystobasidiomycetes L4050 (A), of uncultured Erythrobasidiaceae L3243 (G), of *Lichenozyma pisutiana* L3041 (H); mature cells and polar budding cells of uncultured Cystobasidiomycetes L4050 (B, C) and L4045 (D, K), of *L. pisutiana* L3041 (I, J); mature cells and polar budding cells strained with Phloxin B of uncultured Cystobasidiomycetes L4045 (E, F), of uncultured Erythrobasidiaceae L3243 (L). Scale bars: 1,5 cm (A, G, H), 10 µm (B-D, L), 5 µm (E, F, I-K).

Microbotryomycetes (Fig. 10) – The two strains (L4070 and L4072) in the lineage of *Colacoglea* spp., grew as pale orange colonies (Figs. 10a, b). Their cells were ellipsoid (6 × 3 µm) and budding polar cell were present (Figs. 10c-e). The strain L4063, which was phylogenetically placed as sister of *Yunzhangia auriculariae,* was characterized by pale orange colonies. Mostly of the cells were ellipsoid (4 × 3 µm) to isodiametric (8 µm; Figs. 10f, h, i, k); budding polar cell were present (Figs. 10f, h-k). Germination cells were observed (Fig. 10g).
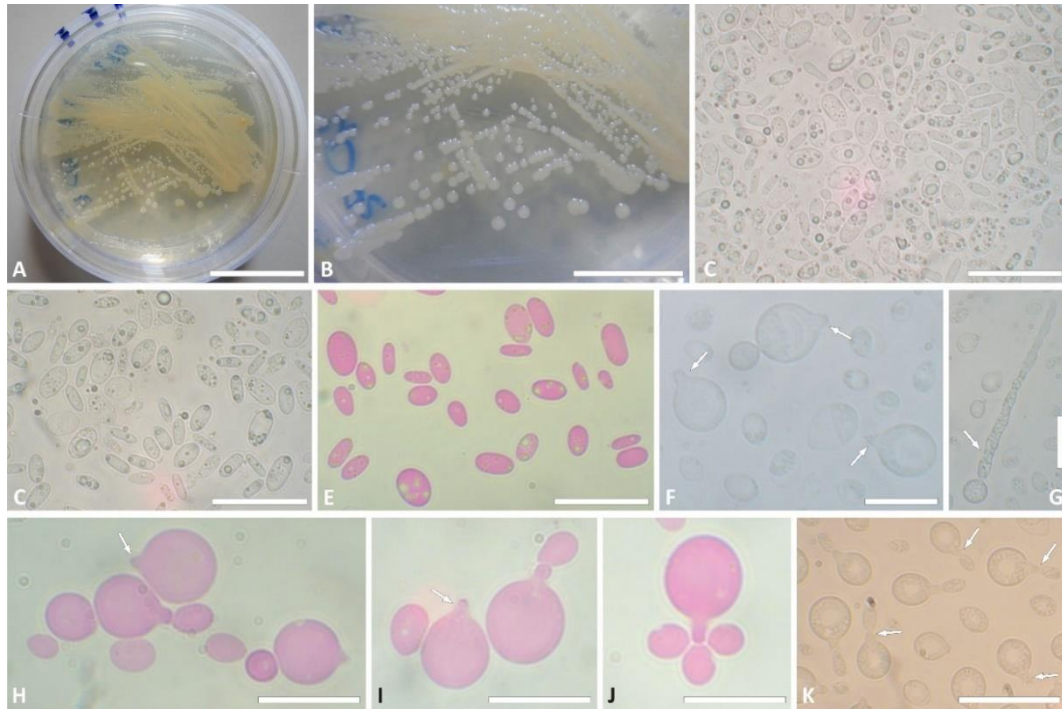
Figure 10. Morphology of six-month old, representative cultured fungal strains belonging to Microbotryomycetes and included in the phylogenetic analysis of Fig. 4. Colony shape of *Colacogloea* sp. L4072 (A, B); mature cells and polar budding cells of *Colacogloea* sp. L4072 (C-D), of *Yunzhangia auriculariae*L4063 (F, K); germination cell of *Y. auriculariae* L4063 (G); mature cells and polar budding cells strained with Phloxin B of *Colacogloea* sp. L4072 (E), of *Y.* auriculariae L4063 (H-J). Scale bars: 1,5 cm (A, B), 20 μm (C-E, G, K), 10 μm (F, H-J).

Tremellomycetes (Figs. 11, 12) – The four strains (L2767, L2770, L2774 and L2781) recognized as *Naganishia albidosimilis*, grew as rosa-orange colonies of about 1.5 cm in diameter (Figs. 11a-d). The cells were isodiametric (6 μm) to ellipsoid (3-6 × 6-9 μm) and budding polar cells were present (Fig. 11e).

The seven strains (L2878, L2889, L4049, L4065, L4087, L4093 and L4102) grouped within the clade of "Yeast Lineage I", in the order of Tremellales, and closely related to a yeast isolated from lichens (KBPY6612), were characterized by pale orange colonies (Figs. 11g, j). Most of the cells were isodiametric (4-7 μm diameter) often with budding polar cells (Figs 11f, h, i). Only the strains L4065 presented germination cells (Fig. 11h). The strain L4103 nested within *Vishniacozyma* was characterized by colonies salmon coloured. The strain L3025, closely related to *Fibulobasidium*, was characterized by colony of 1 cm in diameter, pink-orange coloured. Mostly of the cells were oval (from 5-7 × 3-4 μm) often with budding polar cells (Figs. 11k-m).

The 23 strains (L2615, L2776B, L2779, L2860, L2867, L2885, L2887, L2894, L2895, L3022, L3026, L3029, L3038, L3039, L3046, L3051, L3052, L3738, L3740, L3742, L3743 and L3744) forming the clade "Yeast Lineage II", grew as whitish to orange colonies of about 1.5 cm in

diameter (Figs. 11n, q). Most of the cells were isodiametric (5-8 μm diameter) to oval (7 × 5 μm; Figs. 11p, r-u); polar budding cells were often present; and only the strain L2878 was characterized by multiple budding cells on the mother cell (Fig. 11o). The isolates (L2892, L2898, L3023, L3024 L3027, L3741, L3785, L4075 and L4085) closely related to *T. macrobasidiata* grew as pale pink to orange colonies (Fig. 12a). The cells were isodiametric (4-8 μm diameter) to ellipsoidal (8 × 5 μm; Figs. 12b, k-m); polar, bipolar and multipolar budding cells were presents (Fig. 12b-d, g, k-m). Moreover, many germination cells were observed (Figs. 12e, f, h-k). The eight strains (L4044, L4066, L4067, L4074, L4076, L4077, L4080 and L4091) grouped within *Pseudotremella* were characterized by colony of 2 cm in diameter and creamy to orange (Figs. 12n-p). These strains were dimorphic with both the unicellular and filamentous stages produced in culture. The yeast cells were isodiametric (4-8 μm diameter; Figs. 12q, s-v); polar budding cells were present (Figs. 12s-v); in the filamentous morphology the hyphae had a diameter of about 4 μm (Fig. 12r).

Ustilaginomycetes (Fig. 13) – Four strains recovered within the big lineage of *Tranzschelliella* spp. (L2609, L2891, L2900 and L3062) grew as white to orange colonies of 3 cm in diameter (Figs. 13a-c). These strains were characterized by both the unicellular and filamentous stages (Fig. 13d-i). The yeast cells were isodiametric (3-6 μm diameter) to ellipsoidal (7 × 4 μm); polar and bipolar budding cells were presents (Fig. 13e); germination cells were observed (Figs. 13d-i). The hyphae were 2-4 μm thick and sometimes generate ramifications (Figs. 13e, g, i).
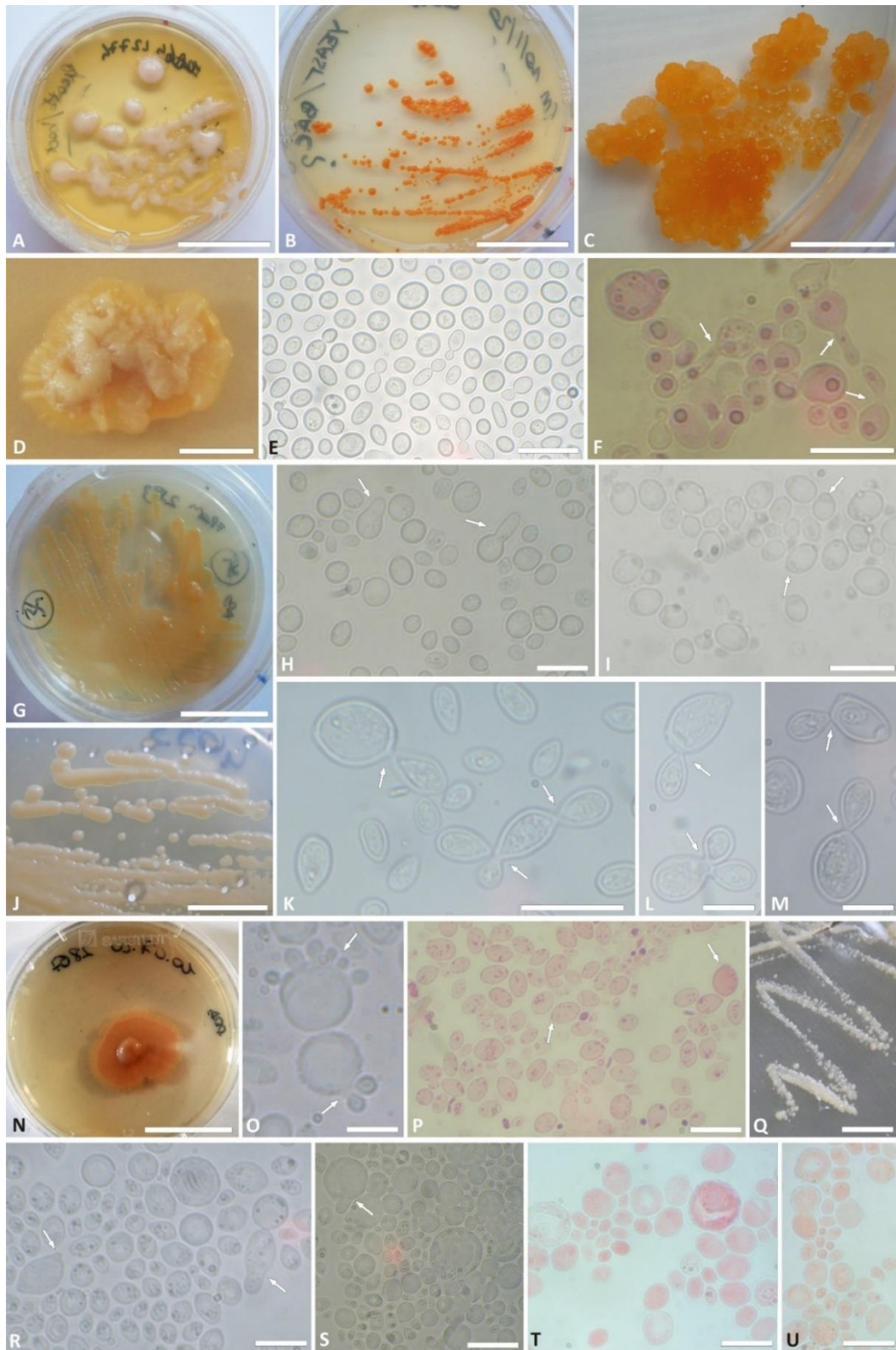
Figure 11. Morphology of six-month old, representative cultured fungal strains belonging to Tremellomycetes and included in the phylogenetic analysis of Fig. 5 and Fig. 6. Colony shape of *Naganishia albidosimilis* L2774 (A), L2781 (B), L2770 (C) and L2767 (D); mature cells and polar budding cells of L2774 are visible in (E). C-olony shape of Saitozyma sp. L2889 (G) and L4093 (J); cells and germination cells strained with Phloxin B of L2889 are visible in (F); mature cells, polar budding cells and germination cells of L4065 (H) and L4049 (I). Colony shape of *Cryptococcus* sp.

L2867 (N) and L2887 (Q); mature cells and polar budding cells of L3025 (K-M) and L2894 (R, S); multipolar budding cells of L2867 (O); cells and polar budding cells strained with Phloxin B of *Cryptococcus* sp. L3051 (P) and L3738 (T, U). Scale bars: 1,5 cm (A, B, G, N), 1 cm (Q), 0,5 cm (C, D, J), 10 μm (E, F, H, I, K, P, U), 5 μm (L, M, O, R-T).
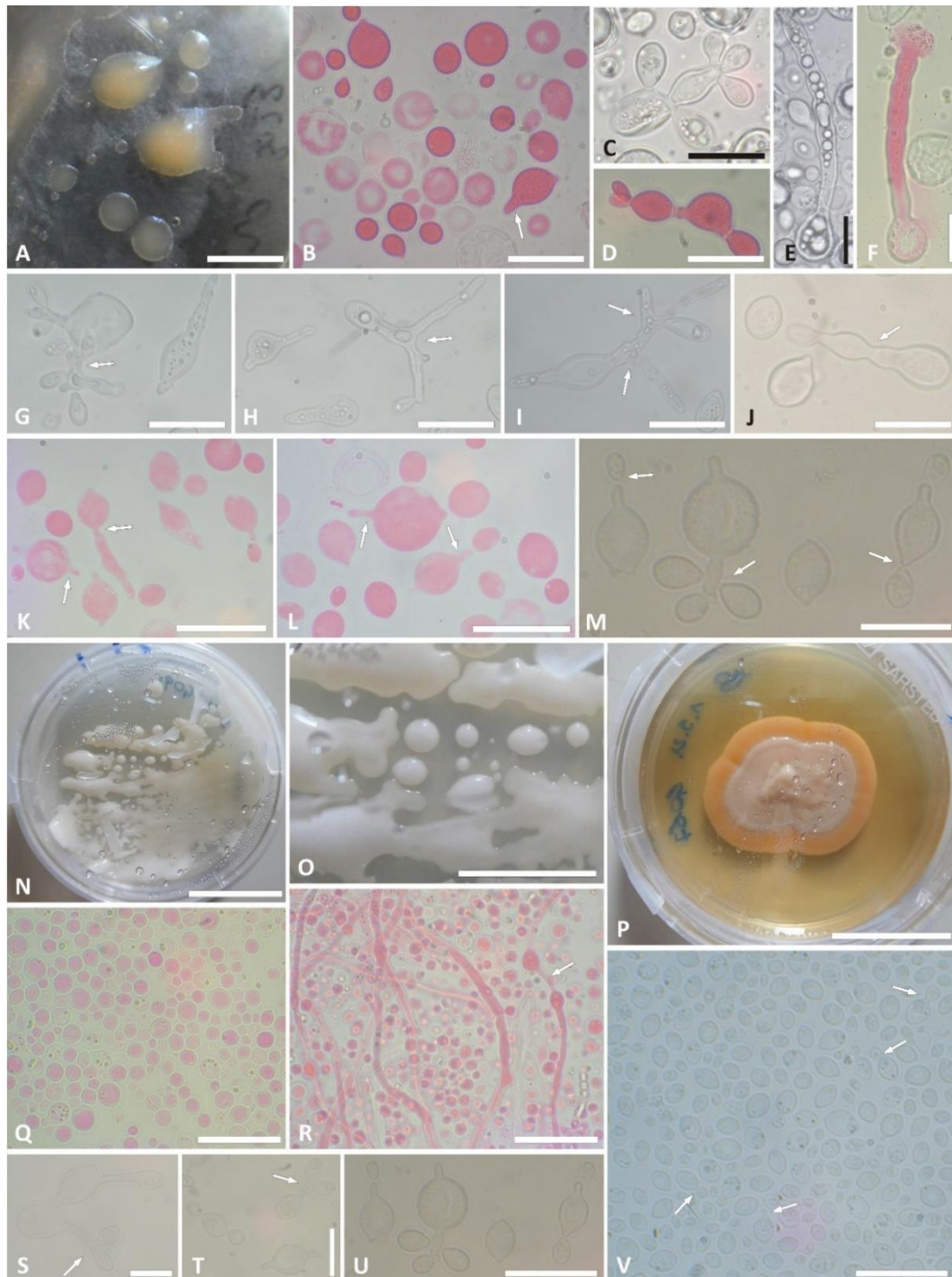
Figure 12. Morphology of six-month old, representative cultured fungal strains belonging to Tremellomycetes and included in the phylogenetic analysis of Fig. 6. Colony shape of *Tremella macrobasidiata* L3023 (A); multipolar budding cells of L3023 (C), L3785 (G) and L4085 (M); germination cells of L3023 (E) and L3785 (H-J); mature cells and budding polar cells strained with Phloxin B of L3023 (B, D) and L4085 (K, L); germination cells strained with Phloxin B of L3023 (F) and L4085 (K). Colony shape of *T. indecorata* L4091 (N, O) and L4067 (P); mature cells and

43

polar budding cells of L4080 (S-V); mature cells strained with Phloxin B of L4067 (Q); hyphae strained with Phloxin B of L4067 (R). Scale bars: 1,5 cm (N, P), 0,5 cm (A, O), 20 µm (Q, R, V), 10 µm (B-D, G-M, U), 5 µm (E, F, O, S).
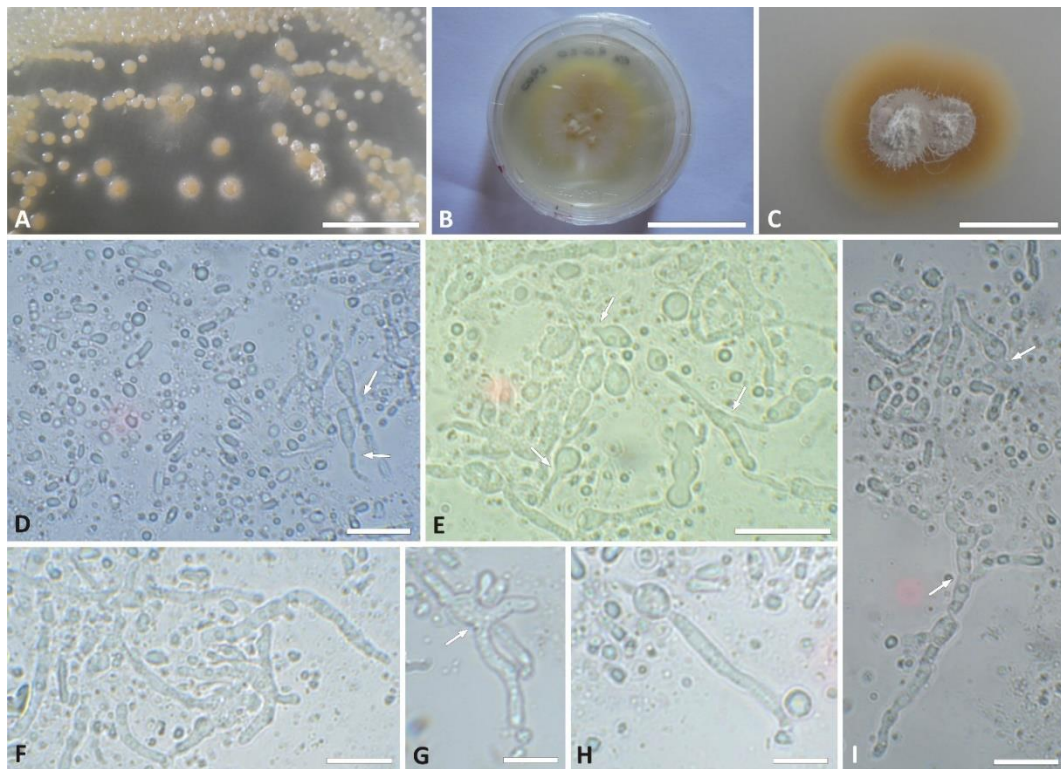

Figure 13. Morphology of six-month old, representative cultured fungal strains belonging to Ustilagomycetes and included in the phylogenetic analysis of Fig. 7. Colony shape of *Tranzschelliella* sp. L2891 (A), L2900 (B) and L3062) (C); unicellular stages, filamentous stages and germination cells of L2900 are visible in (D-I); polar budding cells of L2900 (E). Ramification of hyphae of L2900 are visible in (E, G, I). Scale bars: 0,5 cm (A), 3 cm (B), 1,5 cm (C), 10 µm (D, E, F, I), 5 µm (G, H).

Discussion

In lichens, the major fraction of the mycobiome is composed by ascomycetes belonging to the subphylum Pezizomycotina, while only a minor fraction is represented by basidiomycetes (Zhang et al. 2015, 2016; Fernández-Mendoza et al. 2017; Banchi et al. 2018). Endolichenic ascomycetes were also frequently isolated in cultures (Arnold et al. 2009; Muggia et al. 2016, 2017, 2021) in contrast to basidiomycetes, which are still poorly represented as axenic isolates (Santiago et al. 2015; Zhang et al. 2015; Černajová and Škaloud, 2019, 2020). Recent study by Lendemer et al. (2019) applying metagenomics analyses, suggested that Cystobasidiomycete yeasts represent only a minor fraction of lichen-associated fungi across a comprehensive sampling of lichens (as Cystobasidiomycete yeasts reads were detected only in 2.7% of the analyzed thalli). Similarly, Smith et al. (2020) found little evidence supporting that Cystobasidiales yeasts would always be

present in macrolichens, reporting low abundance reads of basidiomycetes only in samples of *Bryoria* lichens. However, highly specific, *ad hoc* targeted microscopic inspections using fluorescent probes and confocal microscopy consistently identify a high frequency of basidiomycetes yeasts in lichen thalli (Tuovinen et al. 2019, 2021).

Here, we provide a wider perspective into the range of basidiomycete yeasts isolated from lichens and able to grow in axenic culture. The application of HTS and metabarcoding analyses have facilitated a more expansive view into the range of lichen-associated ascomycetes and basidiomycetes. Our study contributes to this body of knowledge by providing axenic culture isolates with accompanying morphological description and DNA sequence data for 76 strains of basidiomycetous yeasts, belonging to five classes and isolated from *Rhizoplaca melanophthalma* and/or *Tephromela atra* collected in boreal, alpine, temperate, humid and arid habitats worldwide. Most of the identified strains were never isolated from lichens before, and some would deserve taxonomic species description, as they either build new own clades or expand clades with other undescribed taxa. The formal descriptions are beyond the aims of this study, and will be tackled instead in a separate, taxonomic manuscript. In this perspective, indeed, we will address *i)* the common procedure to perform assimilation tests/assays for the precise identification of yeast species and *ii)* a complete characterization of the yeast and filamentous phases of the life cycle, when possible. We refer, therefore, to the previously published phylogenies to recognize and temporary name species-level lineages.

Although our results highlight a great diversity of basidiomycetous yeasts, still within individual lichen thalli this diversity seems to be relatively low, as in general one to three yeast taxa could be detected, whereas only from one thallus of *R. melanophthalma* (L2637) up to four different yeast taxa were isolated. A more comprehensive overview of the basidiomycetous yeasts associated to these two lichen species could be revealed in forthcoming metabarcoding sequencing results, which are under analysis.

Interestingly, the only strains recovered in the class Filobasidiales (Tremellomycetes) and nested within *Naganishia* were isolated from thalli of *R. melanophthalma* growing on acidic rocks in South America between 3300 and 3500 m a.s.l.. Yeast strains found only in *R. melanophthalma* form the distinct clade "Yeast Lineage II" (including also four other unidentified samples Fig. 6) in the Tremellales, and are included in the clades of *Fibulobasidium* and *Vishniacozyma* (Tremellomycetes), *Calacogloea* sp. (Microbotryomycetes) and *Tranzscheliella* sp. (Ustilaginales). In contrast, yeast strains isolated only from thalli of *T. atra* form the distinct clade "Yeast Lineage I" (Fig. 6) with two other unidentified samples, others belong to *Pseudotremella* (Tremellomycetes), or have an uncertain position in the Microbotryomycetes. On the other hand, we identified strains

nested within Agaricostilbaceae and Chionosphaeraceae (Agaricostilbomycetes), and *Tremella macrobasidiata* (Tremellomycetes) from both thalli of *R. melanophthalma* and *T. atra*.

Agaricomycotina diversity in R. melanophthalma and T. atra – Tremellomycetes

Our results show that most of the basidiomycetes associated to the two investigated lichen species belong to the class Tremellomycetes (Agaricomycotina), for which so far 72 lichenicolous fungi have been described (Diederich et al. 2018, 2019, 2020; Diederich and Ertz 2020). As introduced before, many Tremellomycetes are characterized by their dimorphism (Bandoni 1995) and this was shown also for some lichen-associated *Tremella* species in thalli of *Lecanora* and *Letharia* (Tuovinen et al. 2019, 2021). Indeed, *Tremella* in lichens are recognizable for the formation of basidiomata which often induce the formation of big deforming galls on the thalli. Only inside the basidiomata, tremelloid haustoria have been observed, and thus the species were considered as mycoparasites of the lichen mycobiont (Spatafora et al. 2017). The interaction between these tremellomycete haustoria and mycobiont hyphae, however, has not been clarified yet (Bauer and Oberwinkler 2008). Whether as yeast or filamentous state, Tremellomycetes detected by the DNA metabarcoding approach are reported for thalli lacking basidiomata (Lindgren et al. 2015; Zhang et al. 2015, Fernández-Mendoza et al. 2017; Banchi et al. 2018; Smith et al. 2020). We isolated the Tremellomycetes strains from thalli which also did not present any galls or deformation: we detected *Tremella macrobasidiata* strains by PCR amplifications from thalli of *R. melanophthalma* and *T. atra* that did not show any symptom of infection. This result strengthens the assumption that the unicellular yeast stage may be common in lichens.

The classification of the Tremellomycetes has been – and still is – problematic. In this study we used the integrated phylogenies published by Liu et al. (2015a,b) to recognize and name the newly identified strains. To better understand species diversity in Tremellomycetes, we analysed our new sequences using two individual datasets of Tremellales and Filobasidiales. Within both orders the newly isolated fungal strains are very closely related to yeasts isolated and described from other rather cold and extreme environments, such as strains 9.L31, LTSP_EUKA_P5H04 and 112_NA3_P32_B23, *Saitozyma* sp., *Vishniacozyma carnescens* and *Naganishia albidosimilis*. In particular, the uncultured fungus LTSP_EUKA_P5H04 and 112_NA3_P32_B23 were obtained from soil collected respectively in British Columbia (Canada) and North American Arctic Transect (NAAT; Hartmann et al. 2009; Timling et al. 2014). Moreover, strain 9.L31 was identified for the first time by Duarte et al. (2016) who isolated it from a thallus of *Usnea antarctica* collected in the South Shetland Island (Antarctica). Our isolates, instead, come from thalli of *R. melanophthalma* collected in South and North America at high elevation (over 3000 m a.s.l.) and in Spain at 1450 m a.s.l..

Strains forming the distinct clade, here indicated as "Yeast Lineage I" (Fig. 6), present the highest sequence similarity with a sample of uncertain position (KBPY6612), which is reported from the lichen *Cladonia rangiferina* from the Altai mountains (Russia) by Kachalkin but still indicated as an unpublished work. Our strains derive from thalli of *T. atra* collected in the Italian Alps, Spain and Tasmania, in an altitudinal range from 500 m to 2000 m a.s.l., likely similar to alpine environments of the Altai mountains.

Only one strain, isolated from *R. melanophthalma* collected in the Alps, is nested within *Vishniacozyma carnescens*, and is recognised to be this species. *Vishniacozyma carnescens* was isolated from Antarctic soil (in South Victoria Land, Ross Sea region and East Ongul Island; Arenz et al. 2006; Connell et al. 2008; Tsuji, 2018) but it is also reported from *Cladonia* lichens collected in Subarctic Russia (Kachalkin et al. 2017), thus justifying its presence also in our lichen sample from a similar environment, such as the Alps.

Tremella macrobasidiata (Zamora et al. 2011; Millanes et al. 2011) was isolated from both *T. atra* and *R. melanophthalma*; while *Pseudotremella* aff. *indecorata* (Millanes et al. 2011; Fan et al. 2021) was isolated from *T. atra* only, from thalli collected in the Alps, on a mountain massif in Spain, and in North and South America. *T. macrobasidiata* is placed in the phylogenetic clade named as Lichenicolous Clade III by Millanes et al. (2011) – and here – together with other lichenicolous *Tremella* and *Biatoropsis* species, while *Pseudotremella* aff. *indecorata* (not lichenicolous) belongs in *Pseudotremella*. *Tremella macrobasidiata* is characterized by a dimorphic lifestyle: the sexual filamentous (hyphae) stage with the formation of basidiomata was observed specifically on the lichen *Lecanora chlarotera* (Zamora et al. 2011, 2016); the yeast stage (asexual) instead was detected in two *Lecanora* species (Tuovinen et al. 2021). Our microscopy analyses confirmed the dimorphism for both species *T. macrobasidiata* and *T. indecorata*, as in the cultured strains we observed either yeasts and budding germination of cells or hyphae growing out of the yeast cells and starting a filamentous mycelium (Fig. 12).

Within the order Filobasidiales, we isolated strains of *Naganishia albidosimilis* from thalli of *R. melanophthalma* collected above 3000 m a.s.l. in Argentina and Chile. *N. albidosimilis* is a psychrophilic basidiomycete isolated from Antarctica (Vishniac and Kurtzman 1992; Scorzetti et al. 2000; Pavlova et al. 2001; Arenz et al. 2006; Connell et al. 2008). Its presence in lichen thalli form dry, cold extreme habitats, such as the localities visited by us, is reasonable.

Pucciniomycotina diversity in R. melanophthalma and T. atra

In our lichen samples, Pucciniomycotina yeasts are represented by the three orders Agaricostilbomycetes, Cystobasidiomycetes and Microbotryomycetes. This phylum is formed by a large group of fungi including plant pathogens (mainly Pucciniales), lichenicolous

heterobasidiomycetes and many other remarkably ecologically and biologically diverse fungi (Aime et al. 2006). Instead, lichen-inhabiting species have been recognized only in the three genera *Crittendenia*, *Cyphobasidium* and *Lichenozyma* (Millanes et al. 2021). Within the class Cystobasidiomycetes, both yeast and dimorphic species having different life strategies are found, such as endophytes, saprophytes, mycoparasites, lichen-associates and fungi adapted to aquatic environments (Boekhout et al. 2011). The lichen-associated yeasts were hypothesized to play a key role in the lichen symbiosis (Spribille et al. 2016), and studies have aimed at uncovering their diversity. Presently, only a few studies have reported on axenically isolated strains of Cystobasidiomycetes. *Cystobasidium psychroaquaticum* was cultured from *Cladonia pocillum* from Svalbard (Zhang et al. 2016); *Cystobasidium* spp. (Cystobasidiales) were isolated from *Usnea aurantiaco-atra*, *U. antarctica*, and *Ramalina terebrata* collected from Antarctic islands (Duarte et al. 2016; Santiago et al. 2015) and from *Umbilicaria arctica* collected from Svalbard (Zhang et al. 2016). Here, we successfully isolated *Lichenozyma pisutiana* (Microsporomycetaceae) from *R. melanophthalma* thalli collected on a mountain massif in central Spain at 1900 m a.s.l.. This yeast was described and isolated for the first time by Černajová and Škaloud (2019) from various *Cladonia* species and *Cetraria ericetorum*. Because *L. pisutiana* does not produce any visible symptoms on the lichen thalli, neither in *Cladonia* nor in our samples, Černajová and Škaloud (2019) proposed that it could represent the anamorphic form of a "still to be discovered" lichenicolous fungus. We isolated *L. pisutiana* from *R. melanophthalma* growing on silicious-granitic boulders, whereas Černajová and Škaloud (2019) isolated it from *Cladonia rei* collected on limestone quarry, suggesting a lack of substrate- and host preference for this yeast species.

We also isolated for the first time in axenic culture two yeast strains whose sequences match most closely with sequences of two fungi sequenced from thalli of *Lecanora pulicaris* and *Cladonia bellidiflora* (Mark et al. 2020; Černajová and Škaloud 2019). The fungus from *L. pulicaris* was detected by Mark et al. (2020) in a metagenomic analysis, that from thalli of *Cladonia bellidiflora* by Černajová and Škaloud (2019) using the specific primers for Cystobasidiomycetes designed by Spribille et al. (2016). As our strains derive from thalli of *T. atra* collected on the Alps, this yeast seems to be rather unspecific for its lichen hosts.

In the class Agaricostilbomycetes we identified two strains within Agaricostilbaceae closely related to *Sterigmatomyces* and *Pseudobensingtonia*, and most of the strains in the family Chionosphaeraceae – closely related to *Kurtzmanomyces nectairei*. Species of *Chionosphaera* are not known to be lichenicolous, as *Chionosphaera apobasidialis* was found on bark of deciduous trees and as the mycoparasite of *Cladosporium herbarum* (Cox 1976), while *C. cuniculicola* grows inside bark beetle galleries (Kirschner et al. 2001). *Chionosphaera apobasidialis* and *C.*

cuniculicola life cycles have been studied in detail in axenic culture by Roberts (1997). The suggestion that these species could complete their life cycle within the lichen thalli has not been supported yet. Our strains, obtained from both *R. melanophthalma* and *T. atra*, were placed in a clade with *Kurtzmanomyces nectairei*, which is evolutionary relatively closely related to the two afore mentioned *Chionosphaera* species (Kwon-Chung 2011; Millanes et al. 2021). The ecological preferences of *Kurtzmanomyces* in nature is not yet clarified: *K. tardus* was isolated from contaminated demineralized water, *K. nectairei* from cheese and *K. insolitus* from the fruit body of a heterobasidiomycete (Sampaio et al. 1999). Some years ago, *Kurtzmanomyces* spp. were also described as saprophytic and isolated from desert soils crusts in northwestern China (Zhang et al. 2013) and from the High Arctic Archipelago Svalbard (Mundra et al. 2016).

Instead, the recognized lichenicolous genus in Agaricostilbomycetes is *Crittendenia* (Millanes et al. 2021), which is not present among our samples. *Crittendenia* includes two described lichenicolous species: *C. coppinsii* growing on thalli of *Melanelixia* and *Melanohalea* species (Blanco et al. 2004; Arup and Sandler Berlin 2011; Divakar et al. 2017), and *C. lichenicola* growing on lichen species of *Micarea* (Millanes et al. 2021). Some other still undescribed *Crittendenia* species parasitize lichen hosts in the families Lecanoraceae, Lobariaceae, Parmeliaceae, Physciaceae, Ramalinaceae and Teloschistaceae (Millanes et al. 2021). Just recently, the new family Crittendeniaceae was established (Diederich et al. 2022).

We found only three strains within the class Microbotryomycetes which are closely related to two genera previously isolated and described from soil, i.e., *Colacogloea* and *Yunzhangia*. The strain isolated from *T. atra* collected on the Alps, appears, although without support, as sister species of *Yunzhangia auriculariae*, a yeast isolated from Antarctic soils and detected in several previous studies (Ray et al. 1989; Pavlova et al. 2001; Buzzini et al. 2012; Shivaji and Prasad, 2019). Instead, the two strains coming from *R. melanophthalma* thalli collected on the Alps are nested within *Colacogloea* spp.. These yeasts were isolated from soil and plant residues in monoterpene-rich environments (Thanh et al. 2004; Pohl et al. 2011) and beech forest soils (Yurkov et al. 2016; Kachalkin et al. 2019), two environments completely different from those visited by us. However, some *Colacogloea* spp. are mycoparasites characterized by septate basidia and colacosomes (Oberwinkler et al. 1990; Bauer and Oberwinkler 1991, 2008). This observation, together with the above reported presence of lichenicolous (our strains) and mycoparasitic species (as *Chionosphaera apobasidialis*) within the same family Chionosphaeraceae seems to be in line with the observation provided by Oberwinkler (2017), who suggested the many yeast taxa in lichens could represent mycoparasites of the lichen mycobiont.

Ustilagomycotina diversity in R. melanophthalma and T. atra

Ustilagomycotina are the least represented in lichens. Here, we isolated only four strains nested within *Tranzscheliella* species from thalli of *R. melanophthalma* collected in North and South America. *Tranzscheliella* spp. are known plant pathogen of 33 genera of grasses (Poaceae) widely distributed around the world (Vánky 2011; Li et al. 2017). Surprisingly, recent metabarcoding analyses report *Tranzscheliella* spp. also from Artic deep-sea sediments (Ogaki et al. 2021).

Conclusions

This is the first study in which an extensive sampling of two reference/model species of cosmopolitan lichens – *Rhizoplaca melanophthalma* and *Tephromela atra* – has been performed to widen our knowledge and characterize the diversity of lichen-associated basidiomycetous yeasts. We report on the successful isolation of 76 yeast strains belonging to the three phyla in Basidiomycota. Phylogenetic analyses helped to identify yeast strains both corresponding to already known lichenicolous basidiomycetous fungi (*Tremella macrobasidiata*, *Tremella indecorata*, *Saitozyma* sp., *Lichenozyma pisutiana*), and to potentially new taxa previously unknown from lichens. Here, we also highlight the importance of understanding the diversity and symbiotic relationships of lichen-associated yeast and the pressing need of a stable, comprehensive taxonomy to recognized them. Bacon and White (2000) suggested that lichens act as protective niches for other microorganisms, helping them in thriving and dispersing in extreme environments, such as the mountain regions where *T. atra* and *R. melanophthalma* were collected. Later, Santiago et al. (2015) introduced the concept of "lichensphere" referring to the surface and those narrow spaces inside lichen thalli offering favorable natural microhabitats for microorganisms. In recent studies it was speculated whether yeasts potentially synthesize secondary metabolites useful for the acquisition of nutrients in lichens (Spribille et al. 2016, 2020; Tagirdzhanova et al. 2021), but there is still no experimental evidence. We noticed a few patterns of host- and substrate preference, thus the yeasts here detected might be ecologically-constrained and forthcoming analyses will clarify their taxonomy and their potential presence in additional lichen species.

Reference and contributing authors of the paper

Cometto, A. [1], Leavitt, S. D. [2], Millanes, A. M. [3], Wedin, M. [4], Grube, M. [5], & Muggia, L. [1*] (2022). The yeast lichenosphere: high diversity of basidiomycetes from the lichens *Tephromela atra* and *Rhizoplaca melanophthalma*. Fungal Biology 126, 587-608.

[1]Department of Life Sciences, University of Trieste, via L. Giorgieri 10, 34127, Trieste, Italy

[2]Department of Biology 4143 LSB, Brigham Young University Provo, Utah, 84602, USA

[3]Departmento de Biología y Geología, Física y Química Inorgánica, Universidad Rey Juan Carlos, Departamental 2. D-22,1 C/ Tulipán s/n, E-28933, Móstoles, Madrid, Spain

[4]Department of Botany, Swedish Museum of Natural History, Box 50007, SE10405, Stockholm, Sweden

[5]University of Graz, Institute of Biology, Holteigasse 6, 8010, Graz, Austria

* Corresponding author: Lucia Muggia. Address: University of Trieste, Department of Life Sciences, via Giorgieri 10, 34127 Trieste, Italy. E-mail: lmuggia@units.it, lucia_muggia@hotmail.com
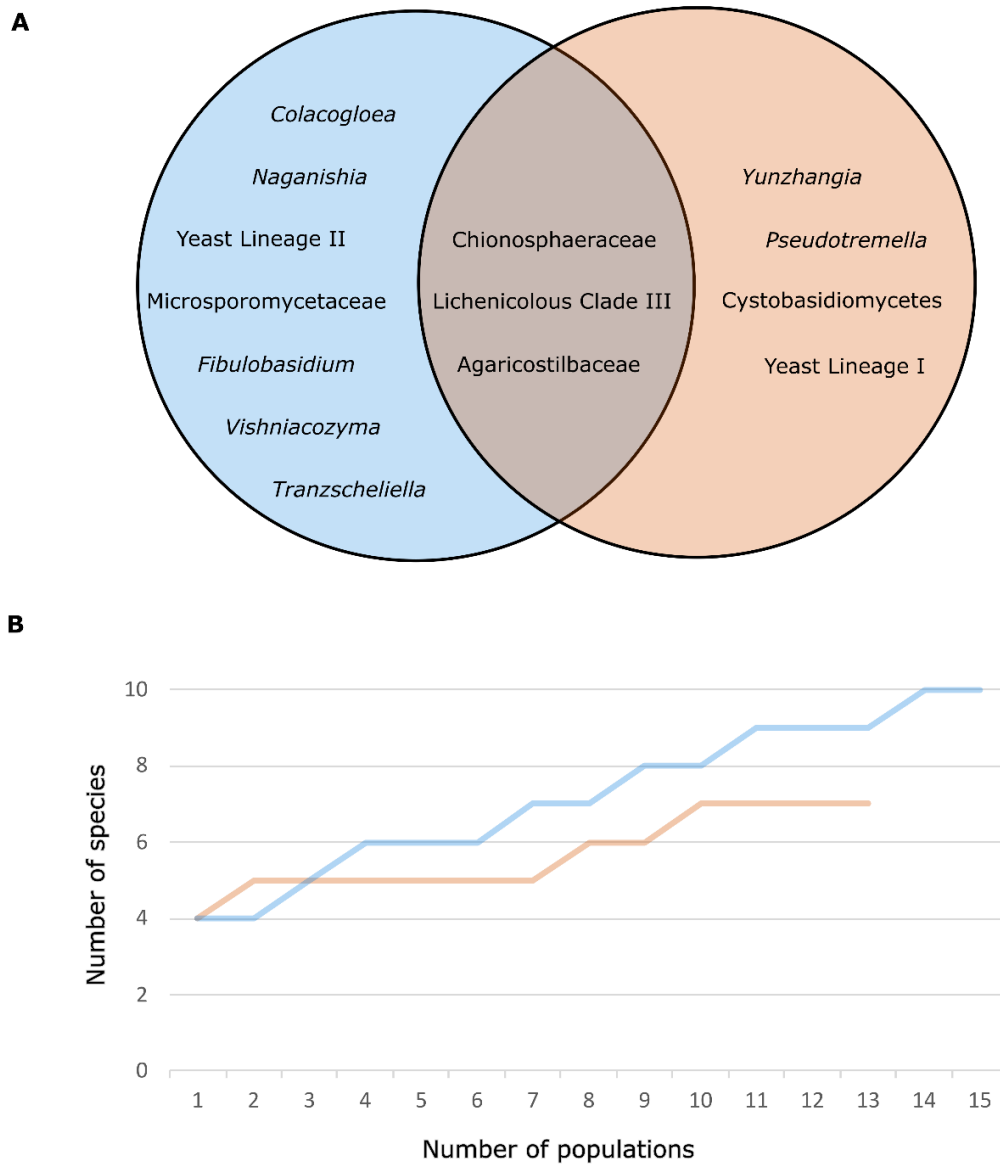
Figure S1. Venn diagram of the yeast strains isolated from *Rhizoplaca melanophthalma* (light blue) and *Tephromela atra* (orange) (A); species accumulation curves for yeast strains isolated from *R. melanophthalma* (light blue) and *T. atra* (orange) (B).
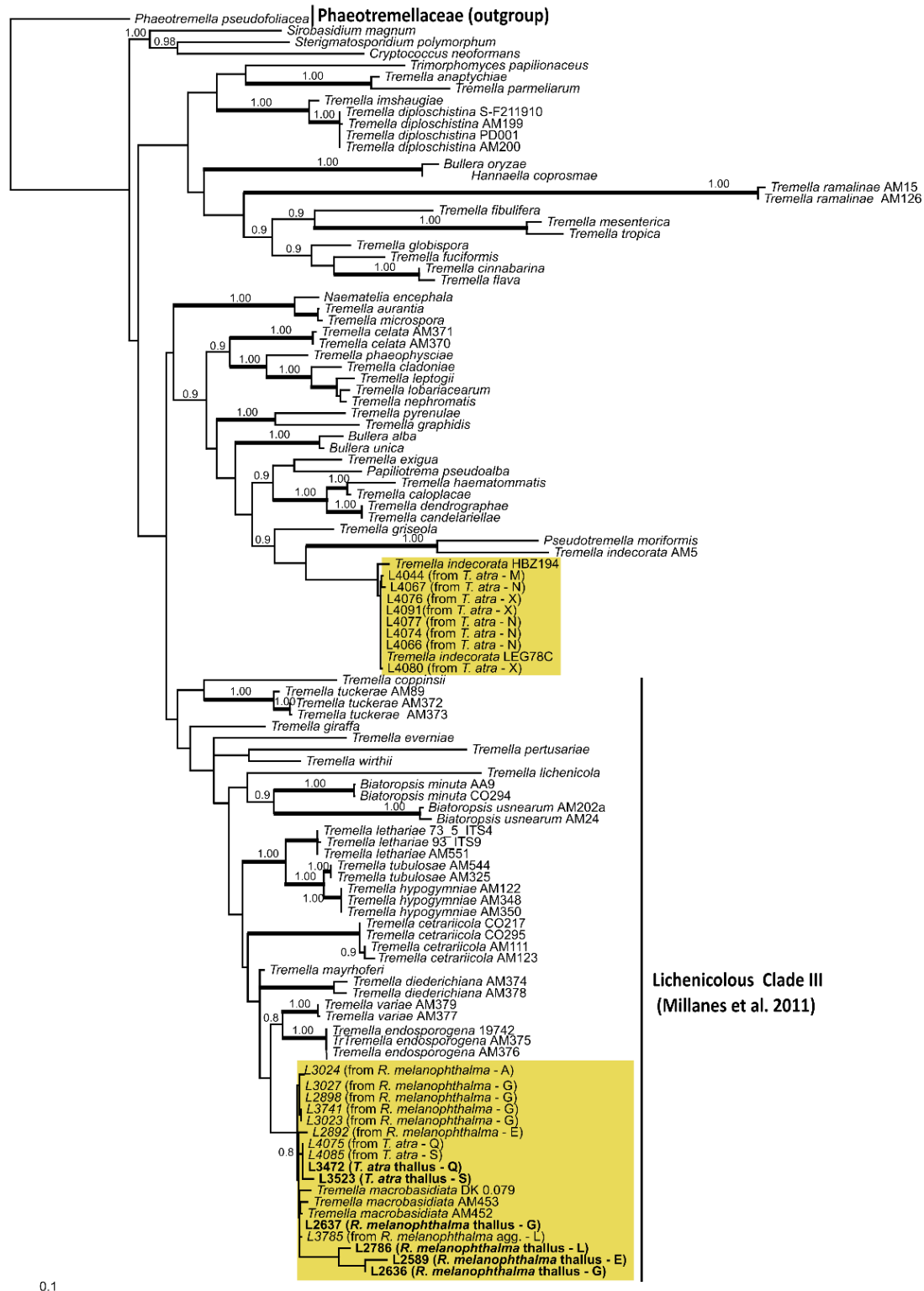
Figure S2. Phylogenetic inference of a reduced group in the Tremellales. *Phaeotroemella foliacea*, sister to the rest of Tremellales, is used as outgroup: Bayesian analysis based on the concatenated nuclear ITS-LSU dataset. Bayesian posterior probabilities ≥0,8 are reported above branches; branches in bold denote RAxML bootstrap support >75%. Newly obtained sequences are in bold and highlighted in yellow; in parenthesis the original lichen species and the sampling locality (according to Table 1) are reported. Clades are named according to the phylogenetic study of Millanes et al. (2011) and Zamora et al. (2017).

Table S1 List of taxa included in the phylogenetic analysis of Agaricostilbomycetes and their NCBI accessions. The type materials included in the database are indicated in bold (see separate external Excel file: https://doi.org/10.5281/zenodo.7245455).

Table S2 List of taxa included in the phylogenetic analysis of Cystobasidiomycetes and their NCBI accessions. The type materials included in the database are indicated in bold (see separate external Excel file: https://doi.org/10.5281/zenodo.7245455).

Table S3 List of taxa included in the phylogenetic analysis of Microbotryomycetes and their NCBI accessions. The type materials included in the database are indicated in bold (see separate external Excel file: https://doi.org/10.5281/zenodo.7245455).

Table S4 List of taxa included in the phylogenetic analysis of Filobasidiales and their NCBI accessions. The type materials included in the database are indicated in bold (see separate external Excel file: https://doi.org/10.5281/zenodo.7245455).

Table S5 List of taxa included in the phylogenetic analysis of Tremellales and their NCBI accessions. The type materials included in the database are indicated in bold (see separate external Excel file: https://doi.org/10.5281/zenodo.7245455).

Table S6 List of taxa included in the phylogenetic analysis of Ustilaginomycetes and their NCBI accessions. The type materials included in the database are indicated in bold (see separate external Excel file: https://doi.org/10.5281/zenodo.7245455).

Table S7 List of taxa included in the phylogenetic analysis of a reduced group within the Tremellales and their NCBI accessions. The type materials included in the database are indicated in bold (see separate external Excel file: https://doi.org/10.5281/zenodo.7245455).

Table S8 Diversity of basidiomycetes strains is shown according to the original lichen thallus from which strains were isolated. Lichen specimens of either *Rhizoplaca melanophthalma* or *Tephromela atra* are listed according to their thallus ID and grouped for localities (as reported also in Table 1). For each culture ID the phylogenetic placement and the BLAST percentages of identity of the ITS and nucLSU sequences, respectively, are reported (see separate external Excel file: https://doi.org/10.5281/zenodo.7245455).

CHAPTER 2

Tackling fungal diversity in lichen symbioses: molecular and morphological data recognize new lineages in Chaetothyriales (Eurotiomycetes, Ascomycota)

Abstract

Lichens have been reappraised as self-sustaining and long-living ecosystems in which a multiplicity of microorganisms are housed, in addition to the main symbiotic partners. Lichen-associated microfungi can frequently occur cryptically, and their species diversity has recently been more fully elucidated by DNA metabarcoding studies and culture isolations. These lichen-associated fungi represent a wide array of major lineages in Ascomycetes and Basidiomycetes, including both filamentous and yeast species. Thanks to culture isolations, the morphology of a subset of the lichen-associated microfungal diversity has been studied. Metabarcoding analyses have shown high diversity of ascomycetous lichen-associated fungi in the two cosmopolitan rock-inhabiting lichens – *Rhizoplaca melanophthalma* Leuckert & Poelt and *Tephromela atra* (Huds.) Hafellner var. *atra* – and many of these taxa were successfully isolated in culture. Based on DNA sequence data and morphological analyses, two new lineages within the family Herpotrichiellaceae in Chaetothyriales were recognized. Both occur in lichens from dry habitats and are described here. Other strains are placed in Pleostigmataceae, Trichomeriaceae, Pleosporales, Mycosphaerellales, Coniochaetales and Hypocreales, further filling gaps of knowledge of the high fungal diversity residing in lichen thalli.

Keywords

Cladophialophora, Dothideomycetes, Herpotrichiellaceae, phylogeny, Sordariomycetes, taxonomy.

Introduction

Lichens have evolved as a life form that develops a particular housing morphology through the interactions of a biotrophic fungus – the mycobiont – with one or more phototrophic organisms – the photobiont (Hawksworth and Honegger 1994). However, in contrast to a simple partnership between the myco- and photobionts, lichens have been reappraised as self-sustaining and long living ecosystems (Hawksworth and Grube 2020), in which a multiplicity of other microorganisms – including other filamentous and yeast microfungi, microalgae and bacteria – are housed (Grube et al. 2009, 2015; Moya et al. 2017; Fernández-Mendoza et al. 2017; Banchi et al. 2018; Molins et al. 2018; Muggia and Grube 2018). The potential functional roles of these complementary microorganisms are a widely discussed subject of research, and they have not been clarified yet

(e.g., Grube et al. 2009, 2015; Moya et al. 2017; Molins et al. 2018; Muggia and Grube 2018; Spribille 2018; Hawksworth and Grube 2020). Furthermore, the overall diversity of these lichen inhabitants/co-symbionts is still largely unknown. Ongoing research has shed light on the geographic and, only in part, ecological distributions of certain groups of microfungi and microalgae associated to lichens (Wang et al. 2016; Williams et al. 2017).

Microfungi identified in/on lichen symbioses were initially discovered in the early 19th century and have been the focus of a wide range of studies. They are commonly known as "lichenicolous fungi" for over a century, and 2319 species are formally recognized (Diederich et al. 2018). These lichen-associated microfungi are mainly represented by Ascomycetes (95%), while only a small fraction appears to be basidiomycetous (Diederich 1996; Lawrey et al. 2007). While lichenicolous fungi usually develop symptoms of infections on their host lichen thalli, some are asymptomatic, occurring mostly as resting spores or hyphal fragments in other lichen species (Arnold et al. 2009; U'Ren et al. 2010, 2012, 2014; Muggia et al. 2016, 2017; Fernández-Mendoza et al. 2017; Banchi et al. 2018; Hafellner 2018). Also, most of the symptomatically occurring lichenicolous fungi show some level of host-specificity, and sometimes even dependency, on the lichen host (Lawrey and Diederich 2003; Hafellner 2018). In contrast to the observed specificity of the lichenicolous fungi, this specificity has been shown in only a few cases in some cryptically occurring taxa (Smith et al. 2020). Furthermore, some studies have shown that certain abiotic factors, such as climate (U'Ren et al. 2012), seasonality, light exposure (Beck et al. 2014), altitude (Wang et al. 2016) and geographic distance (Zhang et al. 2015) may be crucial in shaping the lichenicolous fungal diversity of lichen thalli (Harutyunyan et al. 2008; Arnold et al. 2009; U'Ren et al. 2010; Lagarde et al. 2018; Yoshino et al. 2020).

In general, lichenicolous fungal taxa are phylogenetically distant from the lichen mycobionts and have been found in several lineages within the ascomycete classes Dothideomycetes, Eurotiomycetes, Leotiomycetes and Sordariomycetes (Arnold et al. 2009; U'Ren et al. 2010; Muggia et al. 2016, 2019, 2021; Suryanarayanan and Thirunavukkarasu 2017). Lichenicolous basidiomycetes typically belong to the classes Agaricostilbomycetes, Tremellomycetes and Cystobasidiomycetes (Zamora et al. 2011; Millanes et al. 2011, 2016, 2021; Černajová et al. 2019; Tuovinen et al. 2021; Cometto et al. 2022). The detection of the cryptically occurring lichenicolous species is nowadays feasible by culture isolation and sequence metabarcoding analyses (Arnold et al. 2009; U'Ren et al. 2010: Fernández-Mendoza et al. 2017; Banchi et al. 2018), while lichenicolous yeasts can be more specifically detected by the *ad hoc* combination of fluorescence *in situ* hybridization (FISH) and confocal microscopy (Tuovinen et al. 2019, 2021). However, to

formally characterize new species, axenically isolated strains serving for morphological studies are essential (Lawrey and Diederich 2003).

The lichenicolous fungi which cryptically occur in lichens belong mainly to the classes Eurotiomycetes and Dothideomycetes and are represented by filamentous or yeast-like melanised taxa, which are closely related to the polyphyletic lineages of rock-inhabiting fungi (RIF) and black yeasts (Gueidan et al. 2008; Ruibal et al. 2009; Gostinčar et al. 2012, Quan et al. 2020). These have frequently been reported from epilithic lichens (Harutyunyan et al. 2008; Muggia et al. 2016, 2017, 2021; Muggia and Grube 2018; Quan et al. 2020). Only a few taxa, such as lichenicolous *Phoma* species in Phaeosphaeriaceae (Dothideomycetes), were reported both cryptically and symptomatically occurring in epilithic, epiphytic and soil inhabiting lichens (Lawrey et al. 2012; Muggia et al. 2016, 2017). Recently two lineages within Eurotiomycetes have been formally recognized from Alpine epilithic lichens as the family Pleostigmataceae and the new genus and species *Melanina gunde-cimermaniae* (Muggia et al. 2021). Interestingly, lichenicolous basidiomycetes yeast species that have been described from thalli of the lichen-forming mycobiont genera *Cladonia*, *Lecanora* and *Letharia* (Zamora et al. 2011; Millanes et al. 2011, 2016, 2021; Černajová et al. 2019; Tuovinen et al. 2019, 2021) have been isolated recently from the epilithic lichens *Rhizoplaca melanophthalma* and *Tephromela atra* (Cometto et al. 2022), suggesting that certain taxa of lichenicolous fungi can be unexpectedly widespread in lichens.

In the present study, we deepen our investigation into the diversity of culturable, cryptically occurring lichenicolous ascomycetes from thalli of *Rhizoplaca melanophthalma* Leuckert & Poelt and *Tephromela atra* (Huds.) Hafellner var. *atra*, as these lichens revealed to be important cradles for lichenicolous fungi (Cometto et al. 2022; Cometto et al. under review). Here we report on the successful isolation of 131 ascomycetous strains, 39 of which represent two new lineages within Chaetothyriales and are formally described as new species, while the others belong to already known genera and families of Dothideomycetes, Eurotiomycetes and Sordariomycetes. All together, these and earlier results highlight that certain species of cryptically occurring lichenicolous fungi are more frequent than previously thought and may have their realized ecological niches in lichen thalli.

Materials and methods

Sampling

Lichens representing the *Rhizoplaca melanophthalma* aggregate (Leavitt et al. 2011) have an umbilicate thallus (attached at a single point), whereas lichens representing the *Tephromela atra* group (Muggia et al. 2014a) build a crustose thallus composed of adjacent areoles. Both lichens are

characterized by a worldwide distribution and occur at different ecological conditions and elevations. Also, the mycobiont and photobiont diversity of both lichens has been extensively investigated previously (Muggia et al. 2008, 2010, 2014a; Leavitt et al. 2011, 2016; De Carolis et al. 2022). For the present study, lichen samples were collected in 23 different localities on different rock types (such as schist-sandstone, siliceous, acidic, granitic and calcareous rocks) and at altitudes ranging from 550 to 5100 m above sea level (a.s.l.). The sampling was performed in North America (Utah, Nevada and Idaho), South America (Argentina and Chile), Europe (Spain), Mauritius and Tasmania. In total 20 populations of *R. melanophthalma* and five populations of *T. atra* were analysed (Supplementary Table S1). All the lichen samples were deposited at the herbarium of the University of Trieste (TSB).

Culture isolation

Fungal isolation was performed from four thalli for each population of *Rhizoplaca melanophthalma* and *Tephromela atra* following the protocol of Yamamoto et al. (2002). Approximately 2 mm^2 fragments of lichen thalli were dissected with a sterile razor blade. For *R. melanophthalma*, one marginal lobe and one apothecium were taken, while for *T. atra*, one marginal areole and one apothecium. The fragments were washed three times for 15 minutes with sterile water, followed by 30 minutes of washing with 500 μl of Tween80 diluted 1:10, and a final washing step of 15 minutes for three times 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. Six different media were used to promote the growth of as many different fungi as possible: Lilly and Barnett (LB, Lilly and Barnett 1951), Trebouxia medium (TM, Ahmadjian 1987), Potato Dextrose agar (PDA, ApplChem A5828), Sabouraud's glucose agar base medium (SAB, Pagano et al., 1958), Dichloran/Glycerol agar (DG18, Hocking and Pitt 1980), and Malt Yeast-extract (MY, Lilly and Barnett 1951). Two replicates for each medium were inoculated for a total of 12 inocula from each lichen individual, and incubated in growing chamber (17 °C, 20 µmol × photons m^{-2} × s^{-1}, with a light/dark cycle of 14/10 h). When the inocula developed into a mycelium mass of about 5 mm size (after about three to six months), they were sub-cultured into Petri plates, on the same medium of the original tube.

Molecular analyses: DNA extraction, PCR amplification and sequencing

Small parts of the cultured fungal colonies were taken and put into 1.5 ml reaction tubes, containing three sterile tungsten beads for homogenization, frozen and ground using a TissueLyserII (Retsch). The DNA extractions were performed following the CTAB protocol of Cubero et al. (1999), with minor adjustments. The identity of all fungal strains was studied with sequences of the nuclear

internal transcribed spacers (nucITS) and 5.8S rDNA ribosomal gene amplified with the primers ITS1F (Bruns and Gardes 1993) and ITS4 (White et al. 1990). When ITS sequences were identical (99%-100% identity) among strains sharing the same origin – i.e., isolated from the same lichen thallus, or from thalli coming from the same population – the D1/D2 domain of the 28S nuclear large ribosomal subunit (nucLSU) was further amplified, only for a subset of the strains, with the primers LR0R and LR5 (Vilgalys and Hester 1990; http://www.biology.duke.edu/fungi/mycolab/primers.htm). 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. Negative control reactions were always used to check for potential contamination. PCR amplifications followed the same conditions of previous studies (Muggia et al. 2017; Cometto et al. 2022). 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 bio-tek). Clean amplicons were sent for Sanger sequencing to Macrogen Europe (The Netherlands).

Phylogenetic analyses

The identity of the newly generated sequences was 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 for the phylogenetic analyses. As our sequences showed high similarity with representatives of the classes Eurotiomycetes (particularly the order Chaetothyriales), Dothideomycetes and Sordariomycetes, three separate datasets representing each individual fungal class were prepared (Supplementary Table S2 – S4). The total dataset included the widest spectrum of taxon diversity, as also genera and families closely related to our sequences were selected from previous studies. In particular, the Eurotiomycetes dataset was based on Harutyunyan et al. (2008), Muggia et al. (2016, 2019, 2021) and Quan et al. (2020); that of Dothideomycetes was based on Muggia et al. (2016) and Ametrano et al. (2019); that of Sordariomycetes was based on Muggia et al. (2016). Sequence alignments for each locus (nucITS and nucLSU) and for each fungal class (Eurotiomycetes, Dothideomycetes, Sordariomycetes) were prepared with MAFT v.7 (Katoh and Standley 2013) using the g-ins-I alignment strategy. Ambiguously aligned positions and introns were removed from the alignments using Trimmomatic (Bolger et al. 2014). Single locus phylogenies were inferred with Maximum Likelihood (ML) and Bayesian Inference (BI) approaches. RAxML v.8.2 (Stamatakis 2014) was used for the ML analysis applying GTRGAMMA substitution model and 1000 bootstrap pseudoreplicates. The BI analysis was carried out with the program BEAST v.2.6.7 (Bouckaert et al. 2014) running GTRGAMMA substitution model for 100 million generations. The results were

analysed using the program Tracer v1.7.2 (Rambaut et al. 2018) to check the runs for convergence (burn-in = 10%). TreeAnnotator (included in the BEAST package) was used to summarize the trees in a consensus tree representing the posterior distribution, the first 10% of data were discarded as burn-in. After checking the phylogenetic concordance of the nucITS and nucLSU loci, they were concatenated with MEGA (Kumar et al. 2018) and then analyzed with both RAxML and BEAST with the same settings of the single locus analyses.

To better clarify the phylogenetic placement of two potentially new lineages within Chaetothyriales we performed a further analysis based on a more extended taxon sampling in Herpotrichiellaceae (Supplementary Table S5), selecting six species of Cyphellophoraceae as outgroups (*Cyphellophora olivacea*, *C. pluriseptata*, *C. eucalypti*, *C. laciniata* and *C. sessilis*), and running the phylogenetic analysis using RAxML and BEAST approaches with the same settings explained above.

The phylogenetic trees were visualized using ITOL (Letunic and Bork 2019).

Morphological analysis

Morphological and anatomical characters of 10-month to one-year old cultured fungal strains were analysed with light microscopy considering the following characters: growth form, melanisation, thickness and branching of the hyphae. A tiny part of the colony was removed by a sterile inoculation loop and mounted in water. Digital photos at light microscope were taken with a Zeiss AXIO Imager A2 coupled to a Thorlabs digital camera. The photos were adjusted for colour saturation and sharpness with Adobe Photoshop 7.0 (Adobe System Incorporated, San Jose, CA, USA) and photo-tables were assembled using Inkskape (www.inkscape.org).

Results

Culture isolation

A total of 131 fungal strains were isolated and characterized from 80 *Rhizoplaca melanophthalma* and 20 *Tephromela atra* samples (Fig. 1a): 65 strains belong to Eurotiomycetes (order Chaetothyriales), 53 strains belong to Dothideomycetes and 13 strains belong to Sordariomycetes (Table 1). The Eurotiomycetes (order Chaetothyriales) strains were isolated from 20 *R. melanophthalma* samples collected in 10 localities in South America (Argentina), North America (Utah) and Europe (Spain) and from eight *T. atra* samples collected in five localities in South America (Chile), Tasmania, Mauritius and Europe (Spain). The Dothideomycetes strains were isolated from 20 *R. melanophthalma* samples collected in 13 localities in South America (Argentina and Chile), North America (Utah, Idaho and Nevada) and from seven *T. atra* samples collected in

three localities in South America (Chile) and Europe (Spain). The Sordariomycetes strains were isolated from four *R. melanophthalma* samples collected in two localities in South America (Argentina) and North America (Utah) and from two *T. atra* thalli collected in two localities in Tasmania and Mauritius (Fig. 1b; Table 1; Supplementary Table S1). No fungal strains were obtained from *R. melanophthalma* thalli coming from three populations in Argentina above 3600 m a.s.l. and one in Spain at 2080 m a.s.l.. Only six strains belonging to Phaeosphaeriaceae sp., *Elasticomyces* sp., *Knufia* sp., Pleostigmataceae sp. and *Hyalotiella* sp. were isolated from lichen thalli collected over 3000 m a.s.l. in South America. Six different Eurotiomycetes taxa (identified as *Pleostigma* sp., *Muelerella* sp. and two new lineages in Chaetothyriales) and one Dothideomycetes taxon (belonging to Phaeosphaeriaceae) were isolated from both *R. melanophthalma* and *T. atra*.

All fungal strains grew on the six different culture media except Eurotiomycetes (order Chaetothyriales) which was unable to grow on DG18 media. Eurotiomycetes mainly grew on MY media; Dothideomycetes on LBM and DG18 media; Sordariomycetes on SAB and MY media. In general, MY and LBM were the most suitable media for the isolation of ascomycetes taxa, as 49% of the isolates grew well on them (Fig. 1c; Table 1).
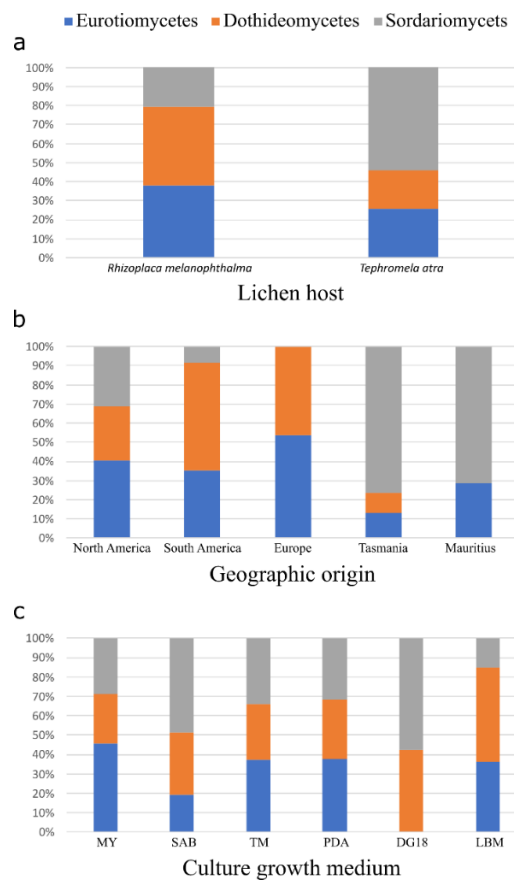
Figure 1. Lichen host (A), geographic distribution (B), growth percentage on the six growth culture media of Eurotiomycetes (blue) (C), Dothideomycetes (orange) and Sordariomycetes (grey).

Table 1. Origin data and sequence accession numbers of Eurothiomycetes, Dothideomycetes and Sordariomycetes strains newly isolated in culture: culture ID, the original lichen host (thalli of *Rhizoplaca melanophthalma* and *Tephromela atra* and their ID), the phylogenetic placement, the geographic origin of the original lichen samples (ID populations as in Table S1), and the new corresponding NCBI accession numbers were reported.

Culture ID	Lichen of origin	Culture medium	Population ID	Phylogenetic placement	ITS	nucLSU
L3809	*R. melanophthalma* L2803	LBM	24	Eurotiomycetes, incertae sedis, Pleostigmataceae	xxxxxxxx	xxxxxxxx
L3810	*R. melanophthalma* L2803	MY	24	Eurotiomycetes, incertae sedis, Pleostigmataceae	xxxxxxxx	-
L3258	*T. atra* L2583	LBM	13	Eurotiomycetes, incertae sedis, Pleostigmataceae	xxxxxxxx	xxxxxxxx
L3819	*T. atra* L2599	MY	15	Eurotiomycetes, incertae sedis, Pleostigmataceae	xxxxxxxx	xxxxxxxx
L3774	*T. atra* L2596	MY	15	Eurotiomycetes, Chaetothyriales, *Muelerella*	xxxxxxxx	xxxxxxxx
L2881	*R. melanophthalma* L2387	LBM	1	Eurotiomycetes, Chaetothyriales, Trichomeriaceae, *Neophaeococcomyces*	xxxxxxxx	-
L2606	*R. melanophthalma* L2390	TM	1	Eurotiomycetes, Chaetothyriales, Trichomeriaceae, *Neophaeococcomyces*	xxxxxxxx	xxxxxxxx
L3238	*R. melanophthalma* L2668	TM	17	Eurotiomycetes, Chaetothyriales, Trichomeriaceae, *Neophaeococcomyces*	xxxxxxxx	xxxxxxxx
L3112	*R. melanophthalma* L2669	MY	17	Eurotiomycetes, Chaetothyriales, Trichomeriaceae, *Neophaeococcomyces*	xxxxxxxx	xxxxxxxx
L3099	*R. melanophthalma* L2670	SAB	17	Eurotiomycetes, Chaetothyriales, Trichomeriaceae, *Knufia*	xxxxxxxx	xxxxxxxx
L3064	*R. melanophthalma* L2786	LBM	23	Eurotiomycetes, Chaetothyriales, Trichomeriaceae, *Knufia*	xxxxxxxx	xxxxxxxx
L3065	*R. melanophthalma* L2786	MY	23	Eurotiomycetes, Chaetothyriales, Trichomeriaceae, *Knufia*	xxxxxxxx	xxxxxxxx
L3103	*R. melanophthalma* L2786	MY	23	Eurotiomycetes, Chaetothyriales, Trichomeriaceae, *Knufia*	xxxxxxxx	-
L3105	*R. melanophthalma* L2802	MY	24	Eurotiomycetes, Chaetothyriales, Trichomeriaceae, *Knufia*	xxxxxxxx	-
L3252	*R. melanophthalma* L2825	TM	25	Eurotiomycetes, Chaetothyriales, Trichomeriaceae, *Knufia*	xxxxxxxx	-
L2876	*R. melanophthalma* L2387	MY	1	Eurotiomycetes, Chaetothyriales, *Melanina gunde-cimermaniae*	xxxxxxxx	xxxxxxxx
L2604	*R. melanophthalma* L2390	TM	1	Eurotiomycetes, Chaetothyriales, *Melanina gunde-cimermaniae*	xxxxxxxx	xxxxxxxx
L2605	*R. melanophthalma* L2390	MY	1	Eurotiomycetes, Chaetothyriales, *Melanina gunde-cimermaniae*	xxxxxxxx	-
L2618	*R. melanophthalma* L2394	LBM	2	Eurotiomycetes, Chaetothyriales, *Melanina gunde-cimermaniae*	xxxxxxxx	-
L2628	*R. melanophthalma* L2398	SAB	2	Eurotiomycetes, Chaetothyriales, *Melanina gunde-cimermaniae*	xxxxxxxx	xxxxxxxx
L3067	*T. atra* L2572	TM	12	Eurotiomycetes, Chaetothyriales, *Melanina gunde-cimermaniae*	xxxxxxxx	xxxxxxxx
L3068	*T. atra* L2572	PDA	12	Eurotiomycetes, Chaetothyriales, *Melanina gunde-cimermaniae*	xxxxxxxx	-
L3233	*T. atra* L2753	MY	22	Eurotiomycetes, Chaetothyriales sp.	xxxxxxxx	xxxxxxxx
L3784	*T. atra* L2755	PDA	22	Eurotiomycetes, Chaetothyriales sp.	xxxxxxxx	xxxxxxxx
L3260	*R. melanophthalma* L2636	PDA	16	Eurotiomycetes, Chaetothyriales, new lineages	xxxxxxxx	-
L3261	*R. melanophthalma* L2636	SAB	16	Eurotiomycetes, Chaetothyriales, new lineages	xxxxxxxx	-
L3262	*R. melanophthalma* L2636	SAB	16	Eurotiomycetes, Chaetothyriales, new lineages	xxxxxxxx	xxxxxxxx
L3763	*R. melanophthalma* L2638	PDA	16	Eurotiomycetes, Chaetothyriales, new lineages	xxxxxxxx	-
L3764	*R. melanophthalma* L2638	MY	16	Eurotiomycetes, Chaetothyriales, new lineages	xxxxxxxx	-
L3765	*R. melanophthalma* L2638	MY	16	Eurotiomycetes, Chaetothyriales, new	xxxxxxxx	xxxxxxxx

L3771	*R. melanophthalma* L2638	MY	16	Eurotiomycetes, Chaetothyriales, new lineages	xxxxxxxx	-
L3779	*T. atra* L2570	MY	12	Eurotiomycetes, Chaetothyriales, new lineages	xxxxxxxx	-
L3780	*T. atra* L2570	MY	12	Eurotiomycetes, Chaetothyriales, new lineages	xxxxxxxx	-
L3781	*T. atra* L2570	TM	12	Eurotiomycetes, Chaetothyriales, new lineages	xxxxxxxx	-
L3782	*T. atra* L2570	PDA	12	Eurotiomycetes, Chaetothyriales, new lineages	xxxxxxxx	xxxxxxxx
L3783	*T. atra* L2570	PDA	12	Eurotiomycetes, Chaetothyriales, new lineages	xxxxxxxx	-
L3776	*T. atra* L2572	MY	12	Eurotiomycetes, Chaetothyriales, new lineages	xxxxxxxx	xxxxxxxx
L3096	*R. melanophthalma* L2734	TM	21	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae	xxxxxxxx	xxxxxxxx
L2863	*T. atra* L2596	TM	15	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae	xxxxxxxx	xxxxxxxx
L2871	*R. melanophthalma* L2389	MY	1	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	xxxxxxxx
L2612	*R. melanophthalma* L2390	LBM	1	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	-
L2614	*R. melanophthalma* L2390	SAB	1	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	xxxxxxxx
L2619	*R. melanophthalma* L2394	LBM	2	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	xxxxxxxx
L3110	*R. melanophthalma* L2421	MY	3	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	-
L2865	*R. melanophthalma* L2568	PDA	14	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	xxxxxxxx
L3059	*R. melanophthalma* L2635	SAB	16	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	-
L3060	*R. melanophthalma* L2635	TM	16	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	xxxxxxxx
L3061	*R. melanophthalma* L2635	MY	16	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	-
L3251	*R. melanophthalma* L2637	MY	16	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	-
L3072	*R. melanophthalma* L2638	LBM	16	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	xxxxxxxx
L3073	*R. melanophthalma* L2638	MY	16	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	-
L3074	*R. melanophthalma* L2638	PDA	16	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	-
L3766	*R. melanophthalma* L2638	MY	16	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	-
L3770	*R. melanophthalma* L2638	PDA	16	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	-
L3106	*R. melanophthalma* L2668	PDA	17	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	-
L3240	*R. melanophthalma* L2668	TM	17	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	-
L3111	*R. melanophthalma* L2669	LBM	17	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	xxxxxxxx
L3098	*R. melanophthalma* L2670	TM	17	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	xxxxxxxx
L3100	*R. melanophthalma* L2670	PDA	17	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	xxxxxxxx
L3264	*R. melanophthalma* L2670	MY	17	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	-
L3057	*T. atra* L2561	LBM	10	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	-
L3058	*T. atra* L2561	LBM	10	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	xxxxxxxx
L2880	*T. atra* L2596	LBM	15	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	-

L2870	*T. atra* L2598	MY	15	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	xxxxxxxx
L3087	*T. atra* L2598	PDA	15	Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, new lineages	xxxxxxxx	xxxxxxxx
L3077	*R. melanophthalma* L2724	LBM	20	Dothideomycetes, incertae sedis	xxxxxxxx	-
L2869	*R. melanophthalma* L2387	PDA	1	Dothideomycetes, Pleosporales	xxxxxxxx	xxxxxxxx
L3091	*T. atra* L2597	TM	15	Dothideomycetes, Pleosporales, Teichosporaceae	xxxxxxxx	xxxxxxxx
L2868	*T. atra* L2598	PDA	15	Dothideomycetes, Pleosporales, Massariaceae, *Paraphaeosphaeria michotii*	xxxxxxxx	xxxxxxxx
L3036	*R. melanophthalma* L2704	DG18	19	Dothideomycetes, Pleosporales	xxxxxxxx	xxxxxxxx
L3021	*T. atra* L2599	LBM	15	Dothideomycetes, Pleosporales, Didymellaceae	xxxxxxxx	xxxxxxxx
L3078	*R. melanophthalma* L2724	MY	20	Dothideomycetes, Pleosporales, Pleosporaceae	xxxxxxxx	xxxxxxxx
L2888	*T. atra* L2545	LBM	10	Dothideomycetes, Pleosporales, Phaeosphaeriaceae	xxxxxxxx	-
L3056	*T. atra* L2569	TM	12	Dothideomycetes, Pleosporales, Phaeosphaeriaceae	xxxxxxxx	xxxxxxxx
L3076	*R. melanophthalma* L2724	MY	20	Dothideomycetes, Pleosporales, Phaeosphaeriaceae	xxxxxxxx	xxxxxxxx
L3042	*R. melanophthalma* L2734	SAB	21	Dothideomycetes, Pleosporales, Phaeosphaeriaceae	xxxxxxxx	xxxxxxxx
L3030	*R. melanophthalma* L2827	TM	25	Dothideomycetes, Pleosporales, Phaeosphaeriaceae	xxxxxxxx	xxxxxxxx
L3094	*T. atra* L2596	TM	15	Dothideomycetes, Pleosporales, Phaeosphaeriaceae	xxxxxxxx	xxxxxxxx
L3037	*R. melanophthalma* L2827	SAB	25	Dothideomycetes, Pleosporales, Phaeosphaeriaceae	xxxxxxxx	-
L3090	*R. melanophthalma* L2454	MY	4	Dothideomycetes, Pleosporales, Phaeosphaeriaceae	xxxxxxxx	xxxxxxxx
L3108	*R. melanophthalma* L2387	DG18	1	Dothideomycetes, Pleosporales, Phaeosphaeriaceae	xxxxxxxx	xxxxxxxx
L2622	*R. melanophthalma* L2398	TM	2	Dothideomycetes, Pleosporales, Phaeosphaeriaceae	xxxxxxxx	-
L2624	*R. melanophthalma* L2398	TM	2	Dothideomycetes, Pleosporales, Phaeosphaeriaceae	xxxxxxxx	-
L2625	*R. melanophthalma* L2398	LBM	2	Dothideomycetes, Pleosporales, Phaeosphaeriaceae	xxxxxxxx	-
L2627	*R. melanophthalma* L2398	LBM	2	Dothideomycetes, Pleosporales, Phaeosphaeriaceae	xxxxxxxx	-
L3107	*R. melanophthalma* L2668	MY	17	Dothideomycetes, Pleosporales, Phaeosphaeriaceae	xxxxxxxx	xxxxxxxx
L3265	*R. melanophthalma* L2670	MY	17	Dothideomycetes, Pleosporales, Phaeosphaeriaceae, *Phoma*	xxxxxxxx	xxxxxxxx
L2897	*R. melanophthalma* L2670	SAB	17	Dothideomycetes, Pleosporales, Phaeosphaeriaceae, *Phoma*	xxxxxxxx	-
L3048	*R. melanophthalma* L2689	DG18	18	Dothideomycetes, Pleosporales, Phaeosphaeriaceae, *Phoma*	xxxxxxxx	xxxxxxxx
L3054	*R. melanophthalma* L2706	PDA	19	Dothideomycetes, Pleosporales, Phaeosphaeriaceae, *Phoma*	xxxxxxxx	xxxxxxxx
L2856	*T. atra* L2572	SAB	12	Dothideomycetes, incertae sedis	xxxxxxxx	-
L2857	*T. atra* L2572	SAB	12	Dothideomycetes, incertae sedis	xxxxxxxx	-
L2890	*T. atra* L2572	MY	12	Dothideomycetes, incertae sedis	xxxxxxxx	xxxxxxxx
L3049	*T. atra* L2572	MY	12	Dothideomycetes, incertae sedis	xxxxxxxx	-
L3776	*T. atra* L2572	DG18	12	Dothideomycetes, incertae sedis	xxxxxxxx	-
L3817	*T. atra* L2572	DG18	12	Dothideomycetes, incertae sedis	xxxxxxxx	-
L3747	*R. melanophthalma* L2385	MY	1	Dothideomycetes, Mycosphaerellales, Mycosphaerellaceae, *Ramularia*	xxxxxxxx	xxxxxxxx
L2879	*R. melanophthalma* L2389	LBM	1	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae	xxxxxxxx	xxxxxxxx
L3239	*R. melanophthalma* L2668	LBM	17	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae	xxxxxxxx	xxxxxxxx
L3270	*R. melanophthalma* L2787	PDA	23	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae	xxxxxxxx	xxxxxxxx

L2773	*R. melanophthalma* L2469	MY	5	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae, *Elasticomyces*	xxxxxxxx	xxxxxxxx
L2775	*R. melanophthalma* L2469	SAB	5	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae, *Elasticomyces*	xxxxxxxx	-
L2777	*R. melanophthalma* L2469	LBM	5	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae, *Elasticomyces*	xxxxxxxx	-
L2784	*R. melanophthalma* L2544	LBM	9	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae, *Elasticomyces*	xxxxxxxx	-
L2785	*R. melanophthalma* L2544	LBM	9	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae, *Elasticomyces*	xxxxxxxx	xxxxxxxx
L2886	*R. melanophthalma* L2528	SAB	9	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae, *Elasticomyces*	xxxxxxxx	-
L3079	*R. melanophthalma* L2687	LBM	18	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae, *Elasticomyces*	xxxxxxxx	-
L3081	*R. melanophthalma* L2687	LBM	18	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae, *Elasticomyces*	xxxxxxxx	-
L3082	*R. melanophthalma* L2687	PDA	18	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae, *Elasticomyces*	xxxxxxxx	xxxxxxxx
L3237	*R. melanophthalma* L2724	PDA	20	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae, *Elasticomyces*	xxxxxxxx	xxxxxxxx
L3104	*R. melanophthalma* L2802	TM	24	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae, *Elasticomyces*	xxxxxxxx	xxxxxxxx
L3115	*R. melanophthalma* L2824	MY	25	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae, *Elasticomyces*	xxxxxxxx	-
L3116	*R. melanophthalma* L2825	LBM	25	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae, *Elasticomyces*	xxxxxxxx	-
L3117	*R. melanophthalma* L2825	MY	25	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae, *Elasticomyces*	xxxxxxxx	-
L3118	*R. melanophthalma* L2825	DG18	25	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae, *Elasticomyces*	xxxxxxxx	xxxxxxxx
L3119	*R. melanophthalma* L2825	PDA	25	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae, *Elasticomyces*	xxxxxxxx	-
L3120	*R. melanophthalma* L2825	PDA	25	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae, *Elasticomyces*	xxxxxxxx	xxxxxxxx
L3253	*R. melanophthalma* L2825	SAB	25	Dothideomycetes, Mycosphaerellales, Theratosphaeriaceae, *Elasticomyces*	xxxxxxxx	-
L3814	*R. melanophthalma* L2635	PDA	16	Sordariomycetes, Xylariales, *Cryptosphaeria*	xxxxxxxx	xxxxxxxx
L3815	*R. melanophthalma* L2635	PDA	16	Sordariomycetes, Xylariales, *Cryptosphaeria*	xxxxxxxx	xxxxxxxx
L3028	*R. melanophthalma* L2827	DG18	25	Sordariomycetes, Xylariales, *Hyalotiella*	xxxxxxxx	xxxxxxxx
L3093	*T. atra* L2596	MY	15	Sordariomycetes, Choniochaetales, *Choniochaeta*	xxxxxxxx	-
L2858	*T. atra* L2598	SAB	15	Sordariomycetes, Choniochaetales, *Choniochaeta*	xxxxxxxx	xxxxxxxx
L2877	*T. atra* L2598	LBM	15	Sordariomycetes, Choniochaetales, *Choniochaeta*	xxxxxxxx	-
L2859	*T. atra* L2598	SAB	15	Sordariomycetes, Hypocreales, *Tolypocladium*	xxxxxxxx	-
L2883	*T. atra* L2598	DG18	15	Sordariomycetes, Hypocreales, *Tolypocladium*	xxxxxxxx	-
L3086	*T. atra* L2598	TM	15	Sordariomycetes, Hypocreales, *Tolypocladium*	xxxxxxxx	-
L3089	*T. atra* L2598	MY	15	Sordariomycetes, Hypocreales, *Tolypocladium*	xxxxxxxx	-
L3114	*T. atra* L2754	TM	22	Sordariomycetes, Hypocreales, *Tolypocladium*	xxxxxxxx	xxxxxxxx
L2896	*R. melanophthalma* L2636	SAB	16	Sordariomycetes, Hypocreales, *Mycrocera*	xxxxxxxx	xxxxxxxx
L3122	*R. melanophthalma* L2638	MY	16	Sordariomycetes, Hypocreales, *Mycrocera*	xxxxxxxx	-

Phylogenetic and morphological analysis

A total of 131 new nucITS and 68 new nucLSU fungal sequences were generated (Table 1). Phylogenetic analyses were performed individually for each taxonomic group – Eurotiomycetes, Dothideomycetes and Sordariomycetes – using the concatenated two-locus datasets (Figs. 2, 4, 6).

ML and Bayesian phylogenetic inference were highly concordant; most of the clades were supported and topologically congruent with previous studies (Harutyunyan et al. 2008; Ametrano et al. 2019; Muggia et al. 2016, 2019, 2021; Quan et al. 2020).

Eurotiomycetes (order Chaetothyriales) (Figs. 2, 3, S1; Table 1) – Four strains were placed in Pleostigmataceae (though this clade was unsupported here as in Muggia et al. 2021): two (L3809 and L3810) isolated from *R. melanophthalma* collected in South America (Argentina) and two (L3258 and L3819) isolated from *T. atra* collected in Europe (Spain) and Tasmania. These strains were characterized by a mycelium composed by a dense aggregate of filamentous hyphae (Fig. 3a). L3809 and L3810 strains were closely related to *Pleostigma frigidum* (Muggia et al. 2021) and had heavy melanized hyphae composed by subcylindrical cells (4 × 14 μm) and by roundish or elliptical cell (5 up to 17 μm diameter; Fig. 3b). L3258 and L3819 strains were sister to *Pleostigma alpinum* and to Chaetothyriales A955. They had melanized and branching hyphae composed by globose cells (4 up to 11 μm diameter) intercalated by rectangular cells (2-4 ×12 μm) from which ramifications generated (Figs. 3c, d).

The '*Muellerella + Lichenidiplis*' clade (*sensu* Muggia et al. 2015) and the family Epibryaceae were found as the most basal lineages in Chaetothyriales. In this clade we found only one strain (L3774) isolated from *T. atra* collected in Tasmania andthe strain Chaetothyriales sp. Pet 5a (Vasse et al. 2017) was also included. The strain L3774 was characterized by heavy melanized elliptical and elongated cells (4 × 8 μm) often constricted at the septa and with rare ramification (Fig. 3e) and conidia-like cells (4 up to 7 μm diameter; Fig. 3f).

Ten strains isolated from *R. melanophthalma* collected in North and South America (Utah and Argentina, respectively) were placed in Trichomeriaceae. Four of them (L2606, L2881, L3112 and L3238) were closely related to *Neophaeococcomyces aloes*, to which *Cladophialophora proteae* had a basal position. The mycelium of these four strains was a dense aggregate of heavily melanized and branching hyphae with irregular margin (Fig. 3g) composed by rectangular cells (4 × 10 μm; Figs. 3i, k) and by conidia and chlamydospore-like cells (4 up to 10 μm diameter) remaining attached to one another (Figs. 3h, j, l). The other six strains (L3064, L3065, L3099, L3103, L3105 and L3252) were related to *Knufia separata*, Fungal sp. CCFEE 5324 and CCFEE 5322 (Selbmann et al., 2013). Their mycelium was a dense aggregate of melanized, moniliform and branched hyphae that build a black-brown to olivaceous black colony with regular margin (Fig. 3m). Their hyphae were formed by globose and sub-globose cells (4 × 6 μm to 11 × 15 μm; Figs. 5o-q) intercalated by rectangular cells (4× 10 μm; Figs. 3n, r). Apically and lateral budding cells (Fig. 3p) and anastomosing hyphae (Fig. 3n) were present.

In the *Melanina gunde-cimermaniae* clade, five (L2604, L2605, L2618, L2628 and L2876) isolates from *R. melanophthalma* collected in South America (Argentina) and two isolates from *T. atra* collected in Europe (Spain) were found together with other two specimen *Capronia* sp. 97003b and *Capronia* sp. 97003a. Our isolates were characterized by a dark grey to black mycelium (Fig. 3s) composed by toruloid hyphae and filaments of conidia (4 up to 10 μm diameter; Figs. 3t-v).

Two isolates (L3233 and L3784) from *T. atra* collected in Mauritius were found alone on individual branches nested with samples of Chaetothyriales sp. [two uncultured samples *Chaetothyriales* sp. FM034.2 (Martos et al. 2012), *Chaetothyriales* sp. NOUTOTU-121 (Qin et al. 2019), and Herpotrichiellaceae sp. MUT 5408 (Gnavi et al. unpublished)], being closely related to the recently described family Paracladophialophoraceae (Wijayawardene et al. 2020) and a monophyletic lineage that was identified here for the first time and we referred to it as "Chaetothyriales sp. 1". The two isolates L3233 and L3784 were characterized by heavy or often slight melanized hyphae in which cylindrical and rectangular cells (4 × 10-15 μm; Fig. 3ac) were intercalated to spherical cells (5 to 15 μm diameter; Fig. 3ad).

The new "Chaetothyriales sp. 1" was represented by 13 newly generated strains [seven isolates – L3260, L3261, L3262, L3763, L3764, L3765 and L3771 – from *R. melanophthalma* collected in North America (Utah) and other six isolates – L3776, L3779, L3780, L3781, L3782 and L3783 – from *T. atra* collected in Europe (Spain)] and several other strains (Fig. S1), including those named *Rhinocladiella* sp. (S1, h2, Sh9, Sh10, Sh12, Sh25, Sh36, L204 and L474, 01001a, 01001b, 04001a, 97001a and 131b) identified in previous studies by Harutyunyan et al. (2008), Wang et al. (only sequences published in NCBI) and Favero-Longo et al. (2015), respectively.

In the largest family Herpotrichiellaceae, 28 new isolates were placed. Two strains (L3096 and L2863), isolated from *R. melanophthalma* collected in North America (Nevada) and from *T. atra* collected in Tasmania, were placed next to *Phaeoannellomyces elegans* and *Exophiala nigra*. They were characterized by a dense mycelium with brown margin that became paler to grey-white in the centre of the colony (Fig. 3w) and by hyphae composed of elongated and rectangular cells (3 × 12 μm) with branches (Fig. 3x). The remaining 26 strains, isolated from both *R. melanophthalma* collected in North and South America (Utah and Argentina, respectively) and Europe (Spain) and from *T. atra* collected in South America (Chile) and Tasmania, were nested with *Cladophialophora* species (Fig. 2, Fig. S1). Twenty-one isolates formed a separate lineage together with black fungi isolated from lichens in previous studies (Harutyunyan et al. 2008, Muggia et al. 2016, 2017, 2021) and named *Cladophialophora* sp. S5, SH8, L359, A1044 and A1069. In the more detailed analysis of Fig. S1 also the samples *Cladophialophora chaetospira, C. nyingchiensis C. parmeliae* and *C.*

tengchongensis were nested within this clade. We referred to this second new lineage as "*Cladophialophora* sp. 2".

The detailed morphological descriptions of the "Chaetothyriale*s* sp. 1" and "*Cladophialophora* sp. 2" were presented below in the "Taxonomy" section.
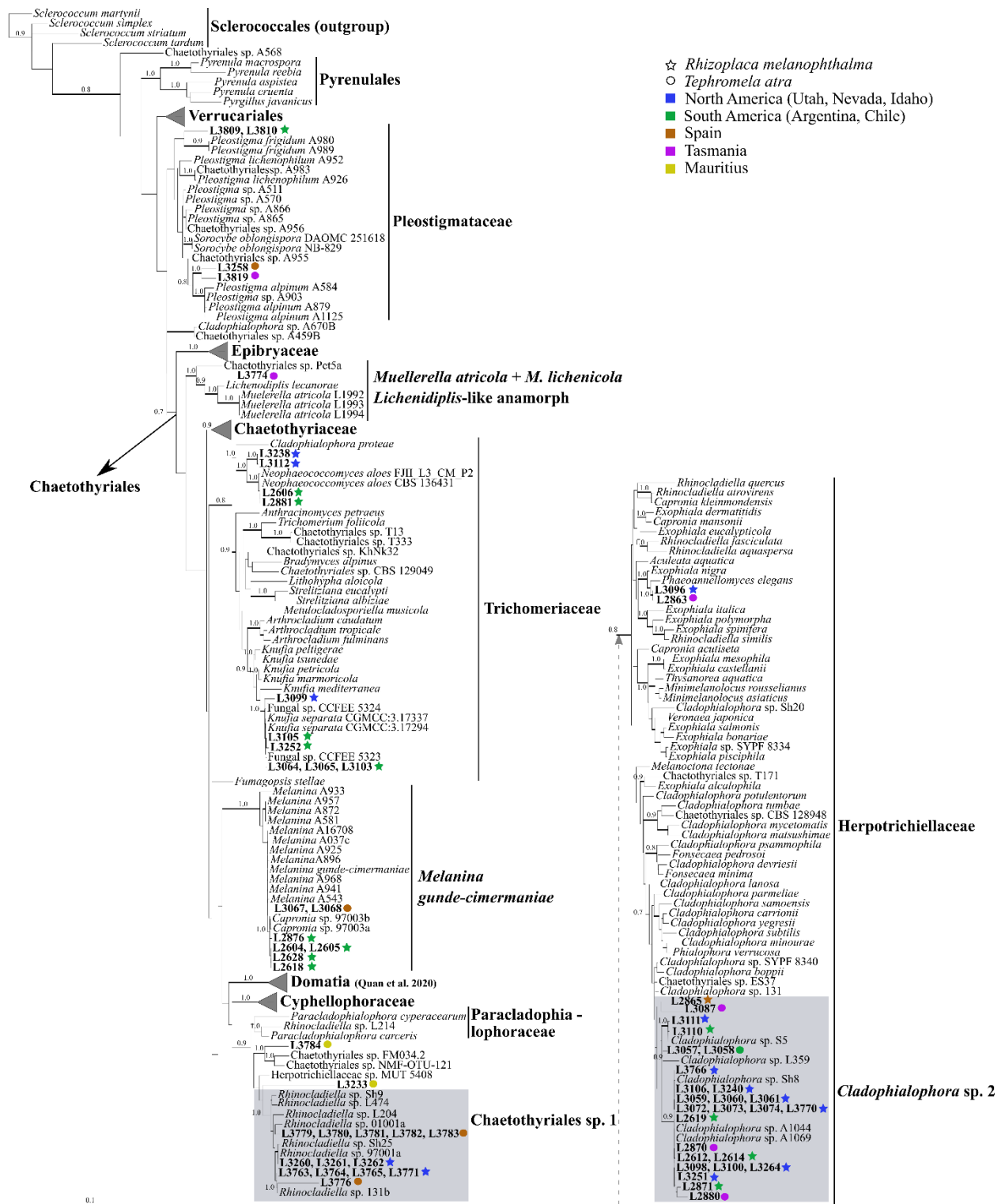
Figure 2. Phylogenetic inference of Eurotiomycetes (Chaetothyriales): Maximum Likelihood analysis based on the concatenated nuclear ITS-LSU dataset; branches in bold denote RAxML bootstrap support ≥75%; Bayesian posterior probabilities ≥0.8 are reported above branches. Newly obtained sequences are in bold and reported in the same line when they were isolated from the same lichen thallus. Symbols and colours indicate the different lichen hosts and the geographic origin from where the strains were isolated, respectively. The new identified lineages are highlighted in grey.

Figure 3. Morphology of six-month to one-year old cultures representative of strains belonging to Eurotiomycetes (Chaetothyriales) and included in the phylogenetic analysis of Fig. 2. Strains L3819 (A); L3809 (B); L3258 (C, D); L3774 (E, F); L2881 (G-I); L3238 (J, K); L3112 (L); L3252 (M); L3052 (N); L3099 (O, P); L3065 (Q, R); L3067 (S, T); L2618 (U); L2876 (V); L3096 (W); L2863 (X); L3262 (Y, Z); L3765 (AA, AB); L3784 (AC, AD); L2865 (AE, AF); L3058 (AG); L3060 (AH); L2870 (AI, AJ). Colony appearance on solid medium after six-month to one year of growth (A, G, M, S, W, Y, AE). Filamentous, septate and melanized hyphae (B, D, P, K, AA, AB, AF, AG). Branching hyphae (C, E, I, O, Q, R, X, AC, AI). Conidia-like cells (Z, H, J, L, T-V, AH, AJ). Anastomosis hyphae (N). Scale bars: 1 cm (A, G, M, S, W, Y, AE); 5 μm (B, F, H-L, N-R, T-V, X, Z-AC, AH); 10 μm (C-E, AF, AG, AI, AJ).

Dothideomycetes (Fig. 4, 5; Table 1, S1). – Fifty-three new strains were found in Dothideomycetes. The strain L3077, isolated from *R. melanophthalma* collected in North America (Idaho), was placed basal to Dothideomycetes, together with *Lichenothelia papilliformis* (Ametrano et al. 2019), Lichenostigmatales sp. A930 (Muggia et al. 2016) and Fungal sp. TRN529 (Ruibal et al. 2009). This strain was morphologically similar to Lichenostigmatales sp. A930, with a yeast-like black mycelium (Fig. 5a), budding hyphae and melanized cells (3 up to 20 μm diameter; Fig. 5b, c). Most strains belonged mainly to the orders Pleosporales and Mycosphaerellales. Twenty-four strains were placed in Pleosporales and belonged to five family level lineages highly supported and fully resolved. In particular, the strain L2869, isolated from *R. melanophthalma* collected in South America (Argentina), was placed in Lophiotremataceae and was characterized by hyaline hyphae (3 μm diameter; not shown). The strain L3091, isolated from *T. atra* collected in Tasmania, was placed in Teichosporaceae and was closely related to an uncultured fungus B3_1986 (found by Vázquez-Nion et al. 2016) and characterized by a grey mycelium with brown margin with septate hyphae (4 × 15 μm; not shown). The strain L2868, isolated from *T. atra* from Tasmania, was placed in the well-supported clade of *Paraphaeosphaeria michotii* and it was characterized by hyaline hyphae (2 μm diameter; not shown).

The strain L3036, isolated from *R. melanophthalma* collected in North America, was nested in a clade sister to *Paraphaeosphaeria michotii* and composed of cultures that were also still unnamed, either cultured – such as Pleosporales sp. A1039 (by Muggia et al. 2016) and *Periconia* sp. – or the uncultured – S241 (Fröhlich-Nowoisky et al. 2009) and L042885-122-065-F09 (Fröhlich-Nowoisky et al. 2012).

The strain L3021, isolated from *T. atra* collected in Tasmania, was placed in the family Didymellaceae and was related to *Phoma herbarum*, *Ampelomyces* sp. and *Didymella* spp. This strain was characterized by a white to grey mycelium (Fig. 5d) with slight melanized hyphae (5 × 15 μm; Fig. 5e).
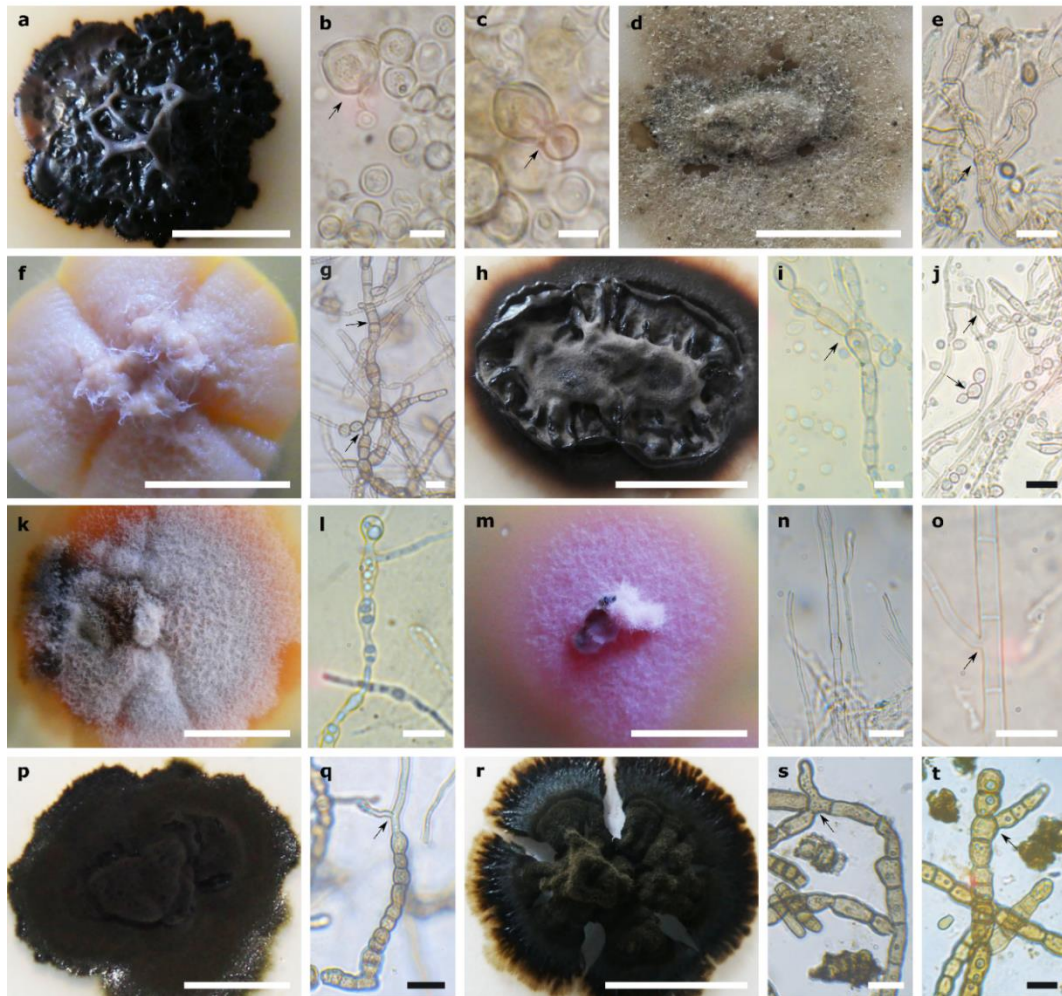
The strain L3078, isolated from *R. melanophthalma* collected in North America was placed in the family Pleosporaceae next to *Pleospora* spp. and *Comoclathris lini*. This strain was characterized by a grey mycelium with brown margin and septate hyphae (4 × 15 μm; not shown).

Eighteen additional strains were found within Phaeosphaeriaceae in five clades. Two strains (L2888 and L3056), isolated from *T. atra* collected in South America (Chile) and in Europe (Spain), built a clade with Capnodiales sp. UFMGCB8750 and *Catenulostroma* sp. UFMGCB8746 (Santiago et al. 2015). They were characterized by a pale pink mycelium with regular margin (Fig. 5f) and slightly melanized hyphae composed by cylindrical and rectangular cells (5 × 15 μm) often branching and constricted at the septa (Fig. 5g). Four strains (L3030, L3042, L3076 and L3094), isolated from *R. melanophthalma* collected in North America (Idaho and Nevada) and South America (Argentina) and from a *Tephromela* thallus collected in Tasmania were closely related to Ascomycota PLC12C, *Leptosphaeria* sp. plC11E (Mouhamadou et al. 2011) and to an uncultured fungus plC11E (Qin et al. unpublished). They were characterized by a black to grey mycelium composed by slight melanized hyphae with rectangular and elliptical cells (5 × 15 μm; Figs. 5h-j) from which the ramifications generate. Conidial cells (5 μm diameter) were observed (Fig. 5j). The strain L3037, isolated from *R. melanophthalma* collected in South America (Argentina), was closely related to two uncultured fungi (G2_CC10, Karst et al. 2013; 99_NA9_P31_O2, Timling et al. 2014) and had a brown to black mycelium with pale pink regular margin (data not shown). The strain L3090, isolated from *R. melanophthalma* collected in South America (Argentina), was placed together with *Jeremyomyces labinae, Melanomma sanguinarium,* Dothideomycetes LTSP_EUKA_P5M163 (Hartmann et al. 2009), an uncultured fungus 112_NA4_P31_N4 (Timling et al. 2014), Pleosporales sp. 19 KB-2015 (Travadon et al. 2015). L3090 was characterized by a grey mycelium with brown margin (data not shown). Six strains (L2622, L2624, L2625, L2627, L3017 and L3108), isolated from *R. melanophthalma* collected in North and South America (Utah and Argentina, respectively), built a separate clade sister to *Didymocyrtis brachylaenae, Phaeosphaeria* sp. SW_0_F12, *Phaeosphaeria* sp. AC (Travadon et al. 2016), *Phaeosphaeria* sp. 1715242 and *Phaeosphaeria* sp.M129 (Berube et al. 2015). These strains had an ochre to pale pink mycelium with hyaline hyphae composed by rectangular cells (4 × 12 μm; not shown). Four strains (L2897, L3048, L3054 and L3265), isolated from *R. melanophthalma* collected in North America (Utah), were nested within *Phoma* species identified from lichens, i.e., *Phoma caloplacae, P. cladoniicola* and several still unidentified *Phoma* sp.. They were characterized by a whitish to pale pinkish mycelia with a pale orange margin and composed by hyaline hyphae distributed to form a dense aggregate (5 × 15 μm) and conidiogenous-like cells (10 μm diameter) (Figs. 5k, l).

Twenty-two strains were found in the order Mycosphaerellales (Abdollahzadeh et al. 2020) and belonged to Mycosphaerellaceae and Teratosphaeriaceae. The strain L3747 isolated from *R. melanophthalma* collected in South America (Argentina) was genetically identical to *Ramularia vizellae* in Mycosphaerellaceae. Within the Teratosphaeriaceae, instead, the newly isolated strains were placed in four separated clades. The strain L2879, isolated from *R. melanophthalma* collected in South America (Argentina) was closely related to Teratosphaeriaceae sp. CPC 12419 (Crous et al. 2008) and sister to *Saxomyces penninicus* and *Teratosphaeria parva.* This strain was characterized by a dark black mycelium (Fig. 5p) composed of melanized and hyaline hyphae with rectangular cells (4 × 10 μm) from which ramification started. Filaments of roundish conidia (7 μm diameter) were observed (Fig. 5q). The strain L3239, isolated from *R. melanophthalma* collected in North America (Utah) was placed in a supported clade together with three unknown fungi labelled as sp. agrD231, agrD244 and agrD242 (Peršoh et al. 2012). This strain was characterized by a blackish mycelium composed by heavy melanized hyphae with rectangular cell (5 × 15 μm) from which ramification started (data not shown). The strain L3270, isolated from *R. melanophthalma* collected in South America (Argentina) was closely related to three unidentified fungi Dothideomycetes sp. AK1125 (U'Ren, 2012), Dothideomycetes sp. PIMO_109 and fungal sp. PIMO_21 (Larkin et al. 2012). Lastly, 14 strains, isolated from *R. melanophthalma* collected in South America (Argentina and Chile) and North America (Utah and Idaho), built a separate clade together with *Elasticomyces elasticus* and four still undetermined fungal samples [i.e. Dothideomycetes sp. s_C03_05.ab (Amend et al. 2010), Dothideomycetes sp. PIMO_446 (Larkin et al. 2012) and two uncultured fungi (127_NA4_P32_L9 and FunN4_01B; Timling et al. 2014; Nemergut et al. 2008)]. These strains were characterized by blackish to greenish mycelia with irregular margin (Fig. 5r) and heavy melanized hyphae composed by cylindrical and rectangular cells (5 × 8-12 μm) with ramification (Fig. 5s, t).

The last six strains (strains L2856, L2857, L2890, L3049, L3776 and L3817), isolated from *T. atra* collected in Europe (Spain), were placed together with Dothideomycetes sp. A931 and A552 (Muggia et al. 2016) in a clade closely related to Venturiales, Lichenocloniales and Abrothallales. These strains had a pink mycelium with regular margin (Fig. 5m) composed by hyaline hyphae built by rectangular and cylindrical cells (2 × 15 μm) from which branches generated (Fig. 5n, o).

Figure 4. Phylogenetic inference of Dothideomycetes: Maximum Likelihood analysis based on the concatenated nuclear ITS-LSU dataset; branches in bold denote RAxML bootstrap support ≥75%; Bayesian posterior probabilities ≥0.8 are reported above branches. Newly obtained sequences are in bold and reported in the same line when they were isolated from the same lichen thallus, respectively. Symbols and colours indicate the lichen hosts and the geographic origin from where the strains were isolated.
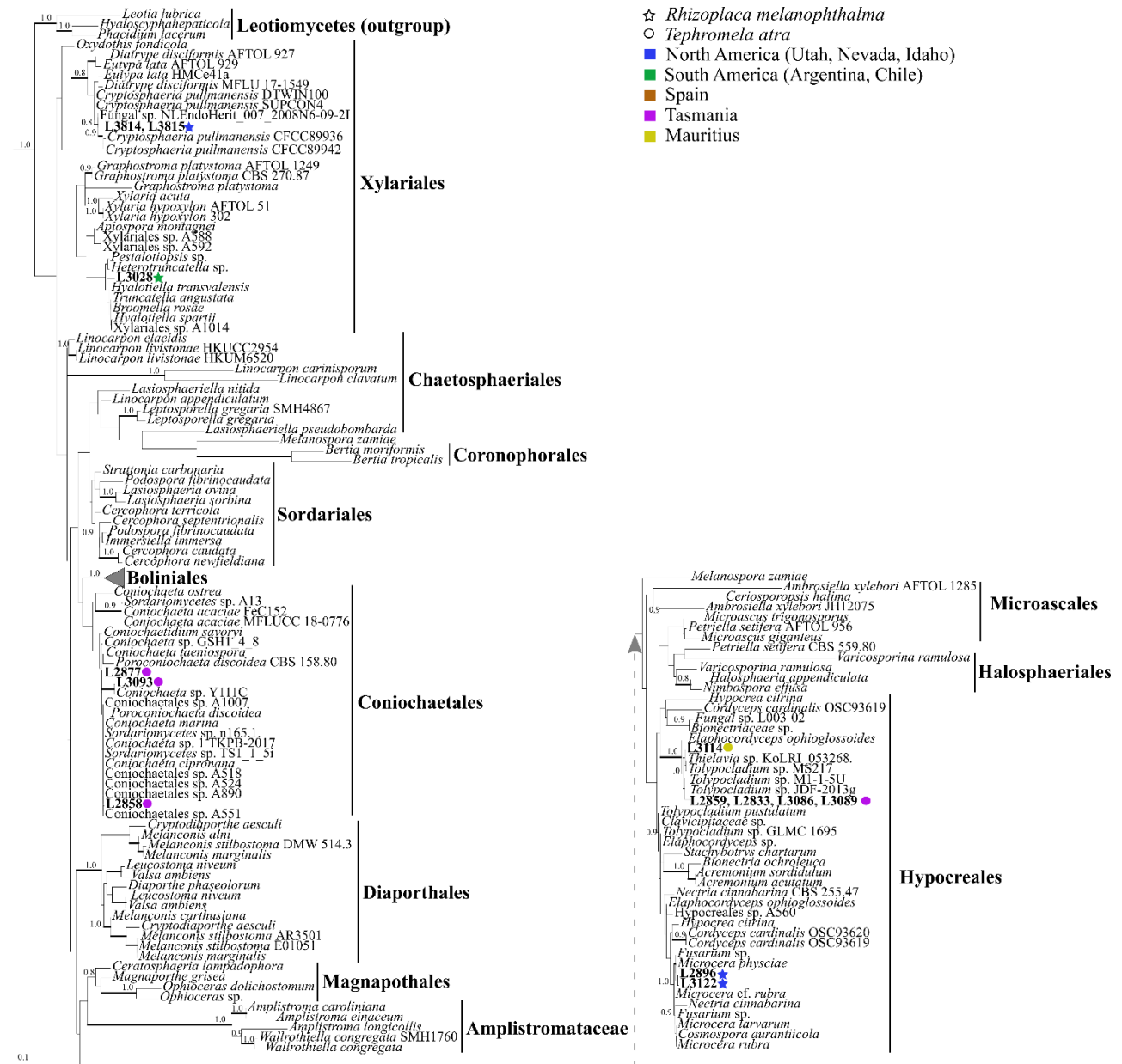
Figure 5. Morphology of six-month to one-year old representative cultured fungal strains belonging to Dothideomycetes and included in the phylogenetic analysis of Fig. 3. Strains L3077 (A-C); L3021 (D, E); L3056 (F, G); L3094 (H-J); L3048 (K, L); L2890 (M); L3890 (N-O); L2879 (P, Q); L3104 (R); L3120 (S-T). Colony appearance on solid medium after six-month to one year of growth (A, D, F, H, K, M, P, R). Yeast-like black mycelium with budding cells (B, C). Filamentous, septate hyphae with branches (E, G, I, J, N, O, S, T). Conidia-like cells (J, L, Q). Scale bars: 1 cm (A, D, F, H, K, M, P, R); 5 μm (B, C); 10 μm (E, G, I, J, N, O, Q, S, T).

Sordariomycetes (Figs. 6, 7; Table 1, S1) – Thirteen strains were found belonging to Xylariales, Coniochaetales and Hypocreales in Sordariomycetes. In Xylariales two strains (L3814 and L3819), isolated from *R. melanophthalma* collected in North America (Utah), were nested in a clade with *Cryptosphaeria pullmanensis* and the fungal sp. NLEndoHerit_007_2008N6-09-2I (Lamit et al. 2014); they were characterized by a blackish mycelium with grey spots (Fig. 7a) formed by slight and hyaline hyphae (3 μm diameter; Figs. 7b, c). One strain (L3028), isolated from *R. melanophthalma* collected in South America (Argentina) was closely related to *Hyalotiella transvalensis, H. spartii, Truncatella angustata, Broomella rosae* and a Xylariales sp. A1014

(Muggia et al. 2016). It was characterized by a reddish mycelium built by hyaline hyphae (3 µm diameter; Figs. 7d, e).

Three strains (L2858, L2877 and L3093), isolated from *T. atra* collected in Tasmania, were nested in Coniochaetales, closely related to *Coniochaeta* sp. Y111c (Muriel et al. 2022) and other still unnamed Coniochaetales sp. [i.e., A518, A524, A551, A890 and A1007 from Muggia et al. (2016), 1 TKPB-2017 (Kowalski and Bilański, 2021), Sordariomycetes sp. n165.1 and TS1_1_5i]. These strains were characterized by a white mycelium (Fig. 7f) composed by hyaline hyphae (3 µm diameter; Fig. 7g).

Seven strains were placed in the order Hypocreales. Five strains (L2859, L2883, L3086, L3089 and L3114), isolated from *T. atra* collected in Tasmania and Mauritius, were closely related to *Tolypocladium* sp. (MS217, M1-1-5U and JDF-2013g; Jiang et al. 2015), *Thielavia* sp. KoLRI_053268 (Yang et al. 2022) and *Elaphocordyceps ophioglossoides*. These strains were characterized by a greyish and white mycelium (Figs. 7h, k) composed of hyaline hyphae (3 µm diameter) with branches (Figs. 7i, j, l). Two strains (L2896 and L3122), isolated from *R. melanophthalma* collected in North America (Utah), were nested within *Microcera* species. These strains were characterized by an orange mycelium (Fig. 7m) made of hyaline hyphae (4 µm diameter) with branches (Figs. 7n, o).

Figure 6. Phylogenetic inference of Sordariomycetes: Maximum Likelihood analysis based on the concatenated nuclear ITS-LSU dataset; branches in bold denote RAxML bootstrap support ≥75%; Bayesian posterior probabilities ≥0.8 are reported above branches. Newly obtained sequences are in bold and reported in the same line when they were isolated from the same lichen thallus. Symbols and colours indicate the different lichen hosts and the geographic origin from where the strains were isolated, respectively.
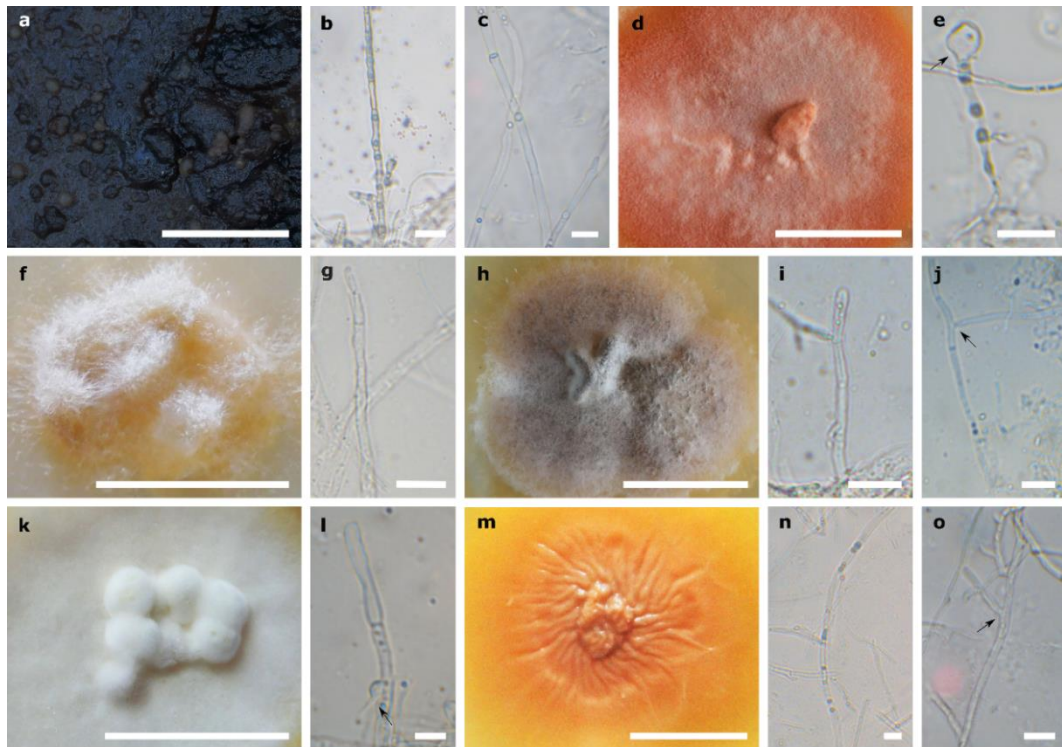
Figure 7. Morphology of six-month to one-year old representative cultured fungal strains belonging to Sordariomycetes and included in the phylogenetic analysis of Fig. 4. Strains L3814 (A); L3815 (B, C); L3028 (D, E); L2877 (F, G); L3114 (H-J); L3086 (K, L); L3028 (M-O). Colony appearance on solid medium after six-month to one year of growth (A, D, F, H, K, M). Slight and hyaline hyphae (B, C, E, G, I, L, N). Branching hyphae (J, O). Scale bars: 1 cm (A, D, F, H, K, M); 10 μm (B, C, E).

Taxonomy

Chaetothyriales sp. 1 Cometto, Leavitt, Grube, De Hoog, Muggia, **sp. nov.** – Mycobank xxxx

Etymology: xxxx

Holotype: L3782, cultured strain, preserved in metabolically inactive state in MY medium (September 2020, date at which they were first identified in culture isolation), isolated from the thallus of *Tephromela atra*.

Description: endolichenic (i.e., cryptically present in lichen thalli), isolates derived likely from hyphal fragments or spores entrapped in the thalline matrix of the lichen hosts, grown *in vitro* rather slowly. Dark gray to black mycelium with a regular margin composed by heavy or often slight melanized hyphae (Fig. 3y). The hyphae have a peculiar shape in which cylindrical and rectangular cells (4 × 10-15 μm; Fig. 3aa) intercalate to spherical cells (5 to 15 μm dimeter; Fig. 3ab). Branching has originated from rectangular (Fig. 3aa). Chain of conidia (2- 5 μm diameter) were observed (Figs. 3z, ab).

Distribution: boreal, isolated from lichens growing on limestone, siliceous and quartzite rocks at about 1260 - 2000 m a.s.l and on basalt rocks from 15 m a.s.l. to 2800 m a.s.l. Isolated so

far from the lichen species *Caloplaca gomerana*, *C. saxicola*, *Lecidella stigmatea*, *Protoparmeliopsis muralis*, *Rhizoplaca melanophthalma*, *Rusawskia elegans*, *Tephromela atra*, *Umbilicaria virginis* and *U. vellea*.

Material examined: NORTH AMERICA, Utah, Rock Canyon, on quartzite rocks, alt. 1700 m a.s.l., endolichenic fungi isolated from *R. melanophthalma* lichen thalli, 2019, S. D. Leavitt, strain numbers L3260, L3261, L3262, L3763, L3764, L3765 and L3771. ASIA, Armenia, Kotayk, Garni gorge, on basalt rocks, alt. 1180 - 2820 m a.s.l., endolichenic fungi isolated from *P. muralis* lichen thalli, 2006, S. Harutyunyan and H. Mayrhofer, strain number Sh9, Sh10, Sh12, Sh25 and Sh36. Armenia, Kotayk, Garni gorge, on basalt rocks, alt. 1180 m a.s.l., endolichenic fungi isolated from *C. saxicola* lichen thalli, 2006, S. Harutyunyan and H. Mayrhofer, strain number h2. EUROPE, Spain, prov. Madrid, Miraflores del la Sierra, Puerto de la Morquera, summit of Pico Najarra, on siliceous-granitic boulders, alt. 2080 m a.s.l., endolichenic fungi isolated from *T. atra* lichen thalli, 2019, L. Muggia and S. Perez-Ortega, strain numbers L3776, L3779, L3780, L3781, L3782 and L3783. Spain, Canary Islands, Tenerife, Punta Roja, on basalt rocks, alt. 15 m a.s.l., endolichenic fungi isolated from *C. gomerana* lichen thalli, 2005, L. Muggia, strain number L204. Austria, Styria, Röthelstein, on limestone rocks, alt. 1260 m a.s.l., endolichenic fungi isolated from *L stigmatea*, 2006, L. Muggia and J. Hafellner, strain number L474.

Cladophialophora sp. 2 Cometto, Leavitt, Grube, De Hoog, Muggia, **sp. nov.** – Mycobank xxxx

Etymology: xxxx

Holotype: L3074, cultured strain, preserved in metabolically inactive state in MY medium (September 2020, date at which they were first identified in culture isolation), isolated from the thallus of *Rhizoplaca melanophthalma*.

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* rather slowly. The mycelium is composed by a dense aggregate of melanized hyphae that builds a blackish-brown colony with irregular margin (Fig. 3ae). Mostly of the hyphae are composed by rectangular and cylindrical cells ($4 \times 12\text{-}17$ μm) from which branches generate (Figs. 3af-ai) and laterally budding cells (Fig. 3ag). Apical muriform conidiogenous cells (10×12 μm, Fig. 3ah) and lateral conidiogenous cell (8×10 μm; Fig. 3aj) were observed.

Distribution: boreal isolated from lichens growing on siliceous-granitic, quartzite, basalt and sandstone rocks at about 1600 - 1900 m a.s.l.; austral, isolated from lichens growing on basaltic and dolorite rocks at about 545 - 2000 m a.s.l.. Isolated so far from the following lichen species:

Gyalolechia fulgida, Lecanora bicincta, L. polytropa, *Protoparmeliopsis muralis, Rhizoplaca melanophthalma*, *Tephromela atra* and *Rusawskia elegans.*

Material examined: SOUTH AMERICA, Argentina, prov. Mendoza, on basalitic boulders, alt. 1450 - 2000 m a.s.l., endolichenic fungi isolated from *R. melanophthalma* lichen thalli, 2019, L. Muggia, strain numbers L2612, L2614, L2619, L2871 and L3110. Chile, Region de Aysén del General Carlos Ibanez del Campo, prov. Capitan Prat, alt. 600 m a.s.l., endolichenic fungi isolated from *T. atra* lichen thalli, 2019, J. Orlando and D. Leiva, strain numbers L3057 and L3058. NORTH AMERICA, Utah, Rock Canyon, and Emery County, on quartzite and sandstone rocks, alt. 1665 - 1700 m a.s.l., endolichenic fungi isolated from *R. melanophthalma* lichen thalli, 2019, S. D. Leavitt, strain numbers L3059, L3060, L3061, L3072, L3073, L3074, L3098, L3100, L3106, L3111, L3240, L3251, L3264, L3766 and L3770. OCEANIA, Tasmania, three Thumbs, on dolorite rocks, alt. 545 m a.s.l., endolichenic fungi isolated from *T. atra* lichen thalli, 2019, G. Kantvilas, strain numbers L2870, L2880 and L3087. ASIA, Armenia, Kotayk, Tsaghkadzor, on rock, alt. 1750 m a.s.l., endolichenic fungi isolated from *R. elegans* lichen thalli, 2005, S. Harutyunyan, strain number S5. Armenia, Kotayk, Geghard, on basalt rocks, alt. 1875 m a.s.l., endolichenic fungi isolated from *P. muralis,* 2006, S. Harutyunyan and H. Mayrhofer, strain number SH8. EUROPE, Spain, prov. Madrid, Miraflores del la Sierra, Puerto de la Morquera, summit of Pico Najarra, on siliceous-granitic boulders, alt. 2080 m a.s.l., endolichenic fungi isolated from *R, melanophthalma* lichen thalli, 2019, L. Muggia and S. Perez-Ortega, strain number L2865. Italy, Sardegna, Nuoro, Mt. Albo Massiv, on limestone rocks, alt 1000 m a.s.l., endolichenic fungi isolated from *G. fulgida* lichen thalli, 2006, L. Muggia, strain number L359. Austria, between the states Styria and Carinthia, Koralpe mountain, siliceous-schist/ gneissic rocks, alt. 1800 - 2100 m a.s.l., endolichenic fungi isolated from *Lecanora bicincta* and *L. polytropa* lichen thalli*,* 2012, L. Muggia, strains number A1044 and A1069.

Discussion

The culture approach applied in the present study captured a great diversity of the microfungi associated with two cosmopolitan, epilithic lichens – *Rhizoplaca melanophthalma* and *Tephromela atra* – collected in a range of harsh environmental conditions. Here, we detected both already known fungal lineages and two new lineages which seem to be recurrently associated to lichen thalli. Indeed, many strains belonging to the new lineages were previously detected in local communities of alpine (Fleischhacker et al. 2015; Muggia et al. 2016, 2017) and Mediterranean lichens (Harutyunyan et al. 2008). Although fungi can adapt to diverse environments and develop

different lifestyles and evolutionary strategies, some lineages seem to preferentially reside in lichen thalli and might develop a certain specificity to their hosts.

Phylogenetic relationships of the 131 new fungal isolates were inferred, among which 65 belong to Eurotiomycetes, 53 to Dothideomycetes and 13 to Sordariomycetes. Furthermore, isolates representing most of the phylogenetic lineages were morphologically characterized. We found that these microfungi grew on six different culture media, and the percentage of their growth success corresponded well to that reported by Muggia et al. (2017). We obtained fungal isolates from almost all the lichen samples, with the exception of those thalli of *R. melanophthalma* collected in three localities in the Argentinian Andes at altitudes ranging from 3600 to 5100 m a.s.l., and in Spain at 2080 m a.s.l.. The fact that no fungal strains could be isolated from these lichen thalli may be explained by selective constraints in which the lichen grew, that were not simulated in the culture conditions. Thus, the culture conditions applied seem to have not favoured *in vitro* the development of mycelia, particularly of those that would be more extremophilic fungi. In fact, the inocula were incubated at 17 °C and constant humidity in growth chambers, while the original thalli experienced harsher conditions of drought stress and radiation in the environments. An alternative explanation may be found in the fact that these lichen thalli presented a lower and different mycobiome diversity in the DNA metabarcoding analyses (Cometto et al. under review), which would likely support the lack of culturable strains within the species-poor pool of fungi. Extremophilic fungi, indeed, grow extremely slowly *in vitro*, if not at all, and their isolation often demands specific requirements and extended amounts of incubation time (Urzı and De Leo 2001; Selbmann et al. 2014).

Interestingly, 50% of the isolates obtained in this study had already been reported for lichens from arid Mediterranean and alpine habitats (Harutyunyan et al. 2008; Muggia et al. 2016, 2017) collected on soil and trees (Harutyunyan et al. 2008; Peršoh et al. 2012; Muggia et al. 2016, 2017). The other identified strains form either small, still unnamed (because still too poorly represented) lineages or were closely related to species of rock-inhabiting fungi and plant endophytes within each of the three classes. In fact, strains corresponding to the rock-inhabiting genera *Knufi*a (Eurotiomycetes, Chaetothyriales, Trichomeriaceae), *Elasticomyces* (Dothideomycetes, Teratosphaeriaceae), or the endophytic *Neophaeococcomyces* (Eurotiomycetes, Chaetothyriales, Trichomeriaceae), *Paraphaeosphaeria* (Dothideomycetes, Pleosporales), or *Cryptosphaeria* (Sordariomycetes, Xylariales) were identified here.

Our data show that most of the isolated strains are members of the Eurotiomycetes. Within this class, the majority of microfungi belong to families in Chaetothyriales, an order which also includes many saprophytic and opportunistic pathogens on humans and cold-blooded vertebrates

(de Hoog et al. 2011; Teixeira et al. 2017, Quan et al. 2020). The newly isolated strains share the characteristic traits of the melanised polyextremotolerant fungi already described for rock-inhabiting fungi, pathogens and lichen-associated lineages (Gostinčar et al. 2012, 2018). Indeed, three strains represent *Pleostigma* species, one *Muellerella* and another seven correspond to the recently described species *Melanina gunde-cimermaniae*. *Muellerella* is a well-known genus of symptomatic lichenicolous fungi which was successfully isolated from ascospores and conidia but occurs also cryptically in lichens (Muggia et al. 2015, 2019, 2021). The strain L3774 which is in the clade '*Muellerella + Lichenodiplis*' was indeed isolated from a thallus of *Tephromela atra* (which is the original lichen host species of *Muellerella atricola* and *Lichenodiplis lecanorae*; Atienza et al. 2009; Muggia et al. 2015, 2019), but morphological inspections confirmed that this thallus is devoid of any perithecia or sporodochia, thus supporting the cryptic occurrence of the *Muellerella* fungus.

The genera *Pleostigma* – together with the corresponding family Pleostigmataceae – and *Melanina* were recently described by Muggia et al. (2021) to allocate black fungal strains that were isolated from alpine lichens (i.e. *Aspicilia caesiocinerea*, *A. simoensis*, *Aspicilia* sp., *Lecanora intricata*, *L. polytropa*, *Lecidea lapicida*, *Lecidea* sp., *Rhizocarpon geographicum*, *Schaereria fuscocinerea* and *Umbilicaria cylindrica*; Muggia et al. 2021) and a few fungi that were identified by Ruibal et al. (2009) from calcareous rocks in the Mediterranean basin. Finding here both *Pleostigma* spp. and *Melanina* also in thalli collected at diverse altitudes and across a worldwide geographic range supports their specific endolichenic lifestyle and likely their ubiquitous distribution in lichens.

The most important outcome is that the present research conducted on such a broad scale allowed us to recognize additionally two new lineages of lichen-associated fungi in Chaetothyriales. Our phylogenetic inference is topologically congruent with those of Crous et al. (2016, 2018), Quan et al. (2020) and Wijayawardene et al. (2020). However, "Chaetothyriales sp. 1" might be still of *incertae sedis* in Chaetothyriales, as its relationship with the next closely related Paracladophialophoraceae (Wijayawardene et al. 2020), Cyphellophoraceae and "Domatia" clade *sensu* Quan et al. (2020) is unsupported (Fig. 2). "Chaetothyriales sp. 1" is well represented by a variety of strains isolated here form both *R. melanophthalma* and *T. atra* (n. 15), in addition to lichens collected in the Mediterranean basin (i.e., *Caloplaca gomerana, Lecidella stigmatea, Protoparmeliopsis muralis, Protoparmeliopsis muralis, Umbilicaria virginis* and *U. vellea*; Harutyunyan et al. 2008). Members of this lineages occur at different altitudes and on different rock substrates (basalt, calcareous and siliceous). Harutyunyan et al. (2008) assigned these strains to the genus *Rhinocladiella* because at that time only a few sequences were available for comparison. Later *Rhinocladiella* was included in Herpotrichiellaceae (Teixeira et al. 2017), while the still

undescribed strains provisionally maintained this genus name. Also at that time, Harutyunyan et al. (2008) suggested that these strains could be facultative lichen colonisers because of the absence of a specific lichen host and the infection symptoms. However, the present results strengthen the idea that "Chaetothyriales sp. 1" has developed a certain preference for the lichen-associated lifestyle and offer additional data to support its formal recognition.

The second new lineage – "*Cladophialophora* sp. 2" – is placed within Herpotrichiellaceae, and its topology is mostly congruent with that of Muggia et al. (2017, 2021). In addition to our 26 strains isolated from both *R. melanophthalma* and *T. atra*, this new clade includes other *Cladophialophora* samples previously isolated from other lichen species (*Gyalolechia fulgida, Lecanora polytropa*, *P. muralis* and *Rusavskia elegans*; Harutyunyan et al. 2008; Muggia et al. 2016). Herpotrichiellaceae is the largest family in Chaetothyriales which includes ecologically very diverse fungi (e.g., human opportunists, rock-inhabiting fungi and lichenicolous fungi; Crous et al. 2007) and our results are not unexpected – the lichen-associated lifestyle is ancestral in this order (Quan et al. 2020, Quan et al. 2022 under review). Interestingly, the lichen associated Herpotrichiellaceae are mainly found to be likely *Cladophialophora* species, a genus known to be involved in the aromatic hydrocarbon degradation (Prenafeta-Boldú et al. 2006; Badali et al. 2008) and supposed to be able also to take benefits from the secondary metabolites found in lichens (Harutyunyan et al. 2008). The strains forming "*Cladophialophora* sp. 2" are strictly lichen-associated fungi, while the closest related species are *C. chaetospira*, *C. nyingchiensis* and *C. tengchongensis*, the latter two recently described as microcolonial melanised rock-inhabiting fungi (Sun et al. 2020).

Significant diversity of lichen-associated microfungi is also found in Dothideomycetes, although here the isolated strains are phylogenetically more heterogeneous. In Dothideomycetes, our samples were related to fungi with different lifestyles (Schoch et al. 2009; Hyde et al. 2013; Wijayawardene et al. 2014), as well as lichenicolous fungi such as *Phoma caloplacae* and *P. cladonicola* (Lawrey et al. 2012). Interestingly, most of the isolates retrieved in Dothideomycetes are related to fungi previously isolated from lichens or to rock-inhabiting fungi from extreme environments, such as *Elasticomyces elasticus* or *Saxomyces penninicus* (Selbmann et al. 2008, 2013; Ruibal, 2011; Muggia et al. 2016, 2017). All the new strains placed in Teratosphaeriaceae were isolated from *R. melanophthalma* collected in relatively high altitude (about 2000 - 4300 m a.s.l.) in Argentina, Chile and Utah, allowing a hypothesis that their choice to live endolichenically enhances protection from the high irradiation and the continuous fluctuation of temperature that characterize high-altitude mountain environments. However, one exception is the strain L3239, isolated from *R. melanophthalma* which (in Teratosphaeriaceae) forms a clade together with fungi

isolated from lichens in the association of *Letharietum vulpinae* (Peršoh et al. 2012). Four strains are nested with *Phoma cladoniicola*, *P. caloplacae* and many other *Phoma* samples isolated from alpine epilithic lichens (Muggia et al. 2016). This result strengthens the hypothesis of Lawrey et al. (2012), who suggested that the same *Phoma* species can be isolated from a variety of lichens, in contrast to a previous assumption that *Phoma* species were highly selective for their hosts (Hawksworth and Cole 2004; Diederich et al. 2007).

In Sordariomycetes, some new microfungal strains also correspond to previously isolated fungi from lichens, in particular within Coniochaetales, Hypocreales and Xylariales. Five strains isolated from *T. atra* collected in Tasmania and Mauritius are related to *Tolypocladium* sp. and *Elaphocordyceps* sp. isolated from other lichen species (Jiang et al. 2015; Yang et al. 2022). Interestingly, *Tolypocladium* is a genus of fungicolous fungi (Sun et al. 2019), and its presence in lichens may hint to his parasitic behaviour towards the lichen mycobiont. Furthermore, we also recovered two strains related to *Microcera physciae*, recently isolated and described from the lichen *Physcia tenella* (Crous et al. 2021).

In conclusion, lichens are long-lived symbiotic systems and serve as suitable niches for many cryptically occurring fungi, some of which have likely specialized to them, finding in these systems a kind of protection and realized niche. These fungi do not germinate further and do not exit the lichen thalli when environmental conditions are too harsh and unfavourable, remaining even undetectable by morphological inspections. However, most of these fungi, that do not readily grow in the natural habitat, may start to grow when isolated as axenic culture under suitable culture conditions. The remaining unculturable/uncultivated fraction of lichenicolous fungi seems to demand further efforts to be evidenced and morphologically studied.

Acknowledgements

Reference and contributing authors of the paper

Cometto, A. [1], Leavitt, S. D. [2], Grube, M. [3], De Hoog, S.[4], & Muggia, L. [1*] (202X). Tackling fungal diversity in lichen symbioses: molecular and morphological data recognize new lineages in Chaetothyriales (Eurotiomycetes, Ascomycota). Under submission to Mycological Progress.

[1] Department of Life Sciences, University of Trieste, via L. Giorgieri 10, I-34127 Trieste, Italy

[2] Institute of Biology, University of Graz, Holteigasse 6, A-8010 Graz, Austria

[3]Department of Biology, Brigham Young University, 84602 Provo, Utah, USA

[4]Center of Expertise in Mycology of Radboud University Medical Center / Canisius Wilhelmina Hospital, Geert Grooteplein 10 Zuid, 6525GA Nijmegen, The Netherlands

* Corresponding author: Lucia Muggia. Address: University of Trieste, Department of Life Sciences, via Giorgieri 10, 34127 Trieste, Italy. E-mail: lmuggia@units.it, lucia_muggia@hotmail.com

Supplementary information

Figure S1. Phylogenetic inference of Herpotrichiellaceae (Chaetothyriales): Maximum Likelihood analysis based on the concatenated nuclear ITS-LSU dataset; branches in bold denote RAxML bootstrap support ≥75%; Bayesian posterior probabilities ≥0.8 are reported above branches. Newly obtained sequences are in bold. Symbols and colours

indicate the different lichen host and the localities from where the fungal strains were isolated, respectively. The new generated lineages were highlighted in grey.

Table S1. Samples of *Rhizoplaca melanophtalma* and *Tephromela atra* were reported with their thallus ID and the geographic origins; localities are identified by numbers (1 - 25) (see separate external Excel file: https://zenodo.org/badge/DOI/10.5281/zenodo.7311356.svg).

Table S2. List of taxa included in the phylogenetic analysis of Eurotiomycetes (Chaetothyriales) and their NCBI accessions (see separate external Excel file: https://zenodo.org/badge/DOI/10.5281/zenodo.7311356.svg).

Table S3 List of taxa included in the phylogenetic analysis of Dothideomycetes and their NCBI accessions (see separate external Excel file: https://zenodo.org/badge/DOI/10.5281/zenodo.7311356.svg).

Table S4. List of taxa included in the phylogenetic analysis of Sordariomycetes and their NCBI accessions (see separate external Excel file: https://zenodo.org/badge/DOI/10.5281/zenodo.7311356.svg).

Table S4. List of taxa included in the phylogenetic analysis of Herpotrichiellaceae (Chaetothyriales) and their NCBI accessions (see separate external Excel file: https://zenodo.org/badge/DOI/10.5281/zenodo.7311356.svg).

CHAPTER 3

Developing mycobiont-specific blocking primers for metabarcoding studies of lichen associated fungi

Abstract

Lichens are complex organisms which host diverse fungal communities (i.e. the lichen mycobiomes) of species closely related to taxa with different lifestyle and ecological strategies. To date, High-Throughput Sequencing (HTS) has become a powerful approach to characterize the mycobiome diversity in lichen thalli. However, the most abundant DNA extracted form a lichen thallus is that of the lichen mycobiont, and this likely affect the amplification and the sequencing depth of all the other fungi of the mycobiome present in a much less amount. This can lead to misestimation of the fungal community richness and composition in the thalli. The use of blocking primers which would reduce specifically the amplification of the non-target mycobiont DNA could overcome this problem. This experimental work aimed at developing and testing two sets of blocking primers specifically designed for the two mycobiont species *Rhizoplaca melanophthalma* Leuckert & Poelt and *Tephromela atra* (Huds.) Hafellner var. *atra*. The experimental setup is presented and the suitability and the application of the blocking primers on lichen samples is discussed.

Keywords

Metabarcoding, blocking, mycobiome, diversity.

Introduction

High Throughput Sequencing (HTS) technologies have provided unprecedented insights into fungal diversity and ecology (Garlapati et al. 2019; Nilsson et al. 2019). In this context, many studies have recently focused their investigation on the diversity of lichen-associated fungi (lichen mycobiome) using a culture-independent approach that allows to catch the highest fungal diversity as possible (U'Ren et al. 2012, 2014; Fernández-Mendoza et al. 2017; Banchi et al. 2018; Smith et al. 2020, Yang et al. 2022). These studies have highlighted that the main limit to applying a HTS methods to investigate the lichen mycobiome is represented by the predominant presence of the lichen-forming fungus, i.e. the lichen mycobiont, which is amplified during the library construction. Indeed, Fernández-Mendoza et al. (2017) and Banchi et al. (2018) showed that up to 99% of the reads obtained by HTS of the environmental DNA extracted from lichen thalli belonged to the

mycobionts. The consequence is that the sampling depth of the lichen mycobiome seems to be negatively affected, potentially leading to a loss of information and a misinterpretation of the whole fungal diversity of the lichen mycobiome. To avoid this problem a cost-effective strategy consists in the exclusion or reduction of the mycobiont DNA during the PCR amplification. There are different approaches to reduce the amplification of the non-target DNA in the environmental samples. One method is to remove the non-target DNA before or after the PCR amplification using restriction enzyme (Blankenship and Yayanos 2005). A second approach consists in the use of non-target specific primers or probes to reduce or suppress PCR amplification of the mycobiont (e.g. "blocking PCR" or "PCR-clamping"; Egholm et al. 1993; Ørum et al. 1993; Karkare and Bhatnagar 2006; Vestheim and Jarman 2008) or the use of primers with a suboptimal annealing temperature to non-target DNA (Easterday et al. 2005).

Blocking primers are synthesized in the same way as conventional amplification primers but are *i)* highly specific to the non-target DNA to be blocked and *ii)* modified with the addition of a C3-CPG spacer (3 hydrocarbons) at 3'-end which inhibits the enzymatic elongation of the primer and consequently reduce the non-target DNA amplifications. Furthermore, blocking primers can be modified at 3'-end adding a phosphate group, a phosphate ester or using 3'-3' linkage (Liles et al. 2003). However, in comparison to C3-CPG spacer, these last methods are not so effective due to side reaction during the deprotection of the oligonucleotide (Cradic et al. 2004; Dames et al. 2007). Blocking primers have been already successfully applied in many different systems, such as to study the presence of parasite DNA in blood samples (Bensch et al. 2004), the rare mutations in clinical samples (Sidransky 1997), for food DNA in dietary samples (Deagle et al. 2005) or to detect rare bacterial sequences in environmental samples (Wintzingerode et al. 1997; Leray et al. 2013; Piñol et al. 2015).

In this study – within the broader context of the project investigating the mycobiome diversity associated to two lichen species collected worldwide, i.e. *Rhizoplaca melanophthalma* Leuckert & Poelt and *Tephromela atra* – we developed and tested a few blocking primer pairs to be potentially used in a DNA metabarcoding study to block the amplification of the lichen mycobiont.

**Material and methods**

Material used

Two lichen species, *Rhizoplaca melanophthalma* and *Tephromela atra*, were chosen as study systems. *R. melanophthalma* is characterized by an umbilicate thallus (attached at a single point), whereas *T. atra* builds a crustose thallus composed of adjacent areoles. Lichen thalli of both species were collected in different localities along their ecological and geographical distributional ranges

and at different elevations (from 500 m up to 5100 m a.s.l.) worldwide.

The blocking primers were tested on the DNA of six specimens for each lichen species and on axenic culture of the mycobiont *R. melanophthalma* and *T. atra*, which were already available in our laboratory. For each lichen species two individuals, each from three populations were selected: the samples of *R. melanophthalma* came from Argentina (Provincial of Mendoza, samples L2383 and L2383), Europe (Spain, samples L2587 and L2588) and North America (Utah, samples L2668 and L2669); those of *T. atra* came from Chile (Region de Aysén, samples L2545 and L2546), Europe (Spain, samples L2571 and L2576) and Australia (Tasmania, samples L2596 and L2598).

Design of blocking primer

Three blocking primers specific to *R. melanophthalma* and *T. atra* sequences were designed: one blocking primer forward and one reverse specific for *R. melanophthalma*, and a single forward blocking primer for *T. atra*. Blocking primers were designed using an alignment of 150 sequences of *R. melanophthalma* and another of 76 sequences of *T. atra*. The alignments consisted of both newly generated sequences obtained from selected samples of several collected populations and sequences retrieved from GenBank. Blocking primers were designed at the site next to the binding site of the universal primer for the ITS2 region, i.e. the forward primers ITS-u2 (Cheng et al. 2016) or ITS3 (White et al. 1990) and the reverse primer ITS4f (Banchi et al. 2020). This site is particularly conserved in either lichen mycobionts, and any match with mycobiont species closely related to our two targets, i.e. *R. melanophthalma* and *T. atra*, was also avoided (Fig. 1, Table 1). The designed blocking primer are characterized by a C3 spacer at 3'-end and an unspecific oligonucleotide at 5'-end.
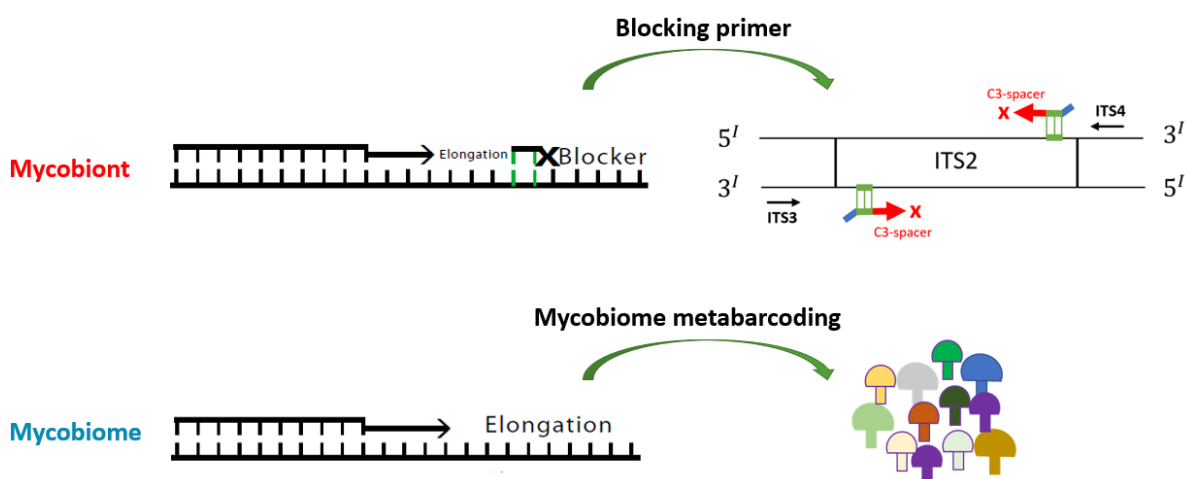
Figure 1. Schematic illustration of the action mechanism of blocking primers. Blocking non-target amplification, only target sequences amplified using universal PCR primers. The blocker is a modified DNA oligonucleotide that does not

prime amplification: it binds specifically to the non-target DNA (green) and it is characterized by C3 spacer at 3'-end (red) and an unspecific oligonucleotide at 5'-end (blue).

Table 1. List of the primers used in the blocking primers PCR test. The additional unspecific oligonucleotides at 5'-end of the blocking primers are written in bold blue.

Primers	Oligonucleotide sequence (5'→3')	References
Blocking primer forward *R. melanophthalma*	**CCATC**YAGCTTGGTATTGGGTCTTCG	this study
Blocking primer reverse *R. melanophthalma*	**GAGC**TTGAAAGATTTGGGCRTTKA	this study
Blocking primer forward *T. atra*	**TTTAC**CGAAAAGYAGTGGCGGTCCG	this study
Universal primer forward ITS-U2	GAAYCATCGARTCTTTGAACGC	Cheng et al. (2016)
Universal primer forward ITS3	CATCGATGAAGAACGCAGC	White et al. (1990)
Universal primer reverse ITS4f	CCGCTTATTGATATGCTTAAG	Banchi et al. (2020)
Universal primer forward ITS1F	CTTGGTCATTTAGAGGAAGTAA	Bruns and Gardes (1993)
Universal primer reverse ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)

Molecular analyses: DNA extraction, PCR amplification

The thallus DNA extractions were performed using approximately 2 mm^2 fragments of lobes and areolas of *R. melanophthalma* and *T. atra*, respectively. The fragments were washed three times for 15 minutes with sterile water, followed by 30 minutes of washing with 500 μl of Tween80 diluted 1:10. A final washing step was performed rinsing the thallus fragments three times for 15 minutes with sterile water. Moreover, small parts of the cultured mycobionts colonies were taken with a sterile inoculation loop and transferred into 1.5 ml reaction tubes, containing three sterile tungsten beads for homogenization, frozen and ground using a TissueLyserII (Retsch). The DNA extraction followed the C-TAB protocol (Cubero et al. 1999) with small adjustment.

The efficiency of the blocking primers was tested on the DNA extracted both from the thalli and the cultured mycobionts with real-time PCR (CFX 96™ PCR System; Bio-Rad) analysis. Blocking primers were tested in addition to the universal primer pairs ITS-u2 (Cheng et al. 2016) **/** ITS4f (Banchi et al. 2020) and ITS3 (White et al. 1990) / ITS4f (Banchi et al. 2020), that amplify the nuclear internal transcribed spacer 2 region (nucITS2), and ITS1F (Bruns and Gardes 1993) / ITS4 (White et al. 1990) that amplify the entire nuclear internal transcribed spacers region (nucITS1, the 5.8S rDNA ribosomal gene and nucITS2; Table 1). Moreover, we tried to use a ratio of blocking primers/ universal primers in a value of 20:1 and 10:1 and three different annealing temperatures (i.e. 62 °C, 58 °C and 55 °C; Table 2). All the experiments were performed using as positive controls PCR reactions containing only the universal primers and comparing the results with the PCR amplifications obtained with the supplementary blocking primers. A negative control was also included to verify the absence of non-specific amplification products.

Table 2. Summary of the universal primer pairs and the PCR condition tested with the blocking primers.

Universal primers pairs	ITS-u2 / ITS4
	ITS3 / ITS4f
	ITS1F / ITS4
Blocking primers: universal primers ratio	20:1
	10:1
Annealing temperature	62 °C
	58 °C
	55 °C

Library preparation and sequencing

After determining the best blocking primers conditions, we carried on the library preparation. Amplicons for HTS sequencing were obtained with two sequentially PCRs: the first PCR (primary PCR) allows the amplification of the DNA; the second PCR is necessary to attach the barcode for the Ion Torrent sequencing. We used as universal primer ITS-u2 (Cheng et al. 2016) and ITS4f (Banchi et al. 2020) to promote the amplifications of both basidiomycetes and ascomycetes of the lichen mycobiomes. We used a ratio 20:1 of blocking primers: universal primer pairs. The first PCR reaction contained 2 μL DNA template (about 3 ng DNA), 12.5 μl AccuStart II PCR ToughMix, 1 μl EvaGreen™ 20× (Biotium), 0.4 μl forward primer ITS-u2 (10 μM), 0.4 μl reverse primer ITS4f_R (10 μM) and 1.6 μl of blocking primer (50 μM) in a final volume of 20 μl. As control, to compare the efficiency of the blocking primers, an unblocked DNA template obtained by the amplification with only the universal primers (i.e., without the addition of the blocking primer to the PCR mix) was used. The PCR amplifications were performed in a CFX 96™ PCR System (Bio-Rad) with the following cycling profile: 94 °C for 3 min and 35 cycles at 94 °C for 10 sec, 60 °C for 30 sec and 72 °C for 30 sec. The reaction mix of the second PCR contained 1 μl of the first PCR product, 12.5 μl of AccuStart II PCR ToughMix, 0.75 μL of TrP1 adaptor, 1 μl EvaGreen™ 20× (Biotium) and 0.75 μL of the barcode in a final volume of 25 μl. The PCR amplifications were performed in a CFX 96™ PCR System (Bio-Rad) with the following cycling profile: 94 °C for 3 min and 12 cycles at 94 °C for 10 sec, 60 °C for 10 sec and 72 °C for 30 sec. The quality of the amplicons was checked in a 2% agarose gel stained with Green Safe Gel. After that, the PCR products were purified with Mag-Bind® Normalizer Kit (Omega Biotek) and their concentration was measured with Qubit™ Fluorimeter. All 24 amplicons were pooled together in equimolar concertation (4 ng/ μL) and sequenced with an in-house Ion Torrent Personal Genome Machine™ (PGM, Thermo Fisher Scientific) using a 314™ chip (Thermo Fisher Scientific) for a maximum read length of 400 bp.

Bioinformatic analyses and statistics

CLC Genomics Workbench v.12 (Qiagen) was used to trim (removal of primer and adapter sequence parts), and quality filter (minimum length 200 bp, minimum average quality score 20) the raw reads. The operational taxonomic units (OTUs) were obtained by clustering at 97% similarity the reads. Taxonomy was assigned to OTUs in Microbial Genomics module (CLC Genomics Workbench v.12; Qiagen) using UNITE as reference database (Kõljalg et al. 2013). BLAST identity search was applied in the case UNITE did not succeed in the taxonomic assignment (N/A).

To estimate the differences in the amplification with and without blocking primers we calculated for each sample the fractions of the OTUs abundance belonging to the mycobiont on the total of the OTUs abundances. The two distributions were compared in Prism v. 5 using a non-parametric paired Wilcoxon statistical test.

Results and discussion

Blocking primer development

We designed two blocking primers for *R. melanophthalma* (one forward and one reverse), and only one blocking primer forward for *T. atra.* Indeed, we identified two conserved regions as potential target for the blocking primers for *R. melanophthalma* (Figs. 2a, b). Instead, in *T. atra* the final part of ITS2 region was more variable and prevented the design of the specific reverse blocking primer (Fig. 2c).
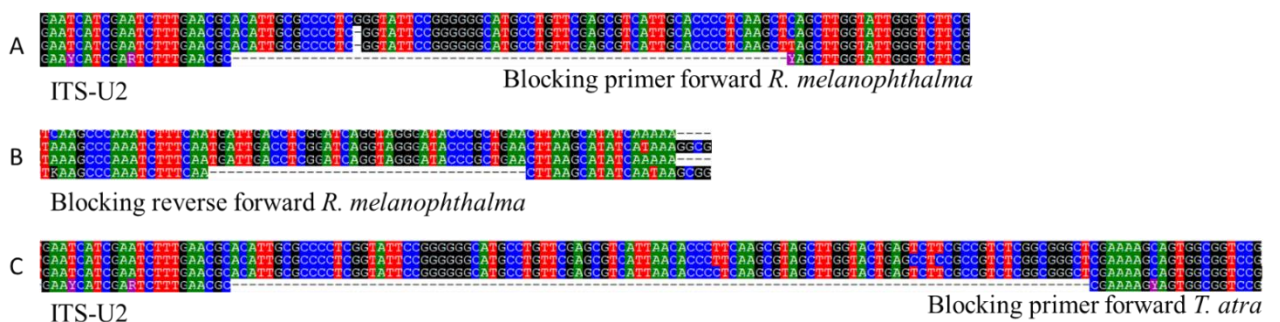

Figure 2. Sequences of *R. melanophthalma* (A, B) and *T. atra* (C) aligned with the respective specific blocking primers (only three representative sequences of the alignments of either mycobiont are shown).

Blocking primer performance

We did not observe any differences in the PCR profile of the amplification with and without blocking primers, either when we tested the blocking primers with the three different universal primer pairs (ITS-u2/ITS4f; ITS3/ITS4f; ITS1F/ITS4), or in cases when blocking primers/universal primers were added in a ratio 20:1 and 10:1 and using different annealing temperatures.

Furthermore, the melting curves of the amplicons (with and without blocking primers) were the same in the amplification with and without blocking primers, of both the cultured mycobionts and the lichen thalli of *R. melanophthalma* and *T. atra*. Because of this, we decided to use for the library preparation the universal primers ITS-u2 (Cheng et al. 2016) and ITS4f (Banchi et al. 2020) that amplify the ITS2 region. This region is usually used as target for fungal characterization because of its hight interspecific variability and conserved primer sites, being part of the established fungal barcode region (Schoch et al. 2012; Blaalid et al. 2013). No differences in the amplicon fluoresces was observed in the agarose gel stained with Green Safe Gel (Figs. 3, 4). In particular, the amplifications of the thalli generated three (in the sample L2382) or two amplicons (in all the other samples but L2382), with different lengths (as visible in the agarose gel in Fig. 4), the lower bands were about 270 bp long and the upper bands about 320 bp.
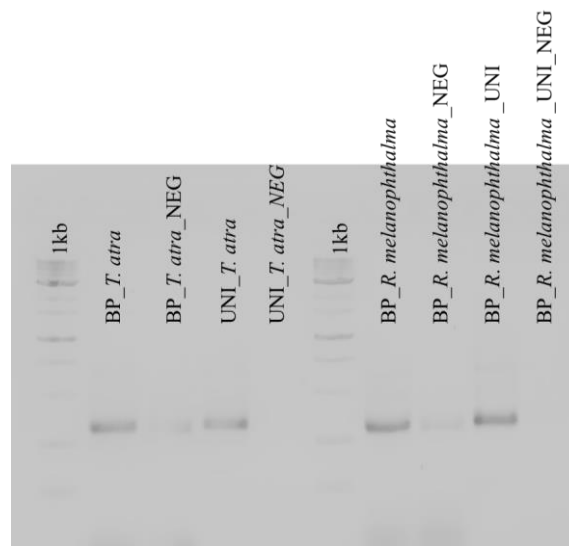
Figure 3. Agarose gel strained with Green Safe Gel of the amplification with (BP) and without (UNI) blocking primers of the cultured mycobionts *R. melanophthalma* and *T. atra*. 'NEG' is the negative control. The universal primer pair ITSU2/ITS4 was used for amplification. 1KB is the DNA ladder.
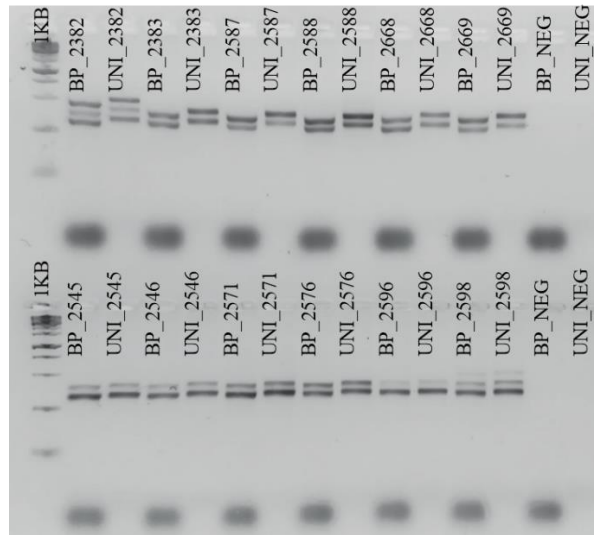
Figure 4. Agarose gel strained with Green Safe Gel of the amplification with (BP) and without (UNI) blocking primers. Upper row: samples L2382, L2383, L2587, L2588, L2668 and L2669 are *R. melanophthalma*; lower row: samples L2545, L2546, L2571, L2576, L2596 and L2598 are *T. atra*. 'NEG' is the negative control. The universal primer pair ITSU2/ITS4 was used for amplification. 1KB is the DNA ladder.

DNA sequencing and data analysis

A total of 197,613 raw reads were generated. 183,540 reads were retained after the quality filtering, and they had an average length of 266 bp. 1,997 OTUs were identified after denoising and singletons exclusion. Most of the reads of both lichen species belong to their mycobionts (46% for *R. melanophthalma* and 77% for *T. atra*) and to their major photobionts, i.e. *Trebouxia* species (48% in *R. melanophthalma* and 19% for *T. atra*). Indeed, since the universal primers we used to amplify the ITS2 region were designed on a conserved region of the fungal DNA that differ for one or two nucleotides from the photobiont sequences, the unspecific match and the amplification of the photobiont fraction in lichens was expected (Beiggi and Piercey-Normore 2007). As further confirmation of this, the reads with two different lengths (visible in the agarose gel of the ITS2 amplification from the lichen thalli, Fig. 4) were separated bioinformatically: the reads with an average length of 265 bp mostly belong to the fungal fractions of *R. melanophthalma* and *T. atra* and those with an average length of 312 bp belong to different species of *Trebouxia*.

The statistical analysis confirmed the results previously obtain by the PCR profile experiments: the amplifications with the blocking primers failed and no statistical differences with the amplification with only the universal primers are evident (Fig. 5). This could be due to the inability of the blocking primers to bind the mycobiont sequences: it may happen that the unspecific oligonucleotides added at the 5'-end of the blocking primers prevent the binding with the mycobiont sequences because of a possible steric hindrance of the unspecific oligonucleotides
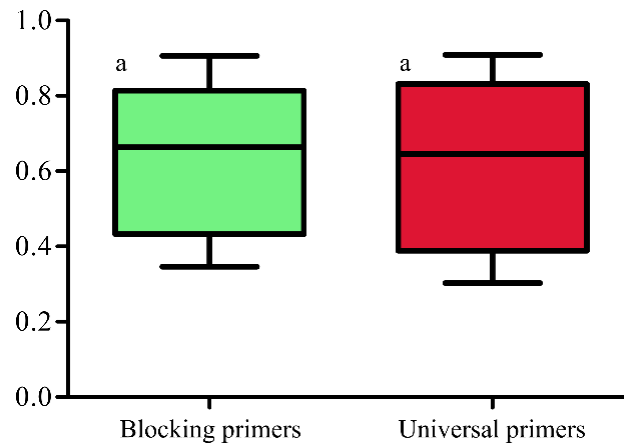
Figure 5. Boxplot of the mycobionts fractions obtained by the amplification with (green) and without (red) blocking primers. Values followed by the same letter are not statistically different according to Wilcoxon test.

Conclusions

We could not develop successful blocking primers to be used in the subsequently planned metabarcoding experiments, but we are confident that additional trials, such as testing several sets of blocking primers on different fugal genetic markers are worthy and would lead to the development of this approach also for lichens. This approach indeed would critically ease the study of species diversity of lichen mycobiomes and lead to a more reliable estimation and identification of the fungal taxa associated to lichens.

Acknowledgments

CHAPTER 4

Largely transient mycobiomes shape fungal diversity in two globally distributed lichens

Abstract

Lichens are multi-kingdom symbioses in which fungi, algae and bacteria interact to develop a stable selection unit. In addition to the mycobiont forming the symbiosis, fungal communities associated with lichens represent the lichen mycobiome, but their taxonomic and functional diversity across the range of their host lichens is still largely unknown. We aim to characterize the diversity of the lichen mycobiome in two cosmopolitan lichens, i.e. *Rhizoplaca melanophthalma* Leuckert & Poelt and *Tephromela atra* (Huds.) Hafellner var. *atra* as study models/references. Their mycobiomes were investigated across their entire geographic range using both a culture-dependent approach and environmental DNA. The variation of the mycobiomes associated with the two lichen species is extremely high, and a stable species-specific core mycobiome is hardly identifiable. Most of the mycobiome taxa are present in low frequency of occurrence. No taxon is ubiquitously present in neither lichen species. The mycobiomes of *R. melanophthalma* and *T. atra* are thus composed of transient lichenicolous fungi. A fraction of these mycobiomes is also generalist, as it associates with both lichens. A fraction of the two lichen mycobiomes is detectable only by the culture-dependent approach and escapes PCR amplification. Lichen structures rather are reservoirs of other fungi than representing their functional partners.

Keywords

Ascomycota, Basidiomycota, community, DNA metabarcoding, ecology, symbiosis.

Introduction

Symbioses are self-sustaining interactions between organisms of different kingdoms, known as symbionts, which result in new structures and metabolic activities (De Bary 1878; Frank 1887; Douglas 1994). The symbionts interact to develop a stable unit of selection in which they are interlinked by a mutualistic-antagonistic continuum of relationships, thus resulting in a superorganism termed 'holobiont' (Margulis and Fester 1991; Douglas and Werren 2016). The holobiont is determined by the availability of the different partners and their specialization in environmental conditions (Rosenberg et al. 2010; Rosenberg and Zilber-Rosenberg 2011; Rafferty et al. 2015; Douglas and Werren 2016; Chomicki and Renner 2017).

The term symbiosis was originally introduced to study lichens (Frank 1887) which are, among the terrestrial symbioses, iconic examples of the living together of the main fungus (the mycobiont) and populations of photosynthetic green algae or cyanobacteria (the photobionts; Hawksworth and Honegger 1994; Honegger 2009). These two partners shape the phenotypic outcome of the symbiosis, i.e., the lichen thallus. The lichen thallus is indeed a holobiont, because it contains a multitude of associated microorganisms as well, such as prokaryotes, microalgae and microfungi (e.g., Arnold et al. 2009; Grube et al. 2009; Aschenbrenner et al. 2017; Moya et al. 2017; Muggia and Grube 2018; Spribille 2018; Hawksworth and Grube 2020). All lichen-associated microorganisms potentially contribute to the symbiotic outcome on its whole, being acknowledged as the microbiome (bacteria; Grube et al. 2009), the mycobiome (fungi; Fernández-Mendoza et al. 2017) and the phycobiome (green microalgae; Barreno et al. 2022); however, their functional and taxonomic diversity is still largely unknown (Spribille 2018; Hawksworth and Grube 2020).

In particular, the lichen mycobiome (Fernández-Mendoza et al. 2017; Banchi et al. 2018; Muggia and Grube 2018), is represented by microfungi which seem to develop diverse trophic relationships with the main lichen symbionts. They can be parasitic, commensals, or saprotrophic either on the lichen mycobiont or photobionts (Lawrey and Diederich 2003). These fungi have been discovered in the early 19th century and have been traditionally referred "lichenicolous fungi" for over a century. They were – and still are – recognized by infection or fertile structures on the thalli (Lawrey and Diederich 2003; Diederich et al. 2018). Some years ago, however, also the presence of fungi cryptically occurring in lichen thalli was highlighted (e.g., Harutyunyan et al. 2008; Arnold et al. 2009; Muggia et al. 2016). In analogy with endophytism, these taxa became known as "endolichenic fungi" (Arnold et al. 2009; U'Ren et al. 2010; Muggia et al. 2016). Owing to ambiguities in distinguishing the two groups of fungi, as later studies shown that also "lichenicolous fungi" can occur cryptically (Fernández-Mendoza et al. 2017; Banchi et al. 2018), Hafellner (2018) proposed a re-definition of lichenicolous fungi as "all lichen-inhabiting fungi, both non-lichenized and lichenized, either obligate or facultative, with a colonization inducing symptoms on the host or not." He recognized three subgroups of fungi: 1) lichenicolous fungi s.str. (living exclusively on lichens; about 1800 described species; Diederich et al. 2018); 2) endolichenic fungi (endophytes of lichens, resulting from primary non-lichenized lineages of Dothideomycetes, Eurotiomycetes, Leotiomycetes and Sordariomycetes; Arnold et al. 2009; Muggia et al. 2016, 2019, 2021), which are still little known, they are hardly detectable with standard microscopic techniques and their study requires molecular and culture-dependent approaches; 3) lichen epiphytes, fungi usually lichenized that grow on the lichen thalli (Hafellner 2018).

While lichenicolous fungi s.str. *sensu* Hafellner (2018) were studied more easily based on their morphological traits, the diversity of the endolichenic fungi *sensu* Hafellner (2018) has been uncovered recently and the evolutionary origins were studied only for a few members (Ertz et al. 2009, 2016; Muggia et al. 2016, 2019, 2021). Most of the lichenicolous fungi s.str. seem to be phylogenetic distantly related to the lichen mycobionts (Lutzoni and Miadlikowska 2009) and taxa emerge as new lineages in the fungal tree of life (e.g., Muggia et al. 2021). Arnold et al. (2009) proposed that they could have a key role in the evolution of endophytism and be an important evolutionary link with plant-associated endophytes (U'Ren et al. 2010, 2012, 2014; Fleischhacker et al. 2015; Chagnon et al. 2016; Muggia et al. 2016). The analogies with the plant endophytes are the absence of infection symptoms on lichen thalli, and the biosynthesis of many secondary metabolites (Kellogg and Raja 2017). However, it is still uncertain whether the lichenicolous fungi in general preferentially associate with the algal photobiont rather than the mycobiont (Arnold et al. 2009; Muggia and Grube 2018) and if they specialize on the lichen phenotype.

Morphological microscopic analyses and culture isolations were the main approaches to study the diversity of the lichen mycobiome (Petrini et al. 1990; Crittenden et al. 1995; Girlanda et al. 1997; Lawrey and Diederich 2003; Lawrey et al. 2007; Muggia et al. 2017; Diederich et al. 2018) before the introduction of molecular methods to fungal diversity studies. However, several attempts are required to isolate and grow any lichenicolous fungus in culture, while their inconspicuous, microscopic mycelium hamper their identification inside the lichen thallus. Still, culture isolations are essential for the morphological characterization of taxa, especially when new species are discovered (Muggia et al. 2021). In this context, isolated strains represent only a minor part of the whole lichen mycobiome. The underestimation of the fungal diversity in lichens, obtained by the culture-dependent approach, derives from the fact that many fungi will never grow outside the thalli in axenic conditions (U'Ren et al. 2014; Muggia and Grube 2018; Wijayawardene et al. 2021).

High-throughput sequencing (HTS) technologies data complement the results of culture-dependent approaches and help to uncover more comprehensively the diversity of the lichen associated fungi (Muggia et al. 2020). The understanding of lichen mycobiomes has already benefited from HTS technology already at different scales (Bates et al. 2012; Zhang et al. 2015; Fernández-Mendoza et al. 2017; Banchi et al. 2018; Smith et al. 2020; Yang et al. 2022), and identified three major ecological fractions of the lichenicolous fungi, which in part overlap with the subgroups proposed by Hafellner (2018). These are: i) a "generalist environmental pool" of fungi unspecific to the lichen host; ii) a "lichenicolous/endolichenic pool" that grow and complete their life cycle specifically on their lichen host; iii) a pool of "transient species" likely represented by

fungal propagules or extraneous fungi that do not have a precise ecological role in the lichen thalli (Fernández-Mendoza et al. 2017). Lichen mycobiomes thus are represented by diverse lineages of Ascomycota and Basidiomycota, and both filamentous and yeast species are here recognized. The major representatives belong to the big ascomycetes classes Dothideomycetes, Eurotiomycetes, Leotiomycetes, Sordariomycetes and Lecanoromycetes (Diederich et al. 2018; Muggia and Grube 2018), and in basidiomycetes, Agaricomycetes (Lawrey et al. 2007), Tremellomycetes (Millanes et al. 2011; Tuovinen et al. 2019; Cometto et al. 2022) and Cystobasidiomycetes (Spribille et al. 2016; Černajová and Škaloud 2019, 2020; Cometto et al. 2022). In particular Eurotiomycetes and Dothideomycetes are frequently detected and isolated from rock-inhabiting lichens and are close relatives of melanized rock-inhabiting fungi (RIF, Sterflinger and Krumbein 1995; Wollenzien et al. 1995), plant pathogens and opportunistic fungi (Harutyunyan et al. 2008; Muggia et al. 2016, 2019, 2021; Muggia and Grube 2018; Quan et al. 2020). Leotiomycetes and Sordariomycetes taxa, instead, have been identified from soil- and bark-inhabiting lichens collected in temperate, humid, Antarctic and boreal environments (Arnold et al. 2009; U'ren et al. 2010, 2012), and are represented mainly by lineages closely related to plant endophytes (Arnold et al. 2009; U'Ren et al. 2010; Yu et al. 2018). No taxon is so far known from the classes Arthoniomycetes and Lichinomycetes. Basidiomycetes yeasts are represented by tremelloid (Fernández-Mendoza et al. 2017) and cystobasidioid taxa (Spribille et al. 2016) from lichens growing in alpine, subalpine and boreal habitats. Furthermore, Smith et al. (2020) suggested that also the lichen growth forms build diverse microhabitats and acts as selecting factor of the mycobiome composition.

The present study aims to characterize the range of diversity of lichen mycobiomes to understand the main drivers at a more global scale. We achieve this aim by focusing on two cosmopolitan lichen species, i.e., *Rhizoplaca melanophthalma* Leuckert & Poelt and *Tephromela atra* (Huds.) Hafellner var. *atra* as study models/references. Specific goals are to i) understand if the two target species host significantly different mycobiomes according to their geographic origin, ii) characterize if any 'core mycobiome' can be detected and if it is stable and specific to either lichen species, iii) identify the mycobiome fraction which can be isolated in culture and is also detected by metabarcoding analyses, and which fraction is instead only detected by one approach.

Material and methods

Sampling

The lichens *Rhizoplaca melanophthalma* and *Tephromela atra* were selected as study organisms (Fig. 1). *R. melanophthalma* has an umbilicate thallus (attached at a single point), whereas *T. atra* builds a crustose thallus composed of adjacent areoles (Muggia et al. 2008, 2010, 2014a; Leavitt et

al. 2011, 2016a). Lichen samples were collected in the period 2019-2021 in 56 different localities at an altitude ranging from 350 to 5100 m above sea level (a.s.l.) and on different rock types, such as acidic, granitic, schist-sandstonesandstone, siliceous and calcareous rocks (Supplementary Table S1). The sampling was performed in boreal, alpine, temperate, humid and arid habitats in Europe (Italy, Croatia and Spain), North America (Utah, Nevada and Idaho), South America (Argentina and Chile), Mauritius and Tasmania (Fig. 1). Both lichen species were found together in nine localities (two in Spain and seven in Italy; referred as restricted dataset); in total 34 populations of *R. melanophthalma* and 31 populations of *T. atra* were analyzed (referred as complete dataset). All the lichen samples were deposited at the herbarium of the University of Trieste (TSB).
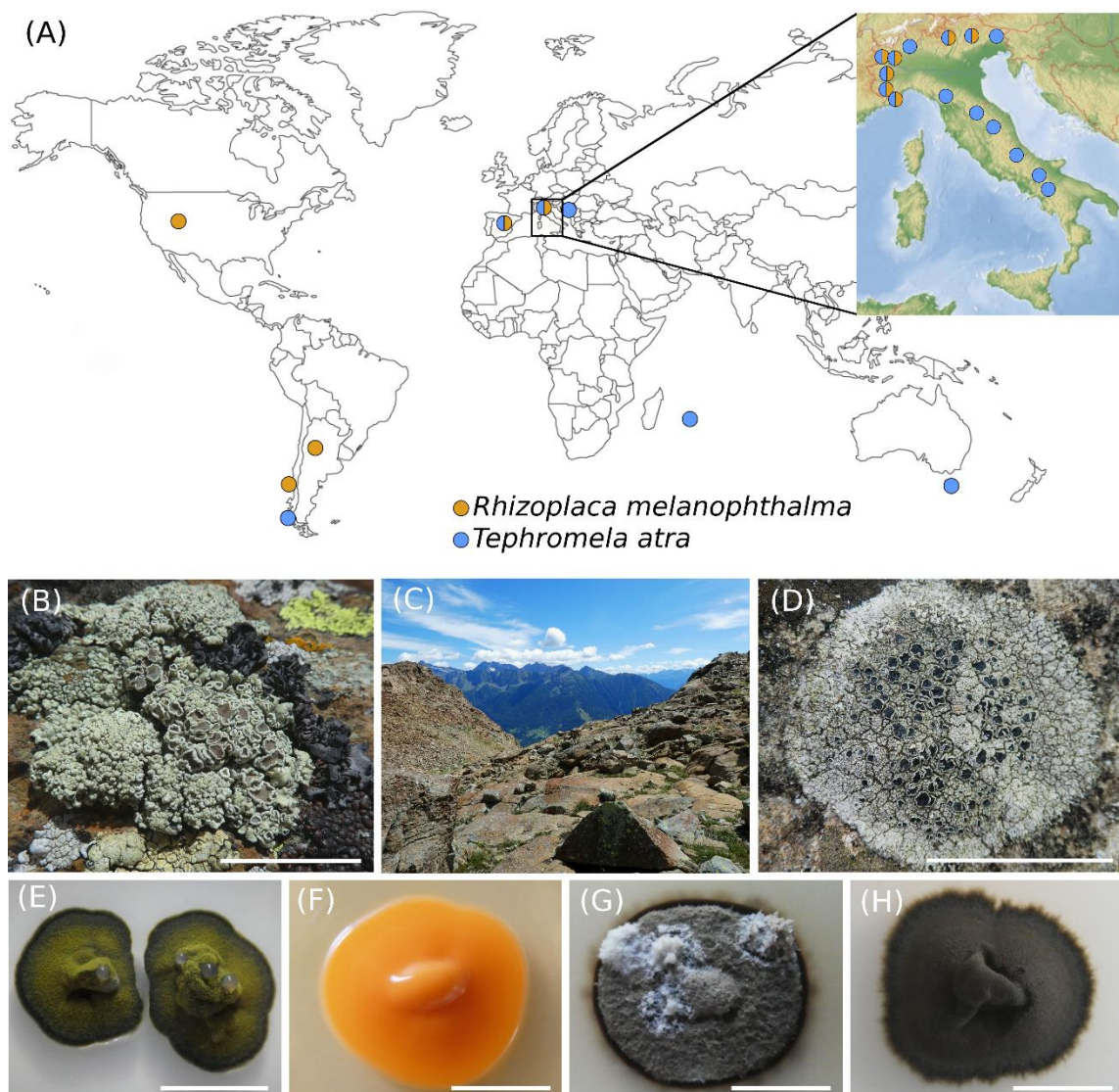
Figure 1. Map of the localities from which *Rhizoplaca melanophthalma* and *Tephromela atra* were collected (A). *R. melanophthalma* collected in the Alps (Italy) (B), collecting localities at Laghetti Sassersa in Italian Alps (Valtellina, Lombardy; Italy) (C), *T. atra* collected in the Apennines (D). Colony shape of Mycosphaerellales sp. L3082 (E) and

Microsporomycetaceae sp. L2343 (F) isolated from *R. melanophthalma*, Coniochaetales sp. L3093 (G) and Chaetothyriales sp. L3059 (H) isolated from *T. atra*. Scale bars: 2 cm (B-D), 1 cm (E-H).

Preparation of lichen materials and DNA extraction

Up to ten (when available) individuals for each population of *R. melanophthalma* and *T. atra* were selected for the molecular analysis. Each lichen thallus was physically cleaned from soil/rock debris and part of it was removed with a sterile razor blade, transferred to 1.5 ml reaction tubes and surface sterilized. For *R. melanophthalma* one marginal lobe and one apothecium were taken, while for *T. atra* one marginal areole and one apothecium. The fragments were washed three times for 15 minutes with sterile water, followed by 30 minutes of washing with 500 µl of a 1:10 dilution of Tween80, and a final washing step of 15 minutes for three times with sterile water. The cleaned samples were let dry under laminar hood and stored at -20 °C until processed for DNA extraction. The DNA extraction was performed following the CTAB protocol of Cubero et al. (1999), with minor adjustment. DNA extractions (~ 50 ng) of ten individuals belonging to the same population were pooled together to represent eventually a single sample in the molecular analyses.

Preparation of mock communities

To better evaluate the sensitivity of our metabarcoding approach two mock communities were artificially created. The two mock communities were prepared using the DNA extracted from fungi and algae that were isolated in axenic culture from the thalli of *R. melanophthalma* and *T. atra* included in the molecular analysis. These fungal and algal strains comprised the two *R. melanophthalma* and *T. atra* mycobionts, the *Trebouxia* spp. photobionts isolated from their thalli (see De Carolis et al. 2022) and other fungal strains (filamentous fungi and yeasts; Figs. 1e-h) which were described in recently published contributions. They were previously identified by Sanger sequencing of their nuclear ribosomal internal transcribed spacer (ITS) and ribosomal large subunit (LSU) genes and by morphological analysis (Cometto et al. 2022; De Carolis et al. 2022). These fungal strains belonged to 11 classes in Ascomycota and Basidiomycota, i.e. Eurotiomycetes, Dothideomycetes, Sordariomycetes, Leotiomycetes, Lecanoromycetes, Taphrinomycetes, Tremellomycetes, Cystobasidiomycetes, Agaricostilbomycetes, Ustilaginomycetes and Microbotryomycetes. The mock community n. 1 was composed of 36 strains isolated from thalli of *R. melanophthalma*, while the mock community n. 2 included 27 strains isolated from thalli of *T. atra.* Either mock community was composed of the mycobiont DNA at the concentration of 5 ng/µl, the photobiont DNA at the concentration of 2.5 ng/µl and the DNA of the other fungi at three different concentrations: 0.5 ng/µl, 0.05 ng/µl and 0.005 ng/µl.

DNA amplification, library construction and sequencing

The fungal ITS2 region was amplified with the forward primer ITS3 (White et al. 1990) and the reverse primer ITS-f4 (Banchi et al. 2020) which were modified at 5'-end with Illumina tails. The annealing temperature was tested using a gradient annealing temperature PCR. Two PCR amplifications were performed to obtain the amplicons for HTS: the first PCR amplifies the target sequence using the universal primers; the second PCR is carried out to attach the dual index i5 and i7 (Glenn et al. 2019a, 2019b) to multiplex samples in the same MiSeq run. The first PCR was performed in triplicates of 15 µl reaction volumes containing 2 µl of DNA (~ 10-20 ng), 7.5 µl of AccuStart II PCR ToughMix, 0.75 µl of EvaGreen™ 20× (Biotium), 0.5 µl forward primer ITS3 (10 µM) and 0.5 µl reverse primer ITSf4 (10 µM). Amplifications were performed with CFX 96™ PCR System (Bio-Rad), stopping the reactions when the amplification reached the exponential phase, under the following conditions: 94 °C for 3 min and about 15 cycles at 94 °C for 20 sec, 54 °C for 30 sec and 72 °C for 30 sec. A negative control was also amplified for about 15 cycles more than the other samples and kept until the sequencing step. The three technical replicates of each sample were pooled together and purified using Mag-Bind® Normalizer Kit (Omega bio-tek). All amplicons were checked for their quality and size by 1.5% agarose gel electrophoresis stained with Green Safe Gel. The second PCR was performed at a final volume of 25 µl containing 2 µl of the first PCR products, 12.5 µl of AccuStart II PCR ToughMix, 1 µl of EvaGreen™ 20× (Biotium), 0.5 µl of each indexed primer (10 µM). The PCR were run under the following conditions: 94 °C for 2 min and 6 cycles at 94 °C for 50 sec, 60 °C for 20 sec and 72 °C for 30 sec. All samples were quantified with Qubit™ Fluorimeter (Thermo Fisher Scientific) and pooled together in equimolar amounts. The final library was purified with Mag-Bind® Normalizer Kit (Omega Bio-tek, Norcross) and checked for its quality by 1.5% agarose gel electrophoresis stained with Green Safe Gel. Amplicons libraries were sequenced with Illumina MiSeq for a maximum read length of 2 × 300 pb (BMR Genomics, Padua, Italy).

Bioinformatic analyses and statistics

Raw Illumina paired-end reads (2 x 300 bp) were demultiplexed, quality checked with FastQC (Andrews, 2010), trimmed by Trimmomatic (Bolger et al. 2014), denoised and dereplicated to Amplicon Sequence Variants (ASV) using DADA2 (Callahan et al. 2016) within the QIIME2 (Bolyen et al. 2018) environment. Taxonomy was assigned to ASVs using the deepest, not conflicting taxonomy between the one assigned by BLAST® and the machine-learning sklearn approach (compare_qiime_taxonomy_pick_deepest.py). Based on this taxonomy, the table containing the features (ASV) and their abundance was filtered from mycobiont, photobionts and

any other non-fungal taxa in QIIME2. Unassigned fungal ASVs were manually checked to ensure no mycobiont ASVs were retained within the mycobiome ASV table; diversity accumulation curves were also plotted. Krona plot (Ondov et al. 2011) were plotted using the ASV table and its taxonomy using Taxon Table Tools (Macher et al. 2021).

The ASV table was normalized using the median (or the geometric mean) of sequencing depth distribution after discarding the samples with less than 500 reads belonging to the mycobiome; the normalized table was used to calculate alpha diversity indexes (ASV richness, Shannon and Simpson). Alpha diversity distributions (complete dataset) of *R. melanophthalma* and *T. atra* samples were plotted as violin plots and compared using a non-parametric t-test (Wilcoxon test). A further comparison was performed only retaining the nine localities where both lichens were collected (restricted dataset).

Beta diversity was assessed with a non-metric Multi-Dimensional Scaling (NMDS) ordination, which was run on the dissimilarity matrix calculated with the Bray-Curtis metric on the normalized ASV tables. Core taxa belonging to the mycobiome of two lichen species were assessed using the ASVs, 99% and 97% similarity clustered ASVs (identified here as Operational Taxonomic Units, OTUs) at various thresholds of ASV relative abundances (0.001, 0.005, 0.010 and 0.050) and sample fractions (percentage of samples containing that ASV or OTU; i.e. 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7). These analyses were performed both on the complete and the restricted datasets. Analyses were performed in R (R Core Team, 2013, metabarcoding_alpha_beta_diversity_core_taxa.R) using packages phyloseq (McMurdie and Holmes, 2013), vegan (Dixon, 2003) and microbiome (Lahti et al. 2017).

ASVs from the mock communities were compared to the original Sanger sequences from the DNA extractions used to build the mock sample itself. Phylogenetic trees from alignments containing both Sanger sequences and ASVs from metabarcoding were calculated with RAxML (Stamatakis, 2014); taxonomy was assigned to ASVs on the trees to ease the visual comparison (assign_qiime_taxonomy_to_fasta_alignment.py).

The correspondence of the mycobiome diversity detected by metabarcoding and culture-dependent approach was examined by BLAST® (sequence similarity of at least 97%). Sanger sequences from the cultures were dereplicated (100% similarity) by cd-hit (Li and Godzik 2006) to avoid the use of the same sequence from multiple fungal isolates. In doing this, all identical isolates in their ITS2 sequences were assumed to be a single strain. The comparison was performed only for those populations for which fungal isolates were obtained in culture, i.e., 25 populations were compared.

Scripts mentioned in the methods and others used through the analyses are available at (https://github.com/claudioametrano/lichen_mycobiome_tiny_scripts).

Results

DNA sequencing and data analysis

Illumina run generated a total of 16.1 x 10^6 reads. The average reads count per sample was 237,000 (st. dev. 77,000). 15.5 x 10^6 reads passed the quality filter, and the average reads count per sample was 228,000 (st. dev. 74,000). After the quality filtering, assembly, denoising and singletons exclusion 1,430 ASVs were identified. ASVs belonging to the mycobionts, the photobionts and any other non-fungal taxa were filtered out and 980 ASVs were retained: 392 AVSs in *R. melanophthalma* and 646 ASVs in *T. atra* samples.

The ASV accumulation curves were generated separately for *R. melanophthalma* and *T. atra* samples: most of them levelled off and reached the plateau with rather small sampling effort (Supplementary Figs. S1-S3). The mean values of mycobiome sampling effort expressed as ASVs abundance was 3326 (95% CI: 2015-4928) and 4884 (95% CI: 2966-7658) for *R. melanophthalma* and *T. atra*, respectively. The median abundance percentage of mycobiome assigned ASVs was 0.89% and 2.65% for *R. melanophthalma* and *T. atra*, respectively.

Mock communities and negative control

In the *R. melanophthalma* mock community (n. 1) 35 out of 36 taxa were detected: only taxon L2882, which was in the mock community at the lowest concentration (0.005 ng/μl), identified by BLAST (99.50% similarity) as *Thermoascaceae* sp. was not detected among the ASVs (Supplementary Fig. S4, Supplementary Table S2). In the *T. atra* mock community (n. 2) 27 out of 28 taxa were detected by HTS sequencing: only taxon L2875, which was the mock community at the lowest concentration (0.005 ng/μl), identified by BLAST (98.25% similarity) as *Teratosphaeriaceae* sp. was not identified among the ASVs (Supplementary Fig. S5, Supplementary Table S3). In some cases (e.g. L3816; Supplementary Fig. S4) multiple ASVs corresponded to a single Sanger sequence from cultures DNA.

The negative control was clean when checked on RT-PCR; its PCR was then run about 15 cycles longer than any other sample. Though, ASVs corresponding to *Capnodiales* sp., *Coniosporium* sp., *Filobasidium* sp., *Malassezia restricta, Pseudeurotium* sp., *Rhizoplaca,* Teloschistaceae sp., *Trebouxia* sp. and *Vuilleminia pseudocystidiata* were detected. ASVs of *Pseudeurotium* sp.; *Rhizoplaca* and *Trebouxia* sp. were also detected in other samples (*Pseudeurotium* sp. in 4 samples, *Trebouxia* sp. in one sample), while the other ASVs were only present in the negative control.

Taxonomic composition of lichen mycobiome

Taxonomy was assigned to ASVs using BLAST® or the machine-learning sklearn approaches (Supplementary Table S4): 33% ASVs taxonomic assignment was congruent (same exact taxonomy was assigned by the two methods), 16% ASVs taxonomic assignment was congruent with BLAST® achieving a deeper taxonomy, 49% ASVs taxonomic assignment was congruent with sklearn achieving a deeper taxonomy. Only 2% ASVs taxonomic assignment was in conflict and in these cases, taxonomy was assigned manually.

The majority of the ASVs of the *R.* melan*ophthalma* mycobiome were Ascomycota (93%), the remainder part was classified as Basidiomycota (6%), unidentified fungi (0,5%), Olpidiomycota (0.2%) and Chytridiomycota (0.02%). At the class level (Fig. 2a), the most represented Ascomycota belonged to Lecanoromycetes (54%), Eurotiomycetes (25%), Dothideomycetes (12%), Leotiomycetes (3%), unidentified class (5%) and Sordariomycetes (0.2%). At the order level the most abundant were Lecanorales (23%), Chaetothyriales (23%), Pleosporales (5%), Capnodiales (5%), Helotiales (2%), Caliciales (1%) and Botryosphaeriales (1%). The most represented Basidiomycota (Fig. 2b) at the class level were Tremellomycetes (94%), Microbotryomycetes (4%), Cystobasidiomycetes (0,7%), while at the order level the most abundant was Tremellales (92%).

The majority of the ASVs of the *T. atra* mycobiome were Ascomycota (81%), the remainder part was classified as Basidiomycota (18%), unidentified fungi (0.5%) and Olpidiomycota (0.1%). At the class level (Fig. 2c), the most represented Ascomycota belonged to Leotiomycetes (24%) Eurotiomycetes (21%), Dothideomycetes (20%), Lecanomycetes (19%), Sordariomycetes (10%) and unidentified class (5%). At the order level the most abundant were Helotiales (21%), Chaetothyriales (19%), Capnodiales (13%), Lecanorales (11%), Pleosporales (4%), Caliciales (3%) and Pertusariales (1%). In Basidiomycota (Fig. 2d) the most represented classes were Tremellomycetes (74%), Agaricomycetes (25%) Microbotryomycetes (0.2%), Cystobasidiomycetes (0,1%), while the most abundant orders were Tremellales (73%), Cantharellales (22%), Corticiales (1%) and Agaricostilbales (0.7%).
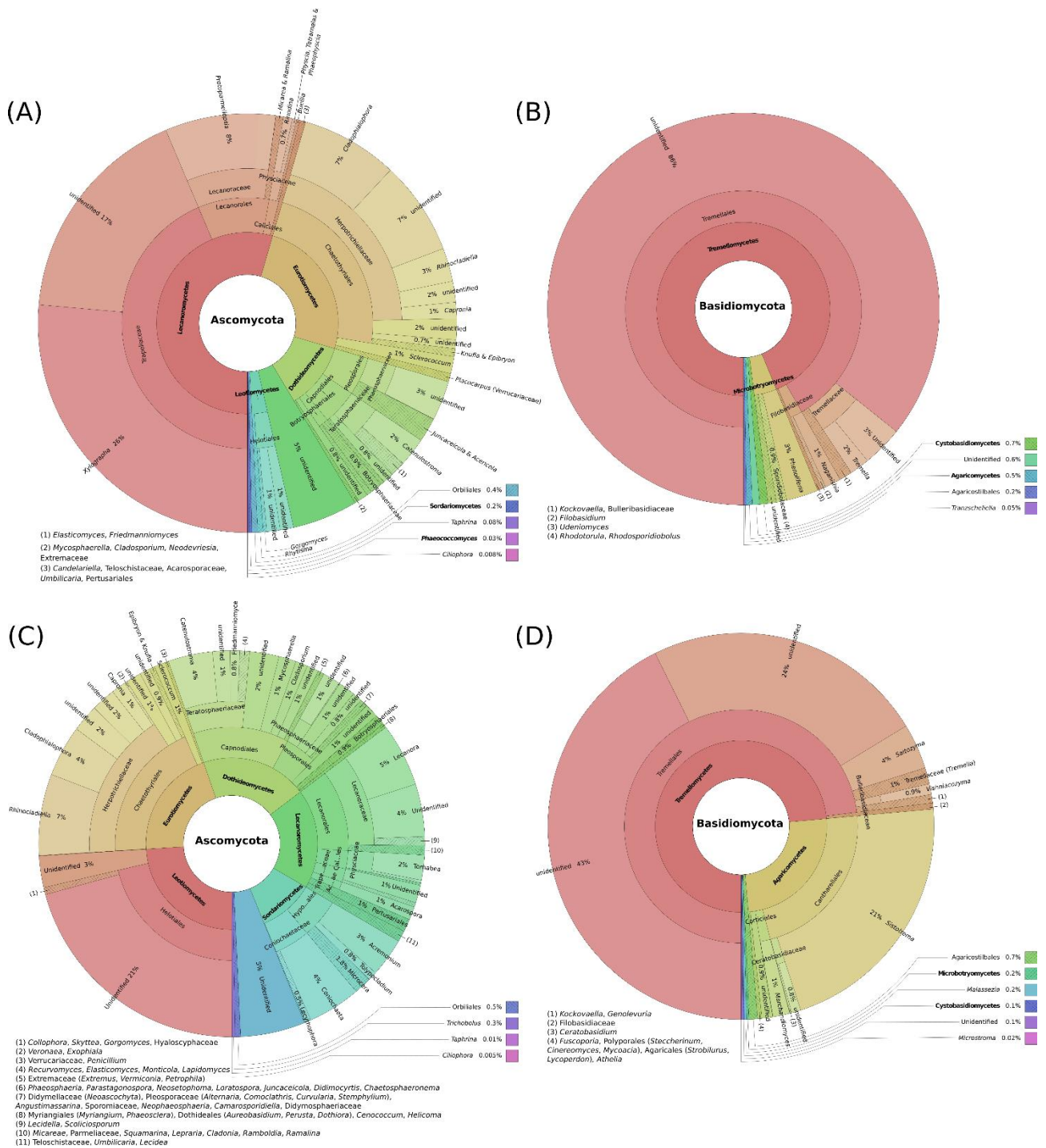
Figure 2. Krona plots describing the diversity and abundance of the main phyla of Ascomycota (A, C) and Basidiomycota (B, D) at the genus level in the mycobiomes of *R. melanophthalma* (A, B) and *T. atra* (C, D) using the non-normalized OTU table. Taxonomy is assigned according to UNITE database (see Methods).

## Alpha, beta diversity and the core mycobiome

After discarding the samples with less than 500 reads assigned to the mycobiome, 29 populations of *R. melanophthalma* and 30 populations of *T. atra* were retained. Six populations (1, 11, 13, 20, 41 and 51) were discarded (Supplementary Table S1). All the applied diversity indices (complete dataset) showed the alpha diversity higher in *T. atra* than in *R. melanophthalma*. The alpha

diversity metrics had median values of 20.4 (95% CI:16.5-24.4) and 30.9 (95% CI: 26.6-35.8) for *R. melanophthalma* and *T. atra*, respectively, using the ASV richness; 1.84 (95% CI: 1.60-1.98) and 2.17 (95% CI: 2.01-2.34) for *R. melanophthalma* and *T. atra*, respectively, using Shannon index; 0.77 (95% CI: 0.64-0.77) and 0.81 (95% CI: 0.74-0.82) for *R. melanophthalma* and *T. atra*, respectively, using Simpson index (Figs. 3a, b). The difference was significant for ASV richness and Shannon index (p < 0.001, Wilcoxon test), while it was not significant using Simpson index (Fig. 3c). The alpha diversity metrics applied to the restricted dataset had median values of 31.0 (95% CI: 22.3-35.1) and 33.0 (95% CI: 26.7-45.6) for *R. melanophthalma* and *T. atra*, respectively, using the ASV richness; 2.22 (95% CI: 1.71-2.40) and 2.27 (95% CI: 2.08-2.50) for *R. melanophthalma* and *T. atra*, respectively, using Shannon index; 0.82 (95% CI: 0.65-0.85) and 0.83 (95% CI: 0.77-0.86) for *R. melanophthalma* and *T. atra*, respectively, using Simpson index. The indices applied to the restricted dataset showed no significant differences between *R. melanophthalma* and *T. atra* distribution, even though the median values of alpha diversity in *T. atra* were still slightly higher (Figs. 3d-f).
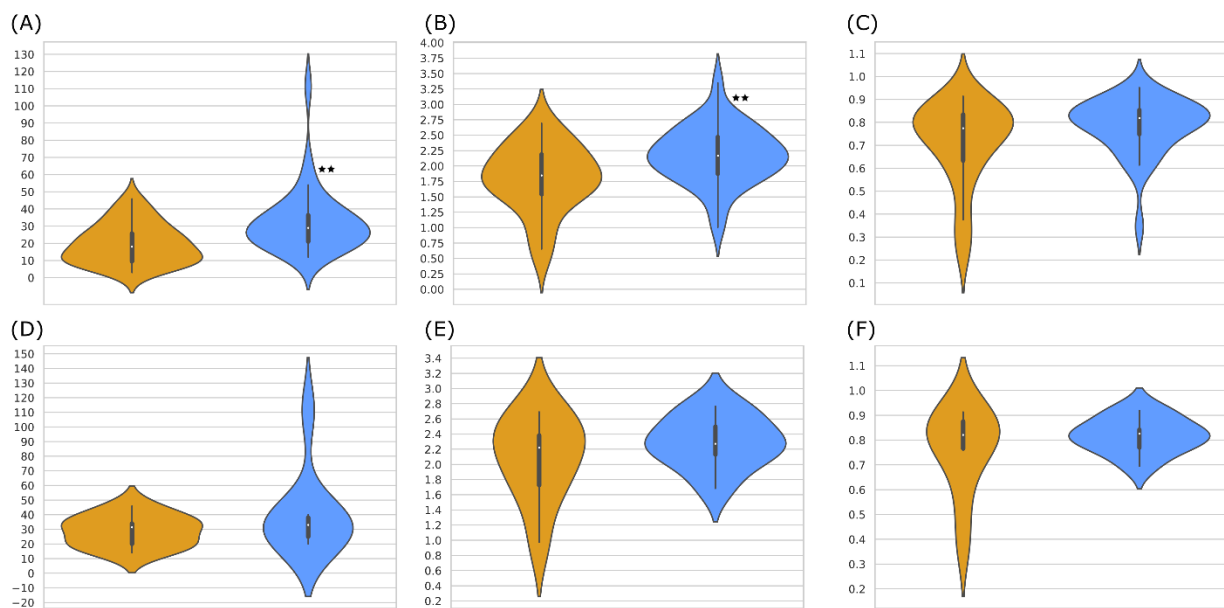
Figure 3. Violin plots of alpha diversity metrics calculate with ASV richness (G, D), Shannon (B, E) and Simpson (C, F) indexes. Complete dataset (A-C), restricted dataset (D-E) for *R. melanophthalma* (orange) and *T. atra* (blue). The violin shape shows the probability density of the distribution; the median value is represented by the white dot, the black bar shows the interquartile range, the black line shows lower/upper adjacent value. Significance detected by Wilcoxon test is indicated by the number of stars: no star is lack of significance, one star is 0.01<p<0.05, two stars are p<0.01.

NMDS plots (Fig 4, Supplementary Fig. S6) showed that the samples and the mock communities were grouped according to the lichen species and geographic origin, although not completely segregating. The ordination also showed that *T. atra* samples from remote localities (sample n. 15 from Tasmania and sample n. 22 from Mauritius) and *R. melanophthalma* samples from USA (samples n. 17-19, 21) were segregated from the European samples. Furthermore, *R. melanophthalma* samples from high elevation (above 3000 m a.s.l.; samples n. 4-9, 24, 25) and *T. atra* samples from calcareous rocks (samples n. 58-63, 65) were also clearly clustered. Six localities, of the nine in which both lichen species were collected (mostly from the Alps), showed an influence of the lichen species on the mycobiome (Fig. 4, line-connected samples). Notably, the negative control sample was completely separated from any other samples, thus strengthening the reliability of the results.
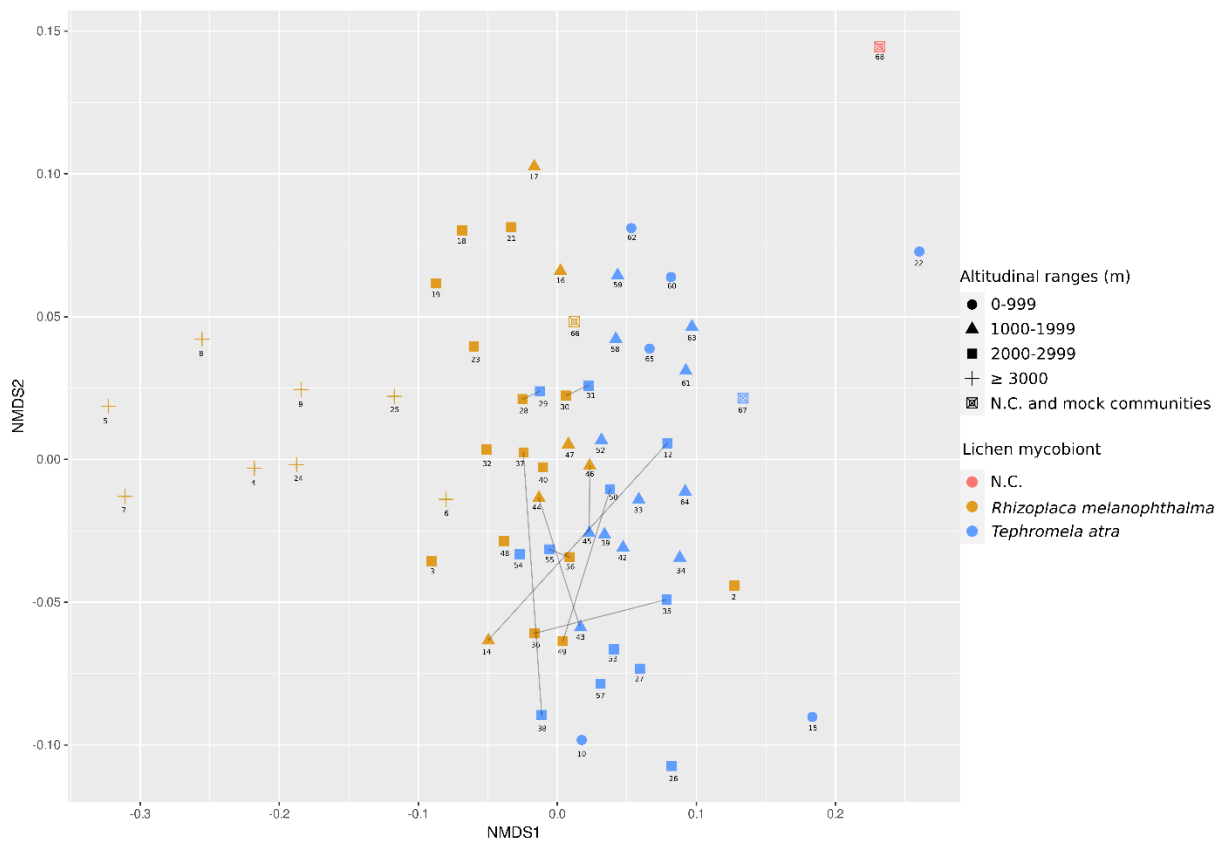
Figure 4. Non-metric Multi Dimensional Scaling (NMDS) ordination based on the Bray-Curtis dissimilarity matrix calculated from the normalized (by median sequencing depth) OTU table. Symbols and colors correspond to altitudinal ranges (m a.s.l.) and lichen species, respectively. Samples n. 66 and 67 correspond to the mock communities created for *R. melanophthalma* and *T. atra*, respectively, and sample 68 to the negative control. Thin grey lines connect the samples of *R. melanophthalma* and *T. atra* coming from the same locality.

Line plots in (Figs. 5a, c, d, f; Supplementary Figs. S7-S10) showed how the core ASVs and OTUs size decreased quickly when stricter (higher) relative abundance and stricter (lower) sample fractions threshold were selected. The 99% similarity OTUs with a relative abundance threshold of 0.001 and sample fraction threshold of 0.1 produced 43 core OTUs for both *R. melanophthalma* and *T. atra* (Figs. 5a-c), while 146 and 179 core OTUs, for *R. melanophthalma* and *T. atra* respectively, were found in the restricted dataset (Figs. 5d-f). Among them 15 were shared between the two cores (53 in the restricted dataset; Fig. 5e). They belonged to the classes Dothideomycetes (Capnodiales sp.*, Catenulostroma* sp., *Cladosporium* sp., *Comoclathris* sp.*,* Didymellaceae sp., *Dothiorella* sp., *Extremus* sp., *Friedmanniomyces endolithicus*, *Helicoma* sp., Pleosporales sp. and Phaesphaeriaceae sp.), Eurotiomycetes (*Cladophialophora* sp., *Epibryon interlamellare*, Herpotrichiellaceae sp., *Knufia* sp., *Rhinocladiella* sp. and *Sclerococcum diminutium*), Lecanoromycetes (Lecanoromycetes sp. and *Candelariella* sp.), Leotiomycetes (*Gorgomyces honrubiae* and Helotiales sp.), Agaricomycetes (*Sistotrema autumnale*), Tremellomycetes (*Filobasidium wieringae*, Tremellales sp., *Tremella anaptychiae* and *T. indecorata*) and Orbilliomycetes (Orbilliales sp.). The number of core OTUs decreased quickly applying higher sample fraction thresholds and (Figs. 5b, e; Supplementary Table S5, S6) using a 0.5 sample fraction threshold (0.7 in the restricted dataset) it reached a single core OTU, *Cladophialophora* sp. (and two OTUs in the restricted dataset, Dothideomycetes sp. and *Xylographa*) for *R. melanophthalma*, and two core OTUs, Dothideomycetes sp. and Tremellales sp. (but none in the restricted dataset) for *T. atra*, while there was no shared core OTUs between the two mycobiomes.

The 97% similarity OTUs with a relative abundance threshold of 0.001 and sample fraction threshold of 0.1 produced 41 core OTUs for *R. melanophthalma* and 45 for *T. atra* (130 and 154 core OTUs, respectively, in the restricted dataset*;* Supplementary Figs. S7, S8). Of these, 16 core OTUs were shared between the two lichen mycobiomes (while 58 core OTUs were shared in the restricted dataset). The shared core composition overlapped the 99% OTU core, except for an unidentified Lecanoromycetes sp. (while in the restricted dataset *Microcera larvarum* and *Sclerococcum parasiticum* were identified). Also, in this case, the core decreased quickly: one core OTU, *Cladophialophora* sp. (and four in the restricted dataset, i.e., *Capronia* sp., Dothideomycetes, Tremellales and *Xylographa* sp.) for *R. melanophthalma* and five core OTUs, i.e., Dothideomycetes sp., Herpotrichiellaceae sp., Tremellales sp. and *Rhinocladiella* sp. (and none in the restricted dataset) for *T. atra* were retained using a 0.5 sample fraction threshold (0.7 in the restricted dataset); there were no shared core OTU (Supplementary Fig. S7, S8 and Supplementary Table S7, S8).

The unclustered ASVs with a relative abundance threshold of 0.001 and 0.1 sample fraction threshold produced 33 core OTUs for *R. melanophthalma* and 36 for *T. atra* (220 and 176 core

OTUs, respectively, in the restricted dataset; Supplementary Fig. S9, S10). Among them 11 OTUs were shared between the two cores (and 49 core OTUs in the restricted dataset). The shared core differed to the 99% and 97% OTU cores for the absence of the OTUs identified as *Cladosporium* sp., *Catenulostroma* sp., Didymellaceae sp. and Lecanoromycetes sp. (while *Microcera larvarum* and *Sclerococcum parasiticum* lacked in the restricted dataset; instead, the new OTU Bothryosphaeriales sp. was present). The number of core OTUs decreased to zero using a 0.5 sample fraction threshold (and 0.7 in restricted dataset; Supplementary Figs. S9, S10 and Supplementary Table S9, S10).
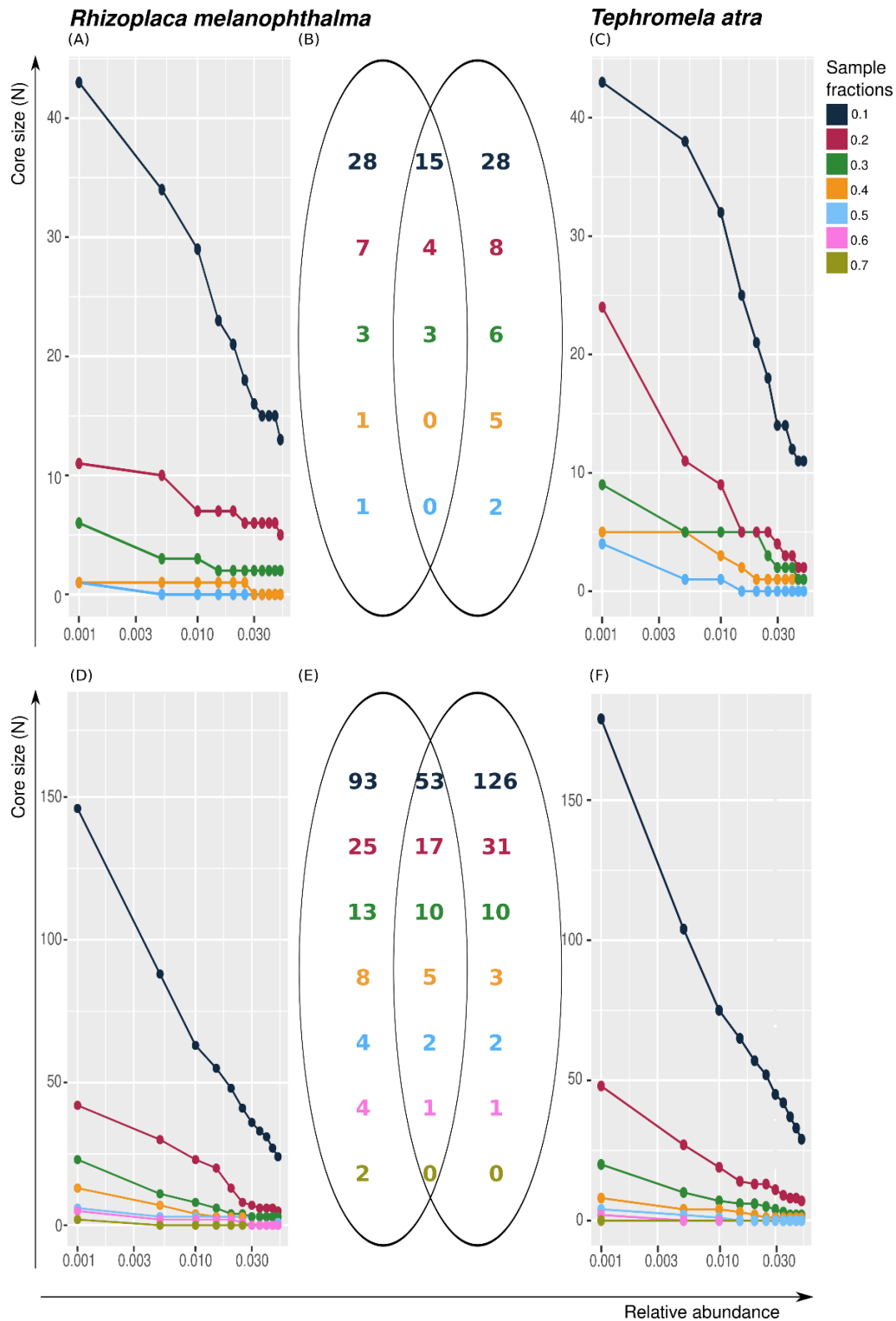
Figure 5. Mycobiome core taxa of *R. melanophthalma* and *T. atra* using 99% similarity clustered ASVs (OTUs) of the complete (A-C) and restricted (D-F) dataset. Line plots (A, C, D, F) show the number of core OTUs retained by varying the read relative abundance and the sample fraction (i.e., percentage of samples containing that OTUs) thresholds. Venn diagram (B, E) shows the number of core OTUs represented by at least 0.001 relative abundance and by at least 0.1 (blue), 0.2 (red), 0.3 (green), 0.4 (yellow) and 0.5 (light-blue), 0.6 (pink) and 0.7 (ocre) sample fractions. Intersection shows the number of shared core OTUs between the two core mycobiomes.

Culture-dependent/independent approach comparison

Starting from 152 fungal isolates from thalli of *R. melanophthalma*, 86 strains were obtained by 100% similarity dereplication. Fifty-seven of these strains corresponded to 32 metabarcoding ASVs; 29 strains were not detected by DNA metabarcoding. In total, 160 ASVs in *R. melanophthalma* were only detected with DNA metabarcoding (Supplementary Fig. 6a, Supplementary Table S11).

Starting from 39 fungal isolates from thalli of *T. atra* 32 strains were obtained by 100% similarity dereplication. Fifteen strains corresponded to nine metabarcoding ASVs; 17 isolates were not detected by DNA metabarcoding. In total, 107 ASVs were only detected with DNA metabarcoding (Supplementary Fig. 6b, Table S12).

(A) **Rhizoplaca melanophthalma**

(B) **Tephromela atra**
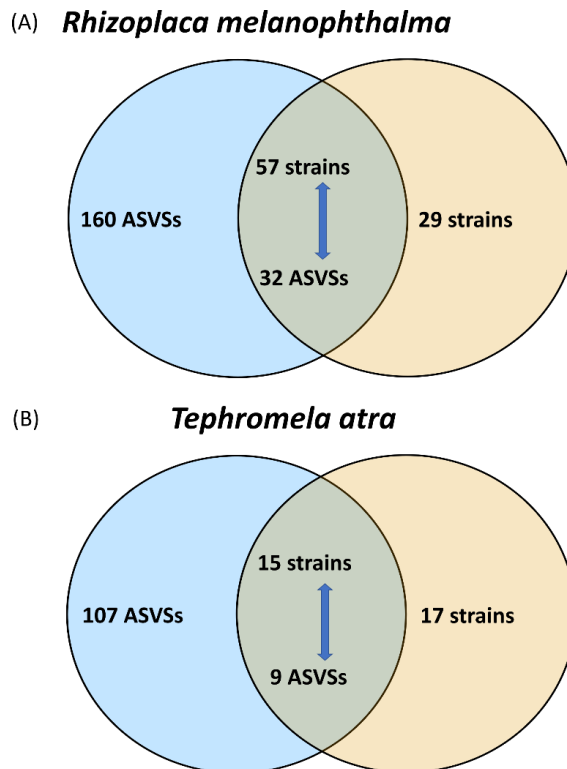
Figure 6. Venn diagrams showing the comparison between culture-independent (metabarcoding, light blue) and culture-dependent (axenic isolation of strains, orange) approaches for *R. melanophthalma* (A) and *T. atra* (B) mycobiomes.

Discussion

We describe the diversity and variation of lichen mycobiomes in the two widespread lichen species *R. melanophthalma* and *T. atra* by the combination of DNA metabarcoding analyses and a culture-dependent approach. We identified fungi that represent the core mycobiome of either lichen. We observed these mycobiomes are variable and comprise taxa occurring with a relatively low

112

frequency (not higher than 70% frequency). No fungal taxon (even recognized as OTU clustered with a permissive 97% similarity threshold) was ubiquitously present across all samples of the two lichens. The mycobiomes of *R. melanophthalma* and *T. atra* are thus largely composed of transient lichenicolous fungi which do not develop any observable structure on the lichen thalli. A fraction of these mycobiomes comprises generalists associated with both lichens. Furthermore, there is a small fraction undetected by PCR and found only by the culture-dependent approach. We assume this could be due to low primer specificity and unavoidable amplification biases.

Lichen mycobiomes are variable and transient

The mycobiomes of the two lichen species *R. melanophthalma* and *T. atra* are diverse and variable. They are mostly composed by Ascomycota, while Basidiomycota represent a less abundant fraction. This agrees with previous studies investigating the mycobiomes of other lichens (Banchi et al. 2018; Fernández-Mendoza et al. 2017; Smith et al. 2020; Yang et al. 2022; Zhang et al. 2015). The mycobiome diversity of *T. atra* is slightly higher than that of *R. melanophthalma,* when either the complete dataset or only the nine sampling locations in which both lichens were growing together are considered. However, only the differences obtained using the complete dataset were significant. For obvious reasons of presence/absence of either one or the other lichen species in most of the localities, the sampling could not be evenly performed across the range of their worldwide distributions. This could hamper the potential consideration that mycobiome diversity can be influenced by the lichen mycobiont species. However, the broad geographic sampling and the number of individuals (lichen thalli) analysed for each population, is enough to distinguish a fair segregation of two groups of lichen mycobiomes, i.e., belonging to *R. melanophthalma* and *T. atra*. The diversity and distinction of the two lichen mycobiomes emerges in the NMDS ordination analysis even more clearly when geographic locations and altitude are considered. Indeed, the mycobiome of *T. atra* collected form Tasmania and Mauritius are very different from the others, being outliers in the ordination. Similarly, *R. melanophthalma* samples collected at high altitudes (over 3000 m a.s.l.) are characterized by mycobiomes with low fungal diversity and comprising different taxa, and segregate from those collected at lower elevations. Also, *R. melanophthalma* samples collected in Utah and Nevada segregate from the South America and European ones. On the other side, the mycobiomes of *R. melanophthalma* and *T. atra* seem to be equalized by the presence of those transient and generalist lichenicolous fungi which would be responsible for the aggregation into that big group of the samples coming from lower altitudes and either from the same location or from close geographic origins.

These transient taxa, retained at low sample fraction thresholds in the two core mycobiomes, are likely only opportunistically present in the lichen thalli or intermingled within them. In this

fraction of ASVs we identified ubiquitous ascomycetes taxa such as *Alternaria* and *Cladosporium*, but also sequences of other lichen mycobionts (e.g., *Aspicilia*, *Caloplaca*, *Lecidea*, *Rhizocarpon*, *Scoliciosporum, Tornabea*) and lichenicolous fungi s.str. (e.g., *Sclerococcum, Skyttea*). The presence of different lichen mycobionts in thalli formed by a certain mycobiont was also reported by Fernández-Mendoza et al. (2017) and suggests thalli are open systems in which potentially multiple lichenizing fungi can grow intertwined. Alternatively, these lichen mycobionts could derive from spores or hyphae from the neighbour thalli. Taxa shared by the two lichen mycobiomes are identified only when relaxed thresholds are applied and are species known to be extremotolerant rock-inhabiting fungi (RIF; Ruibal et al. 2009) such as *Friedmanniomyces endolithicus* (Coleine et al. 2020; Selbmann et al. 2005), *Knufia* (Nai et al. 2013), or various genera including both environmental and pathogenic species, such as *Cladophialophora*, *Rhinocladiella*, *Capronia* (Teixeira et al. 2017) and *Epibryon* (e.g., *Epibryon interlamellare* is a moss pathogen; Davey and Currah 2006). It is worth noting that strains of lichenicolous fungi s. lat., which are phylogenetic closely related to these genera of RIF and pathogens, have been already isolated from lichens (Harutyunyan et al. 2008; Muggia et al. 2016, 2018, 2021) and their occurrence in the here investigated lichen species (from a broader geographic context) is a further confirmation of their lichen-associated life-style.

Basidiomycota, instead, are only 6% and 18% of the detected ASVs in *R. melanophthalma* and *T. atra*, respectively. However, the abundance of Basidiomycota has been variably detected in lichen mycobiomes in general. Indeed, it was reported as low as 0.1% of the total OTUs by Banchi et al. (2018) when sequencing the ITS2 as barcode, whereas Fernández-Mendoza et al. (2017) highlighted a surprisingly 44.6% of Basidiomycota when sequencing the ITS1 as barcode. The two studies of Fernández-Mendoza et al. (2107) and Banchi et al. (2018) analyzed alpine lichen communities composed of diverse species, in which a comparison could be carried out only for *T. atra*, being this species present in all these studies. The presence of basidiomycetes yeasts has been documented within the cortex of lichen thalli by microscopy in some fruticose epiphytic and crustose epilithic lichens (Spribille et al. 2016; Tuovinen et al. 2019, 2021). Here, we detect many basidiomycetes yeast taxa also in the *R. melanophthalma* and *T. atra* mycobiomes, and our results are also supported by the culture-dependent approach (Cometto et al. 2022). The most frequent basidiomycetes yeasts are taxa from the order Tremellales (Tremellomycetes; as also recovered by Banchi et al. 2018; Fernández-Mendoza et al. 2017), while ASVs belonging to Cystobasidiomycetes were recovered sporadically in our analyses. Although Cystobasidiomycetes of the order Cyphobasidiales were hypothesized to be a third key partner in lichen symbioses (Spribille et al. 2016), in accordance with Lendemer et al. (2019) and Smith et al. (2020),

Cystobasidiomycetes seem to be not ubiquitous in the lichen mycobiomes we analysed. Our results place these taxa in the fraction of transient species which presence is occasional in lichen thalli. As, the priming sites of the ITS3-ITSf4 system are conserved in Cystobasidiomycetes (data not shown), our results are less likely to be affected by the lack of amplification of these fungi.

Interestingly only a few taxa seem to be specifically present in the two lichen mycobiomes, as they were consistently found in a high sample fraction from either one or the other lichen species. None of these was shared between the two mycobiomes, even when the relative abundance threshold was kept low (0.001) and permissive 97% similarity OTU was used. These taxa (OTUs) correspond to the *Cladophialophora* sp. Sh17 which is a fungus isolated from lichens from dry habitats many years ago (Harutyunyan et al. 2008), the lichenicolous fungus *Tremella macrobasidiata* AM453 (Millanes et al. 2011; Zamora et al. 2011), and a Dothideomycetes fungus with high BLAST similarity to black fungi isolated from rocks (Gueidan et al. 2011). We thus refrain from proposing a true shared core mycobiome.

The kind of mycobiome diversity that we recover for *R. melanophthalma* and *T. atra* could be a consequence of the different thallus structure of the two lichens, which enable certain fungi (either in form of filamentous mycelia or its fragments, yeasts cells or spores) to remain inside the thalli. However, this hypothesis was not tested here and goes beyond the scope of the present research, deserving further analyses.

Metabarcoding studies in lichens are biased by thallus properties and DNA amplification process
DNA metabarcoding by High-Throughput Sequencing (HTS) uncovers in the most comprehensive way the diversity of fungal communities in diverse environments (Nilsson et al. 2019). However, in lichen systems dominated by the mycobiont as major fungal partner, the use of universal primers comes at the cost of possibly underestimating the diversity of rare fungi (Banchi et al. 2018; Bates et al. 2012; Fernández-Mendoza et al. 2017; Zhang et al. 2015). This results in a low sampling depth of the lichen associated fungi and possibly uneven depth in different lichen species (Banchi et al. 2018; Fernández-Mendoza et al. 2017). Our data also show a relatively low abundance of taxa in the whole mycobiomes and an uneven sequencing depth between *R. melanophthalma* and *T. atra* mycobiomes, having *T. atra* mycobiomes twice the median depth than those of *R. melanophthalma*. This uneven sequencing depth might be due to the lower amount of DNA per mass unit of thallus in *T. atra* than in *R. melanophthalma,* different DNA quality, or actual rDNA copy number per genome affecting amplification. Nevertheless, accumulation curves from both lichens reached the plateau in most samples, highlighting a low fungal diversity (per single lichen population) in the mycobiomes of both lichens.

The selection of a barcode is a determining factor for an extensive detection of fungal taxa, as well as the completeness of the reference database used in the data analyses for the taxonomic assignment. Indeed, discrepancies in PCR amplification of a selected barcode can be due to the universality of the primer systems used. This has been observed for the different detection of Ascomycota or Basidiomycota, when the ITS1 or the ITS2 were chosen as barcodes. In lichens in particular, mycobiomes poorer in Basidiomycota were described when the ITS2 barcode was used (Banchi et al. 2018), while this fungal Phylum was detected in a much higher abundance when the ITS1 was adopted (Fernández-Mendoza et al. 2017). Smith et al. (2020) tried to overcome this issue by mining rDNA sequences from lichen metagenomes, with the advantage of not relying on specific primers and amplified barcodes. In their study the authors included samples of *Rhizoplaca* spp. and detected high-rank taxa that we do not find in our sequencing results, such as Xylonomycetes (Ascomycota), Entomophthoromycetes and Harpellomycetes (Zoopagomycota), Rhizophydiomycetes (Chytridiomycota) and Glomeromycetes (Mucoromycota). While some taxa, such as Xylonomycetes (Ascomycetes), have a very low substitution rate on the priming sites of ITS3-ITSf4 system, other taxa which are basal lineages in the fungal tree of life (Li et al. 2021), such as Entomophthoromycetes (Zoopagomycota), present more divergent sequences at the binding sites of the primers. Thus, the universal primers used in this study may not have been appropriate to efficiently amplify some of these rarer fungi in lichens and let them undetected in the metabarcoding results. Metagenomics is a powerful resource to investigate lichen mycobiomes, however, a successful binning of a specific barcode in a full metagenome and its correct taxonomic assignment, highly depends on the completeness of the genomic references available (Alneberg et al. 2014; Mande et al. 2012). In our case, we refined the taxonomic assignment by applying multiple methods, which, in addition to the constant improvement provided by the update of reference databases (Abarenkov et al. 2010), lowered the amount of unassigned fungal ASVs (5% in Ascomycota, 0.1-0.6% in Basidiomycota).

Using blocking primers designed explicitly for the lichen mycobionts would avoid the yielding amplification of mycobiont reads (U'Ren et al. 2014). We tried this strategy at the beginning of the study and designed blocking primers specific for either lichen mycobiont. However, the developed primers failed to block the amplification of ITS from mycobionts. We refrained from optimizing of this method, also because the universal primers used to catch the widest possible fungal diversity, even in their fungal version (ITSf4), tend to amplify high amount of the abundant algal ITS fragment.

Mock communities, used as positive controls, are a valuable tool to ensure the accuracy of the estimated fungal diversity captured in the sequence metabarcoding, as well as the sensitivity to

rare taxa and probable divergent priming sites (Bakker 2018; Lear et al. 2018; Yeh et al. 2018). Although two of the taxa included at the lowest concentrations (0.005 ng/µl) in the mock communities prepared for this study were not amplified/detected, it was possible to reconstruct the composition of these synthetic communities with high accuracy. Still, biases due to the different quality of genomic DNA (gDNA), unknown rDNA copy numbers in the fungal genomes, and PCR efficiency can impair the accuracy of the taxa abundance recovered in a mock. This issue could be overcome if PCR amplicons at known concentrations were used instead of gDNA (Banchi et al. 2020). However, this approach is unsuitable for estimating the accuracy of taxon abundance if environmental DNA (eDNA) is analysed in the study (Banchi et al. 2020; Lamb et al. 2019).

Our characterization of the core mycobiome of the two lichen species was based on frequency occurrence and relative abundance of the identified taxa. Future analyses will tackle of whether the mycobiome species have any functional role in the *R. melanophthalma* and *T. atra* lichen symbioses. The mycobiome diversity was expressed in ASV, as the smallest unit of diversity in metabarcoding analyses, in which the intraspecific variability is considered (Callahan et al. 2017; Estensmo et al. 2021). This approach convenes when fungal communities are compared in terms of diversity. On the other side, the use of ASVs may lead to an overestimation of the (species) taxonomic diversity, generating a plethora of low abundance taxa, many potentially belonging to the same species, as shown by the results of the mock communities. Therefore, clustering them into OTUs was adopted, when we aimed to identify a potential stable fraction of fungal species associated with the two lichens, i.e. the core mycobiomes. However, any traditionally adopted sequence similarity threshold for ASVs clustering (e.g. 99%), which does not correspond to the species level in many lineages (Hawksworth and Lücking 2017; Lücking et al. 2020), can lead to an error in the opposite direction, that is identifying fewer, more abundant units which include multiple species. Because none of these approaches is bias-free, we applied a gradient of both relative abundance of OTUs (obtained by the clustering of ASVs using different similarity thresholds), of ASVs and sample fractions.

A fraction of the mycobiome is detected only by culture isolation

The comparison between the fungal diversity detected by DNA metabarcoding and the culture-dependent approach on the same samples is seldom reported. In accordance with the few previous studies which performed such comparison (Durán et al. 2021; Pang et al. 2019; Yang et al. 2022), our results show that DNA metabarcoding uncovers the largest part of the diversity of the lichen mycobiome, in which fungi isolated in culture are found. Surprisingly, there are some fungal species which do grow in culture but are not detected by the metabarcoding analysis. We suggest

that this is likely due to several factors distributed along the whole metabarcoding experiment, from the amount and quality of the DNA extracted from the lichen holobiont to its amplification and sequencing. If fungi successfully isolated in culture derive from few spores that outgrow but either their DNA is not easily amplifiable from the thalli (because of low concentrations and competition with more abundant/more efficiently amplifiable template DNA) or are removed by the dataset because singletons in the sequencing results, they will not ever appear as part of the metabarcoded mycobiome.

Conclusions

DNA metabarcoding studies on lichen mycobiome(s) pave the way to understand the diversity and variation of fungi associated to lichens, which may play a role in shaping the phenotypes of lichen thalli and making these symbioses ecologically successful worldwide. Recurrent isolation of lichenicolous fungi from different lichen species and geographic origin, which otherwise are not detected in other types of environmental samples, is a confirmation that lichens are cradles of biodiversity (Arnold et al. 2009). Lichens act as niches in which these fungi may exploit the thallus resources, while only a few may establish more stable trophic relationships within the holobiont. Culture isolates will thus serve for further omics and *ad hoc in vitro* experiments to study the other biological sides of the mycobiome, while future development of species-specific blocking primers may facilitate a more accurate characterization of the mycobiome diversity.

The results presented here raise some important questions about our knowledge of what represents a lichen holobiont and how the holobiont concept could extend the understanding of symbiotic systems. Primary partners detected in lichens contribute to the self-sustaining and maintenance of the long-lived lichen structure, and their roles are fairly-well understood (e.g., Nash 2008), the role of others seems varied. For example, lichen growth can be facilitated by adjacent cyanobacterial colonies (cyanotrophy; Poelt and Mayrhofer, 1988), bacterial colonizers may deliver hormones, vitamins or nutrients to thalli (e.g., Grube et al. 2015), and it might be imagined that some lichenicolous fungi contribute to rejuvenating of thalli by local infections of older parts (Grube and Muggia 2021). Still little is known about the roles of yeasts, which were proclaimed as structurally important partners; it seems very likely that their presence in one species may influence secondary metabolite production and the physiology of the system (Spribille et al. 2016). Even less is known about the activity of the many other fungi that we detect in this study by amplicon sequencing and culturing. Some fungi may just be trapped, others may wait for better conditions. It would perhaps be mere sophistry to automatically assign a working functional role to anything we find in a long-persisting biological structure. So, would such a set of supernumeraries void of

function still be part of the holobiont concept? Perhaps no, since they represent potentially living biomass with possible future roles. In this case, we need to distinguish between the active portion of a holobiont, and the passive part, which is primarily modulated by the overall microbial composition in the habitat.

Reference and contributing authors of the paper

Cometto, A.[1], Ametrano, C. G.[1]*, De Carolis, R.[1], Leavitt, S. D.[2], Grube, M.[3], Pallavicini, A. [1], & Muggia, L.[1] (202X). Largely transient mycobiomes shape fungal diversity in two globally distributed lichens. FEMS Microbiology Ecology, under review.

[1]Department of Life Sciences, University of Trieste, via L. Giorgieri 10, 34127, Trieste, Italy
[2]Department of Biology 4143 LSB, Brigham Young University Provo, Utah, 84602, USA
[3]University of Graz, Institute of Biology, Holteigasse 6, 8010, Graz, Austria

* Corresponding author: Claudio G. Ametrano, Department of Life Sciences, University of Trieste, via L. Giorgieri 10, I-34127 Trieste, Italy; e-mail: claudiogennaro.ametrano@units.it
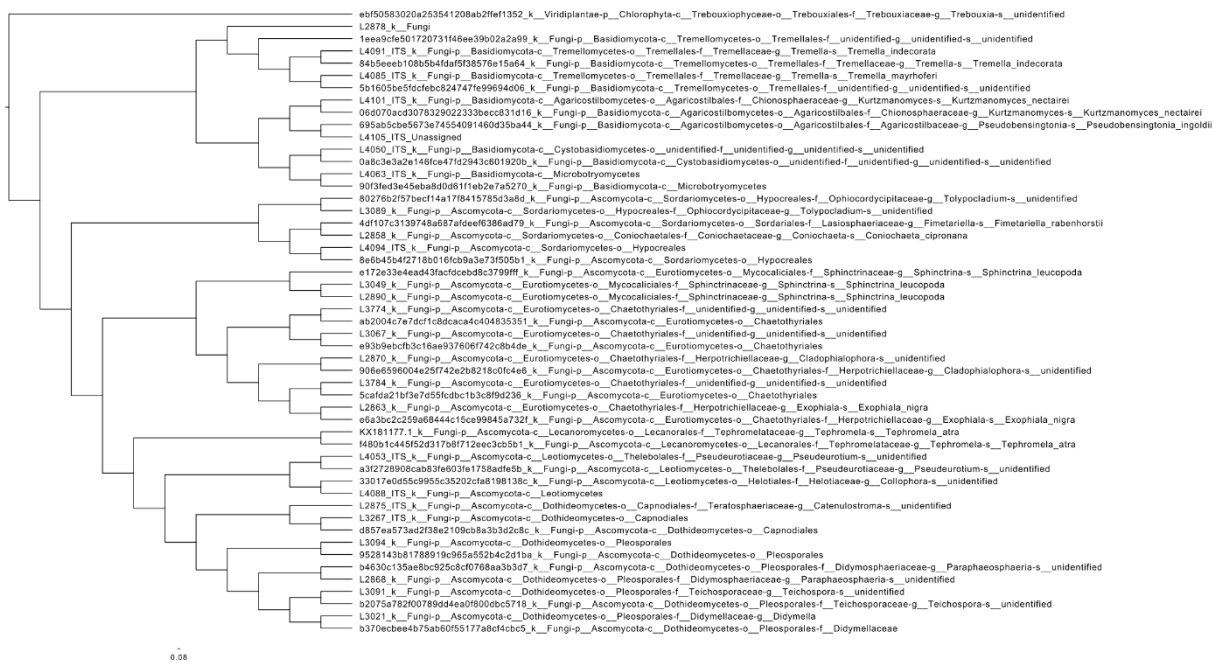
Supplementary information
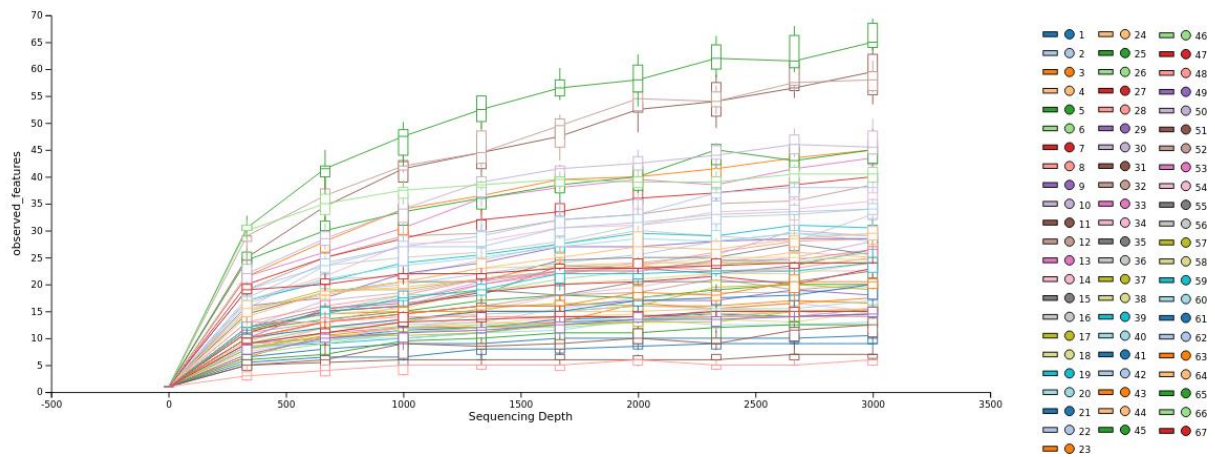
Figure S1. Accumulation curves for each sample of *R. melanophthalma* and *T. atra* (numbers in the legend; Supplementary Table S1) using the unfiltered ASV table.
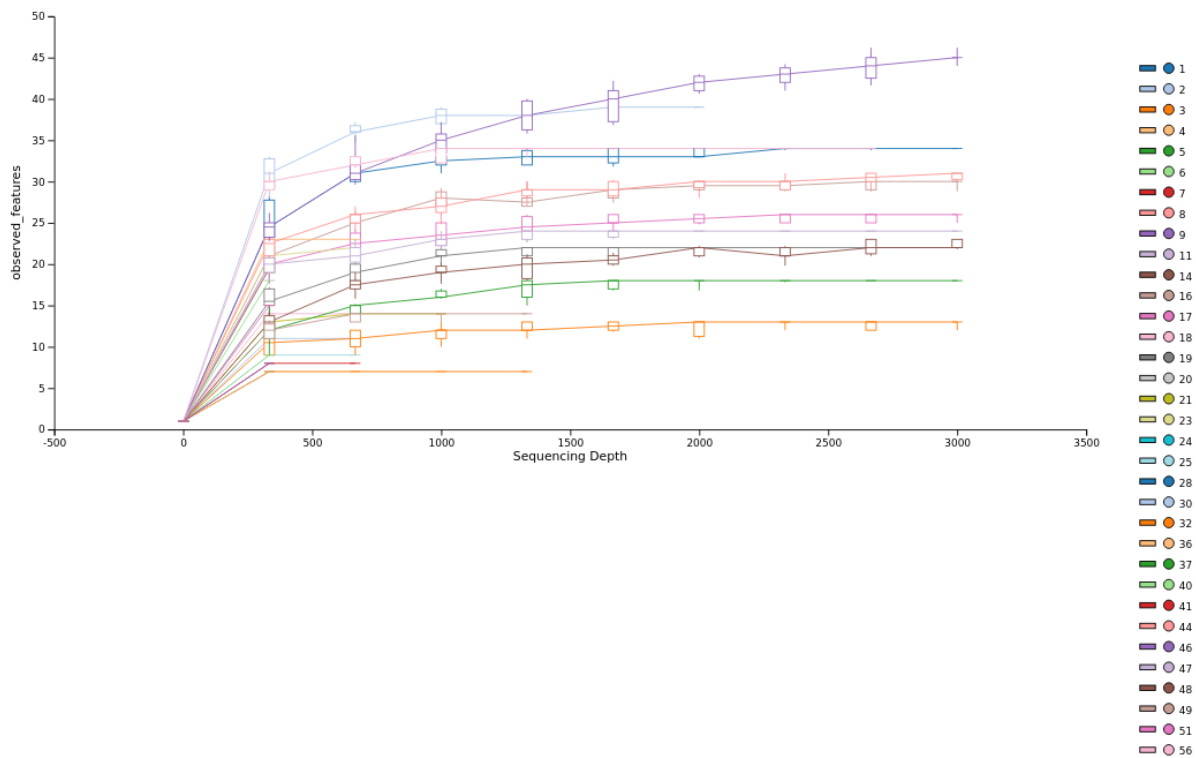

Figure S2. Accumulation curves for each sample of *R. melanophthalma* sample (numbers in the legend; Supplementary Table S1) using ASVs belonging to the mycobiome.

Figure S3. Accumulation curves for each *T. atra* sample (numbers in the legend; Supplementary Table S1) using ASVs belonging to the mycobiome.

Figure S4. Phylogenetic tree showing coupled sequences from Sanger and corresponding ASVs in *R. melanophthalma* mock community.

Figure S5. Phylogenetic tree showing coupled sequences from Sanger and corresponding ASVs in *T. atra* mock community.
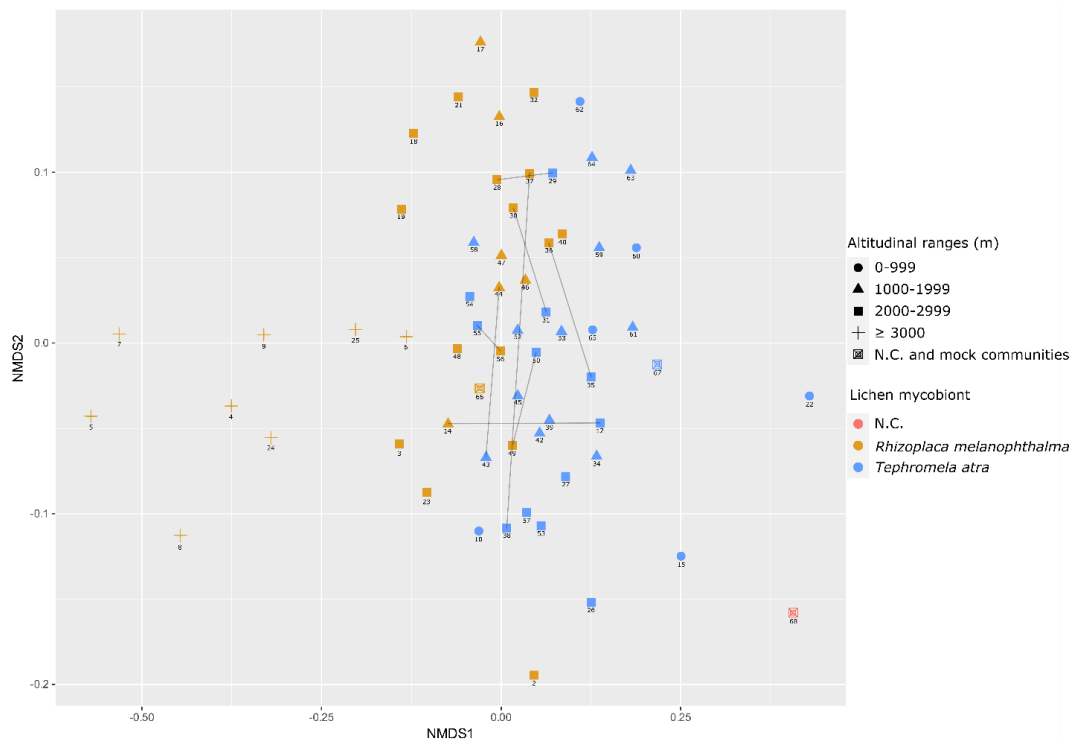

Figure S6. Non-metric Multi Dimensional Scaling (NMDS) ordination based on the Bray-Curtis dissimilarity matrix calculated from the OTU table normalized by geometric mean of sequencing depth. Symbols and colors correspond to altitudinal ranges (m a.s.l.) and lichen species, respectively. Samples n. 66 and 67 correspond to the mock communities created for *R. melanophthalma* and *T. atra*, respectively, and sample 68 to the negative control. Thin grey lines connect the samples of *R. melanophthalma* and *T. atra* coming from the same locality.
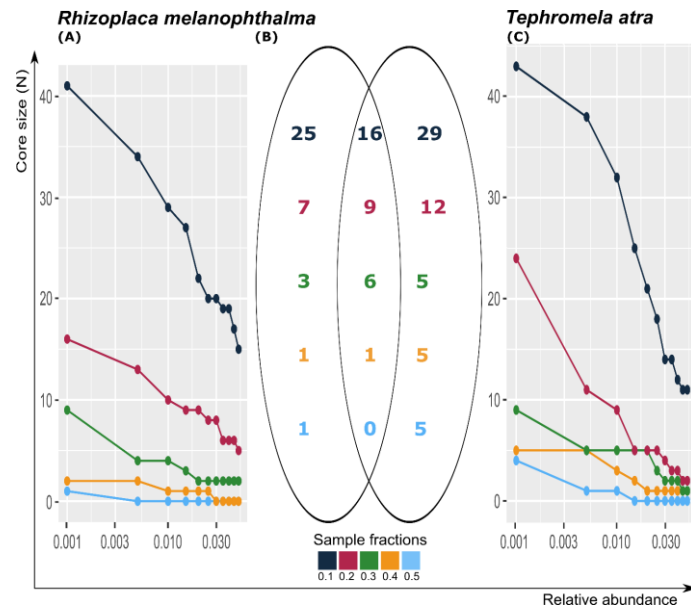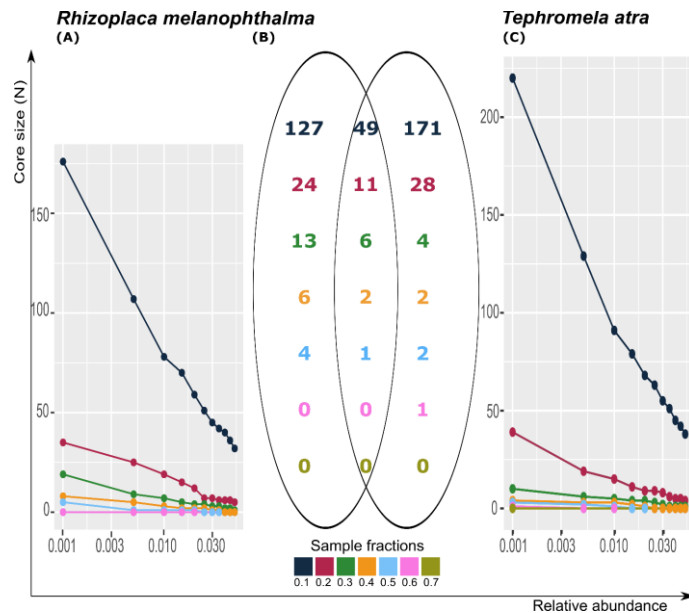
Figure S7. Core taxa in *R. melanophthalma* and *T. atra* using 97% similarity clustered ASVs (OTUs) of the complete dataset. Line plots (A, C) show the number of core OTUs retained by varying the reads relative abundance and the sample fraction (i.e., percentage of samples containing that OTUs) thresholds. Venn diagram (B) shows the number of core OTUs represented by at least 0.001 relative abundance and by at least 0.1 (blue), 0.2 (red), 0.3 (green), 0.4 (yellow) and 0.5 (light-blue) sample fractions. Intersection shows the number of shared core OTUs between the two core mycobiomes.
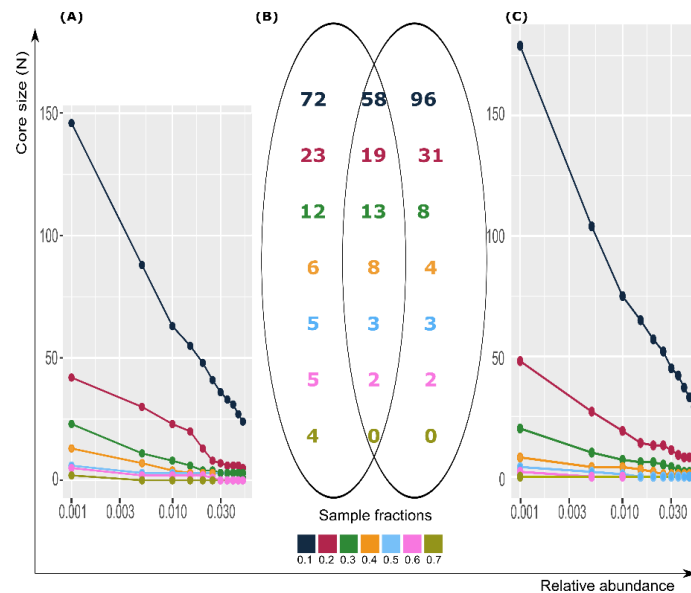
Figure S8. Core taxa in *R. melanophthalma* and *T. atra* using 97% similarity clustered ASVs (OTUs) of the restricted dataset. Line plots (A, C) show the number of core OTUs retained by varying the reads relative abundance and the sample fraction (i.e., percentage of samples containing that OTUs) thresholds. Venn diagram (B) shows the number of core OTUs represented by at least 0.001 relative abundance and by at least 0.1 (blue), 0.2 (red), 0.3 (green), 0.4 (yellow) and 0.5 (light-blue), 0.6 (pink) and 0.7 (ocre) sample fractions. Intersection shows the number of shared core OTUs between the two core mycobiomes.
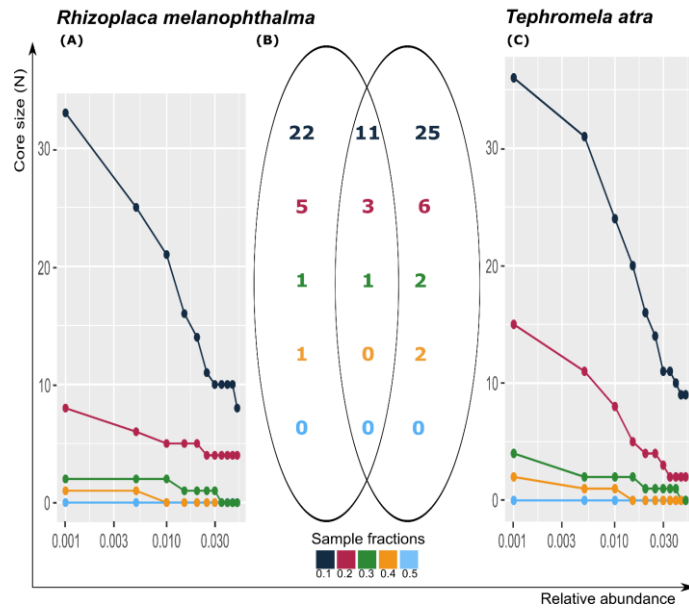
Figure S9. Core taxa in *R. melanophthalma* and *T. atra* using ASVs of the complete dataset. Line plots (A, C) show the number of core OTUs retained by varying the reads relative abundance and the sample fraction (i.e., percentage of samples containing that OTUs) thresholds. Venn diagram (B) shows the number of core OTUs represented by at least 0.001 relative abundance and by at least 0.1 (blue), 0.2 (red), 0.3 (green), 0.4 (yellow) and 0.5 (light-blue) sample fractions. Intersection shows the number of shared core ASVs between the two core mycobiomes.

Figure S10. Core taxa in *R. melanophthalma* and *T. atra* using ASVs of the restricted dataset. Line plots (A, C) show the number of core OTUs retained by varying the reads relative abundance and the sample fraction (i.e., percentage of samples containing that OTUs) thresholds. Venn diagram (B) shows the number of core OTUs represented by at least 0.001 relative abundance and by at least 0.1 (blue), 0.2 (red), 0.3 (green), 0.4 (yellow) and 0.5 (light-blue), 0.6 (pink) and 0.7 (ocre) sample fractions. Intersection shows the number of shared core ASVs between the two core mycobiomes.

Table S1. Samples of *Rhizoplaca melanophthalma* and *Tephromela atra* are reported with their sample ID, thallus ID and the geographic origins. Altitude class 1 (0 - 999 m a.s.l), 2 (1000 - 1999 m a.s.l), 3 (2000 - 2999), 4 (above 3000 m a.s.l) are reported (see separate Excel file: https://doi.org/10.5281/zenodo.7298888).

Table S2. List of the ASVs and their respective ID with their taxonomic assignment (see separate Excel file: https://doi.org/10.5281/zenodo.7298888).

Table S3. *R. melanophthalma* mock communities: comparison between Sanger sequences from the DNA extraction (ID culture) used to build the mock sample itself and ASVs (see separate Excel file: https://doi.org/10.5281/zenodo.7298888).

Table S4. *T. atra* mock communities: comparison between Sanger sequences from the DNA extraction (ID culture) used to build the mock sample itself and ASVs (see separate Excel file: https://doi.org/10.5281/zenodo.7298888).

Table S5. 99% similarity clustered ASVs (OTUs) of the complete dataset retained in the core mycobiome of *R. melanophthalma* and *T. atra* applying by at least 0.001 relative abundance and by at least 0.1, 0.2, 0.3, 0.4 and 0.5 sample fractions (Fig. 5). Where ASVs were clustered the OTUs name was referred to the representative ASV. Numbers next to taxon names correspond to an AVS (Table S2) which represent the ASVs cluster (see separate Excel file: https://doi.org/10.5281/zenodo.7298888).

Table S6. 99% similarity clustered ASVs (OTUs) of the restricted dataset retained in the core mycobiome of *R. melanophthalma* and *T. atra* applying by at least 0.001 relative abundance and by at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 sample fractions (Fig. 5). Where ASVs were clustered the OTUs name was referred to the representative ASV. Numbers next to taxon names correspond to an AVS (Table S2) which represent the ASVs cluster (see separate Excel file: https://doi.org/10.5281/zenodo.7298888).

Table S7. 97% similarity clustered ASVs (OTUs) of the complete dataset retained in the core mycobiome of *R. melanophthalma* and *T. atra* applying by at least 0.001 relative abundance and by at least 0.1, 0.2, 0.3, 0.4 and 0.5 sample fractions (Fig. S7). Where ASVs were clustered the OTUs name was referred to the representative ASV. Numbers next to taxon names correspond to an AVS

(Table S2) which represent the ASVs cluster (see separate Excel file: https://doi.org/10.5281/zenodo.7298888).

Table S8. 97% similarity clustered ASVs (OTUs) of the restricted dataset retained in the core mycobiome of *R. melanophthalma* and *T. atra* applying by at least 0.001 relative abundance and by at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 sample fractions (Fig. S8). Where ASVs were clustered the OTUs name was referred to the representative ASV. Numbers next to taxon names correspond to an AVS (Table S2) which represent the ASVs cluster (see separate Excel file: https://doi.org/10.5281/zenodo.7298888).

Table S9. ASVs of the complete dataset retained in the core mycobiome of *R. melanophthalma* and *T. atra* applying by at least 0.001 relative abundance and by at least 0.1, 0,2, 0,3, 0,4 and 0,5 sample fractions (Fig. S9). Where ASVs were clustered the OTUs name was referred to the representative ASV. Numbers next to taxon names correspond to an AVS (Table S2) which are reported to distinguish between ASVs having the same taxonomy (see separate Excel file: https://doi.org/10.5281/zenodo.7298888).

Table S10. ASVs of the restricted dataset retained in the core mycobiome of *R. melanophthalma* and *T. atra* applying by at least 0.001 relative abundance and by at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 sample fractions (Fig. S10). Where ASVs were clustered the OTUs name was referred to the representative ASV. Numbers next to taxon names correspond to an AVS (Table S2) which are reported to distinguish between ASVs having the same taxonomy (see separate Excel file: https://doi.org/10.5281/zenodo.7298888).

Table S11. Comparison between culture-independent and culture-dependent approach of *R. melanophthalma* mycobiome. Culture ID, ASVs and their respective percentual of identity are reported (see separate Excel file: https://doi.org/10.5281/zenodo.7298888).

Table S12. Comparison between culture-independent and culture-dependent approach of *T. atra* mycobiome. Culture ID, ASVs and their respective percentual of identity are reported (see separate Excel file: https://doi.org/10.5281/zenodo.7298888).

GENERAL CONCLUSION AND FUTURE PERSPECTIVE

The present thesis is the first work addressing the characterization of the lichen mycobiome of two selected species – across their geographic range of distribution worldwide – in which culture-dependent analyses and DNA metabarcoding are combined.

By culture-dependent approach I isolated ascomycetes and basidiomycetes fungi which I characterized on the basis of their morphological and genetic traits. Most of the obtained isolates were ascomycetes belonging to the classes Eurotiomycetes (Chaetothyriales), Dothideomycetes and Sordariomycetes. In particular, I evidenced that several undescribed fungal lineages were present in the two studied species as well as they were detected previously in some other lichens. My results highlighted that certain species of cryptically occurring fungi find in lichens their realized ecological niche and are more frequently present than previously thought. This finding supports the hypothesis of Hafellner (2018) which defined the "lichenicolous fungi s.str" all fungi living exclusively in the lichen thalli. However, as highlighted in this thesis, the lichenicolous fungi s.str are symptomatic only in their specific host even if they are able to occur cryptically in other lichen species. Future researches will aim at investigating which kind of relationship the lichenicolous fungi s.str may establish within the lichen symbiosis and which are the factors that induce these fungi to become symptomatic in certain lichen species. Indeed, these fungi likely reside in thalli as dormant resisting spore finding temporarily shelter, or they might be able to grow and develop distinct asexual and sexual reproductive structures under different ecological conditions and whether these latter would change. Furthermore I found, in accordance with and Hafellner (2018), that lichens are suitable niches for many other cryptically occurring "endolichenic fungi" (Arnold et al. 2009) which are often opportunistic or saprotrophic (e.g. rock-inhabiting fungi, plant-associated endophytes and plant pathogen) and find in lichens a sort of protected natural microhabitat.

I also obtained in culture a relevant fraction of basidiomycete yeasts belonging to the classes Agaricostilbomycetes, Cystobasidiomycetes, Microbotryomycetes, Tremellomycetes and Ustilaginomycetes. Such high diversity of yeast species has never been isolated from lichens in axenic culture before. My analyses did not focus on the detection of their location in the thalli but techniques such as fluorescence in situ hybridization (FISH) coupled with confocal laser scanning microscopy (CLSM), could be applied in the future to address this task. The great diversity of isolates that I obtained represents now a significant potential to set genomics, transcriptomics and symbiotic experiments aiming at understanding the role of these lichenicolous fungi in the lichen symbioses. Mixed culture experiments could be performed in the future to test the specificity that selected lichen-associated fungi show for the mycobiont (or the photobiont) of the host thallus. Also, the metabolic characterization of the isolated fungi could bring new insights into interesting

secondary metabolite patterns, opening new research directions to investigate their biotechnological potentials. Furthermore, many basidiomycete taxa have a complex, dimorphic life-cycle with a haploid yeast phase, less selective in choosing the host as a substrate to live in, and a filamentous stage which develops specifically in a particular host. In this context, the cooperation established with colleagues at the University Rey Juan Carlos in Madrid (Spain; Dr. Ana Millanes, Sandra Freire) and at the Swedish Museum of Natural History of Stockholm (Sweden; Mats Wedin), will center on the study of the life cycles and the mating system of the ubiquitous lichenicolous fungus *Tremella macrobasidiata.* This species was selected because I successfully isolated it from both *R. melanophthalma* and *T. atra*, where it occurred cryptically, while it was previously well known to infect specifically and symptomatically only the lichen *Lecanora chlarotera.*

Many fungal taxa do not grow in axenic culture and this bias estimation of species diversity. However, the complementing DNA metabarcoding analyses offered here a more comprehensive overview and characterization of the lichen mycobiomes of the two species. The broader geographic sampling and the number of individuals analyzed for each populations allowed us to confirm that the selected lichens host a high fungal diversity and that the abiotic factors, especially in presence of harsh conditions (e.g. extreme elevation) and isolated lichen populations, are the major player in the shaping the mycobiome composition.

The recent hypothesis according to which some Cystobasidiomycetes yeasts were proposed as a potential "third symbiont" of the lichen symbioses motivated me to investigate if a core, stable fungal community was present in our two target species. We could not find any possible fungal taxa potentially representing the third symbiont, as only a few taxa occurred with high frequency in *R. melanophthalma* and *T. atra* and none of them were shared between the two lichen mycobiomes. However, further studies based on a higher number of lichen species could be worth to better understand if some members of the lichen mycobiome are ubiquitously present in other lichen thalli. Moreover, since the limitation of the taxonomic-based classification systems to determinate the functional role of the lichen mycobiome, a possible alternative to define the community composition and its effect of the host are necessary. The "omics"-based methods for functional classification of the lichen mycobiome using metagenomics and metabolomics could be performed in the future to better understand the host-mycobiome interactions.

Lichens are proper microniches in which many fungi stand and a few of them might establish more stable relationships with the main symbionts. Further studies are needed to clarify if the core and the ecology-related mycobiome components might play a role in shaping the lichen phenotypes under diverse ecological conditions.

The comparison of the fungal diversity detected by DNA metabarcoding with the one obtained by culture isolation, highlighted that only a small fraction of the isolated fungi was not detected by eDNA. To my knowledge, my present doctoral study together with the work of Yang et al. (2022) are the only two studies in which both culture-dependent and independent approaches were integrated to investigate the diversity of lichen mycobiomes. This combined approach highlighted that the method chosen to study a certain microorganism community can influences substantially the estimation of diversity. Definitely, an integrative approach, based on multiple evidences, is advisable for a thorough understanding of the lichen mycobiome, or of any other community of microorganisms in general.

REFERENCES

Abarenkov, K., Nilsson, R.H., Larsson, K.H., et al., 2010 The UNITE database for molecular identification of fungi - recent updates and future perspectives. New Phytologist 186:281–285.

Abdollahzadeh, J., Groenewald, J.Z., Coetzee, M.P.A., Wingfield, M.J., Crous, P.W., 2020. Evolution of lifestyles in Capnodiales. Studies in Mycology 95, 381–414. https://doi.org/10.1016/j.simyco.2020.02.004

Ahmadjian, V., 1993. The lichen photobiont: what can it tell us about lichen systematics?. The Bryologist 96, 310–313.

Ahmadjian, V., 1987. Coevolution in lichens. Annals of the New York Academy of Sciences 503, 307–315. https://doi.org/10.1111/j.1749-6632.1987.tb40617.x

Aime, M.C., Matheny, P.B., Henk, D.A., Frieders, E.M., Nilsson, R.H., Piepenbring, M., McLaughlin, D.J., Szabo, L.J., Begerow, D., Sampaio, J.P., 2006. An overview of the higher level classification of Pucciniomycotina based on combined analyses of nuclear large and small subunit rDNA sequences. Mycologia 98, 896–905. https://doi.org/10.1080/15572536.2006.11832619

Alneberg, J., Bjarnason, B.S., Bruijn, I.D., Schirmer, M., Quick, J., Ijaz, U.Z., Lahti, L., Loman, N.J., Andersson, A.F., Quince, C., 2014. Binning metagenomic contigs by coverage and composition. Nature Methods 11, 1144–1146. https://doi.org/10.1038/nmeth.3103

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. Journal of Molecular Biology 215, 403–410.

Amend, A.S., Seifert, K.A., Bruns, T.D., 2010. Quantifying microbial communities with 454 pyrosequencing: does read abundance count?. Molecular Ecology 19, 5555–5565. https://doi.org/10.1111/j.1365-294X.2010.04898.x

Ametrano, C.G., Knudsen, K., Kocourková, J., Grube, M., Selbmann, L., Muggia, L., 2019. Phylogenetic relationships of rock-inhabiting black fungi belonging to the widespread genera *Lichenothelia* and *Saxomyces*. Mycologia 111. https://doi.org/10.1080/00275514.2018.1543510

Andrews, S., 2010. FastQC: a quality control tool for high throughput sequence data.

Arenz, B.E., Held, B.W., Jurgens, J.A., Farrell, R.L., Blanchette, R.A., 2006. Fungal diversity in soils and historic wood from the Ross Sea Region of Antarctica. Soil Biology and Biochemistry 38, 3057–3064. https://doi.org/10.1016/j.soilbio.2006.01.016

Arnold, A.E., Miadlikowska, J., Higgins, K.L., Sarvate, S.D., Gugger, P., Way, A., Hofstetter, V., Kauff, F., Lutzoni, F., 2009. A phylogenetic estimation of trophic transition networks for ascomycetous fungi: are lichens cradles of symbiotrophic fungal diversification?. Systematic Biology 58, 283–297. https://doi.org/10.1093/sysbio/syp001

Arup, U., Sandler Berlin, E., 2011. A taxonomic study of *Melanelixia fuliginosa* in Europe. The Lichenologist 43, 89–97. https://doi.org/10.1017/S0024282910000678

Aschenbrenner, I.A., Cernava, T., Erlacher, A., Berg, G., Grube, M., 2017. Differential sharing and distinct co-occurrence networks among spatially close bacterial microbiota of bark, mosses and lichens. Molecular Ecology 26, 2826–2838. https://doi.org/10.1111/mec.14070

Atienza, V., Perez-Ortega, S., Etayo, J., 2009. Two new conidial lichenicolous fungi from Spain indicate the distinction of *Lichenodiplis* and *Minutoexcipula*. The Lichenologist 41, 223–229.

Bacon, C.W., White, J., 2000. Microbial endophytes. CRC press. https://doi.org/10.1201/9781482277302

Badali, H., Gueidan, C., Najafzadeh, M.J., Bonifaz, A., Gerrits van den Ende, A.H.G., De Hoog, G.S., 2008. Biodiversity of the genus *Cladophialophora*. Studies in Mycology 61, 175–191. https://doi.org/10.3114/sim.2008.61.18

Bakker, M.G., 2018. A fungal mock community control for amplicon sequencing experiments. Molecular Ecology Resources 18, 541–556. https://doi.org/10.1111/1755-0998.12760

Banchi, E., Ametrano, C.G., Tordoni, E., Stanković, D., Ongaro, S., Tretiach, M., Pallavicini, A., Muggia, L., Verardo, P., Tassan, F., Trobiani, N., Moretti, O., Borney, M.F., Lazzarin, S., 2020. Environmental DNA assessment of airborne plant and fungal seasonal diversity. Science of the Total Environment 738, 140249. https://doi.org/10.1016/j.scitotenv.2020.140249

Banchi, E., Stankovic, D., Fernández-Mendoza, F., Gionechetti, F., Pallavicini, A., Muggia, L., 2018. ITS2 metabarcoding analysis complements lichen mycobiome diversity data. Mycological Progress 17, 1049–1066. https://doi.org/10.1007/s11557-018-1415-4

Bandoni, R.J., 1995. Dimorphic heterobasidiomycetes: taxonomy and parasitism. Studies in Mycology 38, 13–27.

Barreno, E., Muggia, L., Chiva, S., Molins, A., Bordenave, C., García-Breijo, F., Moya, P., 2022. *Trebouxia lynnae* sp. nov. (former *Trebouxia* sp. TR9): biology and biogeography of an epitome lichen symbiotic microalga. Biology 11, 1196. https://doi.org/10.3390/biology11081196

Bates, S.T., Berg-Lyons, D., Lauber, C.L., Walters, W.A., Knight, R., Fierer, N., 2012. A preliminary survey of lichen associated eukaryotes using pyrosequencing. The Lichenologist 44, 137–146. https://doi.org/10.1017/S0024282911000648

Bauer, R., Oberwinkler, F., 1991. The colacosomes: new structures at the host-parasite interface of a mycoparasitic basidiomycete. Botanica Acta 104, 53–57.

Bauer, R., Oberwinkler, F., 2008. Cellular basidiomycete–fungus interactions. In: Plant Surface Microbiology. Springer, pp 267–279.

Beck, A., Peršoh, D., Rambold, G., 2014. First evidence for seasonal fluctuations in lichen- and bark-colonising fungal communities. Folia Microbiologica 59, 155–157. https://doi.org/10.1007/s12223-013-0278-y

Beiggi, S., Piercey-Normore, M.D., 2007. Evolution of ITS ribosomal RNA secondary structures in fungal and algal symbionts of selected species of *Cladonia* sect. *Cladonia* (Cladoniaceae, Ascomycotina). Journal of Molecular Evolution 64, 528–542. https://doi.org/10.1007/s00239-006-0115-x

Bensch, S., Rez-Tris, J.P., Waldenström, J., Hellgren, O., 2004. Linkage between nuclear and mitochondrial DNA sequences in avian malaria parasites: multiple cases of cryptic speciation?. Evolution 58, 1617–21. https://doi.org/10.1111/j.0014-3820.2004.tb01742.x

Bérubé, J.A., Nicolas, G.G., 2015. Alien fungal species on asymptomatic live woody plant material imported into Canada. Canadian Journal of Plant Pathology 37, 67–81. https://doi.org/10.1080/07060661.2014.986526

Blaalid, R., Kumar, S., Nilsson, R. H., Abarenkov, K., Kirk, P. M., Kauserud, H., 2013. ITS1 versus ITS2 as DNA metabarcodes for fungi. Molecular Ecology Resources 13, 218–224. https://doi.org/10.1111/1755-0998.12065

Blanco, O., Crespo, A., Divakar, P.K., Esslinger, T.L., Hawksworth, D.L., Lumbsch, H.T., 2004. *Melanelixia* and *Melanohalea*, two new genera segregated from *Melanelia* (Parmeliaceae) based on molecular and morphological data. Mycological Research 108, 873–884. https://doi.org/10.1017/S0953756204000723

Blankenship, L.E., Yayanos, A.A., 2005. Universal primers and PCR of gut contents to study marine invertebrate diets. Molecular Ecology 14, 891–899. https://doi.org/10.1111/j.1365-294X.2005.02448.x

Boekhout, T., Fonseca, A., Sampaio, J.P., et al., 2011. Discussion of teleomorphic and anamorphic basidiomycetous yeasts, in: The Yeasts. Elsevier, pp 1339–1372.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., et al., 2018. Reproducible, interactive, scalable, and extensible microbiome data science using QIIME 2. Nature Biotechnology 37, 852–857.

Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C.-H., Xie, D., Suchard, M.A., Rambaut, A., Drummond, A.J., 2014. BEAST 2: a software platform for Bayesian evolutionary analysis. PLoS Computational Biology 10, e1003537.

Bridge, P.D., Spooner, B.M., 2012. Non-lichenized Antarctic fungi: transient visitors or members of a cryptic ecosystem?. Fungal Ecology 5, 381–394. https://doi.org/10.1016/j.funeco.2012.01.007

Bruns, T.D., Gardes, M., 1993. Molecular tools for the identification of ectomycorrhizal fungi-taxon-specific oligonucleotide probes for suilloid fungi. Molecular Ecology 2, 233–242.

Buzzini, P., Branda, E., Goretti, M., Turchetti, B., 2012. Psychrophilic yeasts from worldwide glacial habitats: diversity, adaptation strategies and biotechnological potential. FEMS Microbiology Ecology 82, 217–241. https://doi.org/10.1111/j.1574-6941.2012.01348.x

Callahan, B.J., Mcmurdie, P.J., Holmes, S.P., 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. The ISME Journal 11, 2639–2643. https://doi.org/10.1038/ismej.2017.119

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nature Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869

Černajová, I., Škaloud, P., 2020. Lessons from culturing lichen soredia. Symbiosis 82, 109–122. https://doi.org/10.1007/s13199-020-00718-4

Černajová, I., Škaloud, P., 2019. The first survey of Cystobasidiomycete yeasts in the lichen genus *Cladonia*; with the description of *Lichenozyma pisutiana* gen. nov., sp. Fungal Biology 123, 625–637. https://doi.org/10.1016/j.funbio.2019.05.006

Chagnon, P.L., U'Ren, J.M., Miadlikowska, J., Lutzoni, F., Arnold, A.E., 2016. Interaction type influences ecological network structure more than local abiotic conditions: evidence from endophytic and endolichenic fungi at a continental scale. Oecologia 180, 181–191. https://doi.org/10.1007/s00442-015-3457-5

Cheng, T., Xu, C., Lei, L., Li, C., Zhang, Y., Zhou, S., 2016. Barcoding the kingdom Plantae: new PCR primers for ITS regions of plants with improved universality and specificity. Molecular Ecology Resources 16, 138–149. https://doi.org/10.1111/1755-0998.12438

Chomicki, G., Renner, S.S., 2017. Partner abundance controls mutualism stability and the pace of morphological change over geologic time. Proceedings of the National Academy of Sciences of the United States of America 114, 3951–3956. https://doi.org/10.1073/pnas.1616837114

Coleine, C., Masonjones, S., Sterflinger, K., Onofri, S., Selbmann, L., Stajich, J.E., 2020. Peculiar genomic traits in the stress-adapted cryptoendolithic Antarctic fungus *Friedmanniomyces endolithicus*. Fungal Biology 124, 458–467. https://doi.org/10.1016/j.funbio.2020.01.005

Cometto, A., Leavitt, S.D., Millanes, A.M., Wedind, M., Grube, M., Muggia, L., 2022. The yeast lichenosphere: high diversity of basidiomycetes from the lichens *Tephromela atra* and *Rhizoplaca melanophthalma*. Fungal Biology 126, 587–608. https://doi.org/10.1016/j.funbio.2022.07.004

Connell, L., Redman, R., Craig, S., Scorzetti, G., Iszard, M., Rodriguez, R., 2008. Diversity of soil yeasts isolated from South Victoria Land, Antarctica. Microbial Ecology 56, 448–459. https://doi.org/10.1007/s00248-008-9363-1

Cox, D.E., 1976. A new homobasidiomycete with anomalous basidia. Mycologia 68, 481–510.

Cradic, K.W., Wells, J.E., Allen, L., Kruckeberg, K.E., Singh, R.J., Grebe, S.K.G., 2004. Substitution of 3′-phosphate cap with a carbon-based blocker reduces the possibility of fluorescence resonance energy transfer probe failure in real-time PCR assays. Clinical Chemistry 50, 1080–1082. https://doi.org/10.1373/clinchem.2004.033183

Crittenden, P.D., David, J.C., Hawksworth, D.L., Campbell, F.S., 1995. Attempted isolation and success in the culturing of a broad spectrum of lichen-forming and lichenicolous fungi. New Phytologist 130, 267–297. https://doi.org/10.1111/j.1469-8137.1995.tb03048.x

Crous, P.W., Osieck, E.R., Jurjevi, Ž., Boers, J., Iperen, A.L.V., Starink-Willemse, M., Dima, B., Balashov, S., Bulgakov, T.S., Johnston, P.R., 2021. Fungal planet description sheets: 1284–1382. Persoonia - Molecular Phylogeny and Evolution of Fungi 47, 178–374.

Crous, P.W., Schubert, K., Braun, U., Hoog, G.S.D., Hocking, A.D., Shin, H.D., Groenewald, J.Z., 2007. Opportunistic, human-pathogenic species in the Herpotrichiellaceae are phenotypically similar to saprobic or phytopathogenic species in the Venturiaceae. Studies in Mycology 58, 185–217. https://doi.org/10.3114/sim.2007.58.07

Crous, P.W., Summerell, B.A., Mostert, L., Groenewald, J.Z., 2008. Host specificity and speciation of *Mycosphaerella* and *Teratosphaeria* species associated with leaf spots of Proteaceae. Persoonia - Molecular Phylogeny and Evolution of Fungi 20, 59–86. https://doi.org/10.3767/003158508X323949

Crous, P.W., Wingfield, M.J., Burgess, T.I., Hardy, G.E.S.J., Crane, C., Barrett, S., Cano-Lira, J.F., Leroux, J.J., Thangavel, R., Guarro, J., Stchigel, A.M., et al., 2016. Fungal Planet description sheets: 469–557. Persoonia - Molecular Phylogeny and Evolution of Fungi 37, 218–403. https://doi.org/10.3767/003158516X694499

Crous, P.W., Wingfield, M.J., Burgess, T.I., Hardy, G.E.S.J., Gené, J., Guarro, J., Baseia, I.G., García, D., Gusmão, L.F.P., Souza-Motta, C.M., Thangavel, R., et al., 2018. Fungal Planet description sheets: 716–784. Persoonia - Molecular Phylogeny and Evolution of Fungi 40, 239–392. https://doi.org/10.3767/persoonia.2018.40.10

Cubero, O.F., Crespo, A., Fatehi, J., Bridge, P.D., 1999. DNA extraction and PCR amplification method suitable for fresh, herbarium-stored, lichenized, and other fungi. Plant Systematics and Evolution 216, 243–249.

Dames, S., Margraf, R.L., Pattison, D.C., Wittwer, C.T., Voelkerding, K.V., 2007. Characterization of aberrant melting peaks in unlabeled probe assays. The Journal of Molecular Diagnostics 9, 290–296. https://doi.org/10.2353/jmoldx.2007.060139

Davey, M.L., Currah, R.S., 2006. Interactions between mosses (Bryophyta) and fungi. Canadian Journal of Botany 84,1509–1519. https://doi.org/10.1139/b06-120

Deagle, B.E., Tollit, D.J., Jarman, S.N., Hindell, M.A., Trites, A.W., Gales, N.J., 2005. Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions. Molecular Ecology 14, 1831–1842. https://doi.org/10.1111/j.1365-294X.2005.02531.x

De Bary, A., 1878. "Ueber Symbiose" [On Symbiosis]. Tageblatt für die Versammlung deutscher Naturforscher und Aerzte (in Cassel). Daily Journal for the Conference of German Scientists and Physicians 51, 121–6.

De Carolis, R.D., Cometto, A., Moya, P., Barreno, E., Grube, M., Tretiach, M., Leavitt, S.D., Muggia, L., 2022. Photobiont diversity in lichen symbioses from extreme environments. Frontiers in Microbiology 13, 809804. https://doi:10.3389/fmicb.2022.809804

de Hoog, G.S., Vicente, V.A., Najafzadeh, M.J., Harrak, M.J., Badali, H., Seyedmousavi, S., 2011. Waterborne *Exophiala* species causing disease in cold-blooded animals. Persoonia-Molecular Phylogeny and Evolution of Fungi, 27:46–72. https://doi.org/10.3767/003158511X614258

DePriest, P.T., Ivanova, N.V., Fahselt, D., Alstrup, V., Gargas, A., 2000. Sequences of psychrophilic fungi amplified from glacier-preserved ascolichens. Canadian Journal of Botany 78, 1450–1459. https://doi.org/10.1139/b00-120.

Diederich, P., 1996. The lichenicolous heterobasidiomycetes. Bibliotheca Lichenologica 61, 1–198.

Diederich, P., Common, R.S., Braun, U., Heuchert, B., Millanes, A., Suija, A., Ertz, D., 2019. Lichenicolous fungi from Florida growing on Graphidales. Plant and Fungal Systematics 64, 249–282. https://doi.org/10.2478/pfs-2019-0021

Diederich, P., Ertz, D., 2020. First checklist of lichens and lichenicolous fungi from Mauritius, with phylogenetic analyses and description of new taxa. Plant and Fungal Systematics 65, 13–75. https://doi.org/10.35535/pfsyst-2020-0003

Diederich, P., Kocourková, J., Etayo, J., Zhurbenko, M., 2007. The lichenicolous *Phoma* species (coelomycetes) on *Cladonia*. The Lichenologist 39, 153-163. https://doi.org/10.1017/S0024282907006044

Diederich, P., Lawrey, J.D., Ertz, D., 2018. The 2018 classification and checklist of lichenicolous fungi, with 2000 non-lichenized, obligately lichenicolous taxa. The Bryologist 121, 340–425. https://doi.org/10.1639/0007-2745-121.3.340

Diederich, P., Millanes, A.M., Coppins, B., Wedin, M., 2020. *Tremella imshaugiae* and *T. tubulosae* (Tremellomycetes, Basidiomycota), two new lichenicolous fungi on *Imshaugia aleurites* and *Hypogymnia tubulosa*. Bulletin de la Société des Naturalistes Luxembourgeois 121, 239–246.

Diederich, P., Millanes, A.M., Etayo, J., Boom, P.P.G. van den, Wedin, M., 2022. Finding the needle in the haystack: a revision of *Crittendenia*, a surprisingly diverse lichenicolous genus of Agaricostilbomycetes, Pucciniomycotina. The Bryologist 125, 248–293.

Divakar, P.K., Crespo, A., Kraichak, E., Leavitt, S.D., Singh, G., Schmitt, I., Lumbsch, H.T., 2017. Using a temporal phylogenetic method to harmonize family-and genus-level classification in the largest clade of lichen-forming fungi. Fungal Diversity 84, 101–117. https://doi.org/10.1007/s13225-017-0379-z

Dixon, P., 2003. VEGAN, a package of R functions for community ecology. Journal of Vegetation Science 14, 927–930. https://doi.org/10.1111/j.1654-1103.2003.tb02228.x

Douglas, A.E., Werren, J.H., 2016. Holes in the hologenome: why host-microbe symbioses are not holobionts. MBio 7, e02099-15. https://doi.org/10.1128/mBio.02099-15

Douglas, E.A., 1994. Symbiotic interactions. Oxford University Press. In: Journal of the Marine Biological Association of the United Kingdom 74,148.

Duarte, A.W.F., Passarini, M.R.Z., Delforno, T.P., Pellizzari, F.M., Cipro, C.V.Z., Montone, R.C., Petry, M.V., Putzke, J., Rosa, L.H., Sette, L.D., 2016. Yeasts from macroalgae and lichens that

inhabit the South Shetland Islands, Antarctica. Environmental Microbiology Reports 8, 874–885. https://doi.org/10.1111/1758-2229.12452

Durán, M., Emeterio, L.S., Canals, R.M., 2021. Comparison of culturing and metabarcoding methods to describe the fungal endophytic assemblage of *Brachypodium rupestre* growing in a range of anthropized disturbance regimes. Biology 10, 1246. https://doi.org/10.3390/biology10121246

Easterday, W.R., Ert, M.N.V., Zanecki, S., Keim, P., 2005. Specific detection of *Bacillus anthracis* using a TaqMan® mismatch amplification mutation assay. BioTechniques 38, 731–735. https://doi.org/10.2144/05385ST03

Egholm, M., Buchardt, O., Christensen, L., et al., 1993. PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. Nature 365, 566–568.

Ekman, S., 1999. PCR optimization and troubleshooting, with special reference to the amplification of ribosomal DNA in lichenized fungi. The Lichenologist 31, 517–531.

Ertz, D., Heuchert, B., Braun, U., Freebury, C.E., Common, R.S., Diederich, P., 2016. Contribution to the phylogeny and taxonomy of the genus *Taeniolella*, with a focus on lichenicolous taxa. Fungal Biology 120, 1416–1447. https://doi.org/10.1016/j.funbio.2016.05.008

Ertz, D., Lawrey, J.D., Common, R.S., Diederich, P., 2014. Molecular data resolve a new order of Arthoniomycetes sister to the primarily lichenized Arthoniales and composed of black yeasts, lichenicolous and rock-inhabiting species. Fungal Diversity 66, 113–137. https://doi.org/10.1007/s13225-013-0250-9

Ertz, D., Miadlikowska, J., Lutzoni, F., Dessein, S., Raspé, O., Vigneron, N., Hofstetter, V., Diederich, P., 2009. Towards a new classification of the Arthoniales (Ascomycota) based on a three-gene phylogeny focussing on the genus *Opegrapha*. Mycological Research 113, 141–152. https://doi.org/10.1016/j.mycres.2008.09.002

Estensmo, E.L.F., Maurice, S., Morgado, L., Martin-Sanchez, P.M., Skrede, I., Kauserud, H., Estensmo, C.F.E.L., 2021. The influence of intraspecific sequence variation during DNA metabarcoding: a case study of eleven fungal species. Molecular Ecology Resources 21, 1141–1148. https://doi.org/10.1111/1755-0998.13329

Fan, L.F., Pu, J., Wu, F., Dai, Y.C., 2021. A new species of *Tremella* s.s. (Tremellaceae, Basidiomycota) from southeastern China. Phytotaxa 502, 208–216.

Favero-Longo, S.E., Accattino, E., Matteucci, E., Borghi, A., Piervittori, R., 2015. Weakening of gneiss surfaces colonized by endolithic lichens in the temperate climate area of northwest Italy. Earth Surface Processes and Landforms 40, 2000–2012. https://doi.org/10.1002/esp.3774

Fernández-Mendoza, F., Fleischhacker, A., Kopun, T., Grube, M., Muggia, L., 2017. ITS1 metabarcoding highlights low specificity of lichen mycobiomes at a local scale. Molecular Ecology 26, 4811–4830. https://doi.org/10.1111/mec.14244

Fleischhacker, A., Grube, M., Kopun, T., Hafellner, J., Muggia, L., 2015. Community analyses uncover high diversity of lichenicolous fungi in Alpine habitats. Microbial Ecology 70, 348–360. https://doi.org/10.1007/s00248-015-0579-6

Frank, A.B., 1887. Über die biologischen Verhältnisse des Thallus einiger Krustenflechten. Beitr Biol Pflanzen 2, 123-200.

Fröhlich-Nowoisky, J., Burrows, S.M., Xie, Z., Engling, G., Solomon, P.A., Fraser, M.P., Mayol-Bracero, O.L., Artaxo, P., Begerow, D., Conrad, R., Andreae, M.O., Després, V.R., Pöschl, U., 2012. Biogeography in the air: fungal diversity over land and oceans. Biogeosciences 9, 1125–1136. https://doi.org/10.5194/bg-9-1125-2012

Fröhlich-Nowoisky, J., Pickersgill, D.A., Després, V.R., Pöschl, U., 2009. High diversity of fungi in air particulate matter. Proceedings of the National Academy of Sciences of the United States of America 106, 12814–12819. https://doi.org/10.1073/pnas.0811003106

Garlapati, D., Charankumar, B., Ramu, K., Madeswaran, P., Murthy, M.V.R., 2019. A review on the applications and recent advances in environmental DNA (eDNA) metagenomics. Reviews in Environmental Science and Bio/Technology 18, 389–411. https://doi.org/10.1007/s11157-019-09501-4

Girlanda, M., Isocrono, D., Bianco, C., Luppi-Mosca, A.M., 1997. Two foliose lichens as microfungal ecological niches. Mycologia 89, 531–536. https://doi.org/10.2307/3760987

Glenn, T.C., Nilsen, R.A., Kieran, T.J., Sanders, J.G., Bayona-Vásquez, N.J., Finger, J.W., Pierson, T.W., Bentley, K.E., Hoffberg, S.L., Louha, S., et al., 2019a. Adapterama I: universal stubs and primers for 384 unique dual-indexed or 147,456 combinatorially-indexed Illumina libraries (iTru & iNext). PeerJ 7, e7755. https://doi.org/10.7717/peerj.7755

Glenn, T.C., Pierson, T.W., Bayona-Vásquez, N.J., Kieran, T.J., Hoffberg, S.L., Thomas, J.C., Lefever, D.E., Finger, J.W., Gao, B., Bian, X., et al., 2019b. Adapterama II: universal amplicon sequencing on Illumina platforms (TaggiMatrix). PeerJ 7, e7786. https://doi.org/10.7717/peerj.7786

Gostinčar, C., Muggia, L., Grube, M., 2012. Polyextremotolerant black fungi: oligotrophism, adaptive potential, and a link to lichen symbioses. Frontiers in Microbiology 3, 390. https://doi.org/10.3389/fmicb.2012.00390

Gostinčar, C., Zajc, J., Lenassi, M., Plemenitaš, A., Hoog, S. de, Al-Hatmi, A.M.S., Gunde-Cimerman, N., 2018. Fungi between extremotolerance and opportunistic pathogenicity on humans. Fungal Diversity 93, 195–213. https://doi.org/10.1007/s13225-018-0414-8

Grube, M., Cardinale, M., Castro, J.V.D., Müller, H., Berg, G., 2009. Species-specific structural and functional diversity of bacterial communities in lichen symbioses. The ISME Journal 3, 1105–1115. https://doi.org/10.1038/ismej.2009.63

Grube, M., Cernava, T., Soh, J., Fuchs, S., Aschenbrenner, I., Lassek, C., Wegner, U., Becher, D.R., Riedel, K., Sensen, C.W., Berg, G., 2015. Exploring functional contexts of symbiotic sustain within lichen-associated bacteria by comparative omics. The ISME Journal 9, 412–424. https://doi.org/10.1038/ismej.2014.138

Grube, M., Muggia, L., 2021. 2.5 Complex lichen communities inhabiting rock surfaces. In: Büdel B., Friedl, T. (eds), Life at Rock Surfaces: challenged by extreme light, temperature and hydration fluctuations, Berlin, Boston: De Gruyter, 175–188.

Gueidan, C., Ruibal, C., Hoog, G.S.D., Schneider, H., 2011. Rock-inhabiting fungi originated during periods of dry climate in the late Devonian and middle Triassic. Fungal Biology 115, 987–996. https://doi.org/10.1016/j.funbio.2011.04.002

Gueidan, C., Villaseñor, C.R., Hoog, G.S.D., Gorbushina, A.A., Untereiner, W.A., Lutzoni, F., 2008. A rock-inhabiting ancestor for mutualistic and pathogen-rich fungal lineages. Studies in Mycology 61, 111–119. https://doi.org/10.3114/sim.2008.61.11

Hafellner, J., 2018. Focus on lichenicolous fungi: diversity and taxonomy under the principle "one fungus–one name." Biosystems Diversity 34, 227–243.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, in: Nucleic Acids Symposium Series. [London]: Information Retrieval Ltd., c1979-c2000, pp. 95–98.

Hartmann, M., Lee, S., Hallam, S.J., Mohn, W.W., 2009. Bacterial, archaeal and eukaryal community structures throughout soil horizons of harvested and naturally disturbed forest stands. Environmental Microbiology 11, 3045–3062. https://doi.org/10.1111/j.1462-2920.2009.02008.x

Harutyunyan, S., Muggia, L., Grube, M., 2008. Black fungi in lichens from seasonally arid habitats. Studies in Mycology 61, 83–90. https://doi.org/10.3114/sim.2008.61.08

Hawksworth, D.L., 1982. Secondary fungi in lichen symbioses: parasites, saprophytes and parasymbionts. The Journal of the Hattori Botanical Laboratory 52, 357–366.

Hawksworth, D.L., Cole, M.S., 2004. *Phoma fuliginosa* sp. nov., from *Caloplaca trachyphylla* in Nebraska, with a key to the known lichenicolous species. The Lichenologist 36, 7–13. https://doi.org/10.1017/S0024282904013982

Hawksworth, D.L., Grube, M., 2020. Lichens redefined as complex ecosystems. New Phytologist 227, 1281–1283.

Hawksworth, D.L., Honegger, R., 1994. The lichen thallus: a symbiotic phenotype of nutritionally specialized fungi and its response to gall producers. Systematics Association Special Volume Series 49, 77.

Hawksworth, D.L., Lücking, R., 2017. Fungal diversity revisited: 2.2 to 3.8 million species. Microbiology Spectrum 5, 4–5.

Hocking, A.D., Pitt, J.I., 1980. Dichloran-glycerol medium for enumeration of xerophilic fungi from low-moisture foods. Applied and Environmental Microbiology 39, 488–492. https://doi.org/10.1128/aem.39.3.488-492.1980

Hofstetter, V., Miadlikowska, J., Kauff, F., Lutzoni, F., 2007. Phylogenetic comparison of protein-coding versus ribosomal RNA-coding sequence data: a case study of the Lecanoromycetes (Ascomycota). Molecular Phylogenetics and Evolution 44, 412–426. https://doi.org/10.1016/j.ympev.2006.10.016

Honegger, R., 2012. The symbiotic phenotype of lichen-forming Ascomycetes and their endo-and epibionts. In: Hock, B. (eds), Fungal associations, 287–339. Springer, Berlin, Heidelberg.

Honegger, R., 1997. Metabolic interactions at the mycobiont-photobiont interface in lichens. In: Plant relationships, pp 209–221. Springer, Berlin, Heidelberg.

Honegger, R., 2009. Lichen-forming fungi and their photobionts. In: Deising, H. (eds), Plant relationships, 307–333. Springer, Berlin, Heidelberg.

Hyde, K., Gareth, J.E.B., Liu, J.K, et al., 2013. Families of Dothideomycetes. Fungal Diversity 63, 1–313. https://doi.org/10.1007/s13225-013-0263-4.

Hyvärinen, M., Härdling, R., Tuomi, J., 2002. Cyanobacterial lichen symbiosis: the fungal partner as an optimal harvester. Oikos 98, 498–504. https://doi.org/10.1034/j.1600-0706.2002.980314.x

Jiang, D.F., Zhao, L., Guo, H., Wang, H.Y., 2015. New lineages of *Elaphocordyceps* harboring in lichen thalli. Plant Diversity and Resources 37, 746–750.

Kachalkin, A.V., Glushakova, A.M., Pankratov, T.A., 2017. Yeast population of the Kindo Peninsula lichens. Microbiology 86, 786–792. https://doi.org/10.1134/S0026261717060078

Kachalkin, A.V., Turchetti, B., Inácio, J., Carvalho, C., Mašínová, T., Pontes, A., Röhl, O., Glushakova, A.M., Akulov, A., Baldrian, P., 2019. Rare and undersampled dimorphic basidiomycetes. Mycological Progress 18, 945–971. https://doi.org/10.1007/s11557-019-01491-5

Karkare, S., Bhatnagar, D., 2006. Promising nucleic acid analogs and mimics: characteristic features and applications of PNA. Applied Microbiology and Biotechnology 71, 575–586. https://doi.org/10.1007/s00253-006-0434-2

Karst, J., Piculell, B., Brigham, C., Booth, M., Hoeksema, J.D., 2013. Fungal communities in soils along a vegetative ecotone. Mycologia 105, 61–70. https://doi.org/10.3852/12-042

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular Biology and Evolution 30, 772–780. https://doi.org/10.1093/molbev/mst010

Kellogg, J.J., Raja, H.A., 2017. Endolichenic fungi: a new source of rich bioactive secondary metabolites on the horizon. Phytochemistry Reviews 16, 271–293. https://doi.org/10.1007/s11101-016-9473-1

Kirschner, R., Begerow, D., Oberwinkler, F., 2001. A new *Chionosphaera* species associated with conifer inhabiting bark beetles. Mycological Research 105, 1403–1408. https://doi.org/10.1017/S0953756201005019

Kõljalg, U., Nilsson, R.H., Abarenkov, K., Tedersoo, L., Taylor, A.F.S., Bahram, M., Bates, S.T., Bruns, T.D., Bengtsson-Palme, J., Callaghan, T.M., 2013. Towards a unified paradigm for sequence-based identification of fungi. Molecular Ecology 22, 5271–5277. https://doi.org/10.1111/mec.12481

Kowalski, T., Bilański, P., 2021. Fungi detected in the previous year's leaf petioles of *Fraxinus excelsior* and their antagonistic potential against *Hymenoscyphus fraxineus*. Forests 12, 1412. https://doi.org/10.3390/f12101412

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution 35, 1547–1549. https://doi.org/10.1093/molbev/msy096

Kwon-Chung, K.J., 2011. Chionosphaera Cox (1976), in: The Yeasts. Elsevier, pp. 1395–1401. https://doi.org/10.1016/B978-0-444-52149-1.00106-3.

Lagarde, A., Jargeat, P., Roy, M., Girardot, M., Imbert, C., Millot, M., Mambu, L., 2018. Fungal communities associated with *Evernia prunastri*, *Ramalina fastigiata* and *Pleurosticta acetabulum*: three epiphytic lichens potentially active against *Candida* biofilms. Microbiological Research 211, 1–12. https://doi.org/10.1016/j.micres.2018.03.006

Lahti, L., Shetty, S., Blake, T., Salojarvi, J., et al., 2017. Tools for microbiome analysis in R. Version 1, 28.

Lamb, P.D., Hunter, E., Pinnegar, J.K., Creer, S., Davies, R.G., Taylor, M.I., 2019. How quantitative is metabarcoding: a meta-analytical approach. Molecular Ecology 28, 420–430. https://doi.org/10.1111/mec.14920

Lamit, L.J., L., Lau, M.K., Sthultz, C.M., Wooley, S.C., Whitham, T.G., Gehring, C.A., 2014. Tree genotype and genetically based growth traits structure twig endophyte communities 101, 467–478. https://doi.org/10.3732/ajb.1400034

Larkin, B.G., Hunt, L.S., Ramsey, P.W., 2012. Foliar nutrients shape fungal endophyte communities in Western white pine (*Pinus monticola*) with implications for white-tailed deer herbivory. Fungal Ecology 5, 252–260. https://doi.org/10.1016/j.funeco.2011.11.002

Lawrey, J.D., Binder, M., Diederich, P., Molina, M.C., Sikaroodi, M., Ertz, D., 2007. Phylogenetic diversity of lichen-associated homobasidiomycetes. Molecular Phylogenetics and Evolution 44, 778–789. https://doi.org/10.1016/j.ympev.2006.12.023

Lawrey, J.D., Diederich, P., 2003. Lichenicolous fungi: interactions, evolution, and biodiversity. The Bryologist 106, 80–120. https://doi.org/10.1639/0007-2745(2003)106[0080:lfieab]2.0.co;2

Lawrey, J.D., Diederich, P., 2018. Lichenicolous fungi – worldwide checklist, including isolated cultures and sequences available. http://www. lichenicolous. net

Lawrey, J.D., Diederich, P., Nelsen, M.P., Freebury, C., Broeck, D.V.D., Sikaroodi, M., Ertz, D., 2012. Phylogenetic placement of lichenicolous *Phoma* species in the Phaeosphaeriaceae (Pleosporales, Dothideomycetes). Fungal Diversity 55, 195–213. https://doi.org/10.1007/s13225-012-0166-9

Lear, G., Dickie, I., Banks, J., Boyer, S., Buckley, H.L., Buckley, T.R., Cruickshank, R., Dopheide, A., Handley, K.M., Hermans, S., Kamke, J., Lee, C.K., et al., 2018. Methods for the extraction, storage, amplification and sequencing of dna from environmental samples. New Zealand Journal of Ecology 42, 10. https://doi.org/10.20417/nzjecol.42.9

Leavitt, S.D., Fankhauser, J.D., Leavitt, D.H., Porter, L.D., Johnson, L.A., Clair, L.L.S., 2011. Complex patterns of speciation in cosmopolitan "rock posy" lichens - discovering and delimiting cryptic fungal species in the lichen-forming *Rhizoplaca melanophthalma* species-complex (Lecanoraceae, Ascomycota). Molecular Phylogenetics and Evolution 59, 587–602. https://doi.org/10.1016/j.ympev.2011.03.020

Leavitt, S.D., Grewe, F., Widhelm, T., Muggia, L., Wray, B., Lumbsch, H.T., 2016a. Resolving evolutionary relationships in lichen-forming fungi using diverse phylogenomic datasets and analytical approaches. Scientific Reports 6, 1–11. https://doi.org/10.1038/srep22262

Leavitt, S.D., Kraichak, E., Vondrak, J., Nelsen, M.P., Sohrabi, M., Perez-Ortega, S., Clair, L.L.S., Lumbsch, H.T., 2016b. Cryptic diversity and symbiont interactions in rock-posy lichens. Molecular Phylogenetics and Evolution 99, 261–274. https://doi.org/10.1016/j.ympev.2016.03.030

Lendemer, J.C., Keepers, K.G., Tripp, E.A., Pogoda, C.S., McCain, C.M., Kane, N.C., 2019. A taxonomically broad metagenomic survey of 339 species spanning 57 families suggests Cystobasidiomycete yeasts are not ubiquitous across all lichens. American Journal of Botany 106, 1090–1095. https://doi.org/10.1002/ajb2.1339

Leray, M., Yang, J.Y., Meyer, C.P., Mills, S.C., Agudelo, N., Ranwez, V., Boehm, J.T., Machida, R.J., 2013. A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. Frontiers in Zoology 10, 1–14. https://doi.org/10.1186/1742-9994-10-34

Letunic, I., Bork, P., 2019. Interactive Tree of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Research 47, W256–W259. https://doi.org/10.1093/nar/gkz239

Li, Q., Ren, Y., Xiang, D., Shi, X., Zhao, J., Peng, L., Zhao, G., 2020. Comparative mitogenome analysis of two ectomycorrhizal fungi (*Paxillus*) reveals gene rearrangement, intron dynamics, and phylogeny of basidiomycetes. IMA fungus 11, 1–15. https://doi.org/10.1186/s43008-020-00038-8

Li, W., Godzik, A., 2006. Cd-hit: A fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics 22, 1658–1658. https://doi.org/10.1093/bioinformatics/btl158

Li, W.C., Zhou, J., Guo, S.Y., Guo, L.D., 2007. Endophytic fungi associated with lichens in Baihua mountain of Beijing, China. Fungal Diversity 25, 69–80.

Li, Y., Steenwyk, J.L., Chang, Y., Wang, Y., James, T.Y., Stajich, J.E., Spatafora, J.W., Groenewald, M., Dunn, C.W., Hittinger, C.T., others, 2021. A genome-scale phylogeny of the kingdom Fungi. Current Biology 31, 1653–1665.

Li, Y.-M., Shivas, R.G., Cai, L., 2017. Cryptic diversity in *Tranzscheliella* spp. (Ustilaginales) is driven by host switches. Scientific Reports 7, 1–13.

Liles, M.R., Manske, B.F., Bintrim, S.B., Handelsman, J., Goodman, R.M., 2003. A census of rRNA genes and linked genomic sequences within a soil metagenomic library. Applied and Environmental Microbiology 69, 2684–2691. https://doi.org/10.1128/AEM.69.5.2684-2691.2003

Lilly, V.G., Barnett, H.L., 1951. Physiology of the fungi. Physiology of the fungi. McGraw Hill Book co., New York, p. 464.

Lindgren, H., Diederich, P., Goward, T., Myllys, L., 2015. The phylogenetic analysis of fungi associated with lichenized ascomycete genus *Bryoria* reveals new lineages in the *Tremellales* including a new species *Tremella huuskonenii* hyperparasitic on *Phacopsis huuskonenii*. Fungal Biology 119, 844–856.

Lindsay, W.L., 1869. Memoirs: enumeration of micro-lichens parasitic on other lichens. Journal of Cell Science 2, 342–358.

Liu, X.-Z., Wang, Q.-M., Göker, M., Groenewald, M., Kachalkin, A.V., Lumbsch, H.T., Millanes, A.M., Wedin, M., Yurkov, A.M., Boekhout, T., 2015a. Towards an integrated phylogenetic classification of the Tremellomycetes. Studies in Mycology 81, 85–147. https://doi.org/10.1016/j.simyco.2015.12.001

Liu, X.-Z., Wang, Q.-M., Theelen, B., Groenewald, M., Bai, F.-Y., Boekhout, T., 2015b. Phylogeny of tremellomycetous yeasts and related dimorphic and filamentous basidiomycetes reconstructed from multiple gene sequence analyses. Studies in Mycology 81, 1–26.

Lücking, R., Aime, M.C., Robbertse, B., Miller, A.N., Ariyawansa, H.A., Aoki, T., Cardinali, G., Crous, P.W., Druzhinina, I.S., Geiser, D.M., et al., 2020. Unambiguous identification of fungi: where do we stand and how accurate and precise is fungal DNA barcoding? IMA fungus 11, 1–32. https://doi.org/10.1186/s43008-020-00033-z

Lücking, R., Archer, A.W., Aptroot, A., 2009a. A world-wide key to the genus *Graphis* (Ostropales: Graphidaceae). The Lichenologist 41, 363–452. https://doi.org/10.1017/S0024282909008305

Lücking, R., Lawrey, J.D., Sikaroodi, M., Gillevet, P.M., Chaves, J.L., Sipman, H.J.M., Bungartz, F., 2009b. Do lichens domesticate photobionts like farmers domesticate crops? Evidence from a previously unrecognized lineage of filamentous cyanobacteria. American Journal of Botany 96, 1409–1418. https://doi.org/10.3732/ajb.0800258

Lücking, R., Lumbsch, H.T., 2014. Lichens: fungal farmers. Fungi Magazine 7, 7–12.

Lücking, R., Plata, E.R., Chaves, J.L., Umaña, L., Sipman, H.J.M., 2009b. How many tropical lichens are there... really?. Bibliotheca Lichenologica 100, 399–418.

Lutzoni, F., Miadlikowska, J., 2009. Lichens. Current Biology 19, R502–R503.

Macher, T.-H., Beermann, A.J., Leese, F., 2021. TaxonTableTools: a comprehensive, platform-independent graphical user interface software to explore and visualise DNA metabarcoding data. Molecular Ecology Resources 21, 1705–1714. https://doi.org/10.1111/1755-0998.13358

Mande, S.S., Mohammed, M.H., Ghosh, T.S., 2012. Classification of metagenomic sequences: methods and challenges. Briefings in Bioinformatics 13, 669–681. https://doi.org/10.1093/bib/bbs054

Margulis, L., Fester, R., 1991. Bellagio conference and book. Symbiosis as source of evolutionary innovation: speciation and morphogenesis. conference-June 25-30, 1989, Bellagio Conference Center, Italy., in: Symbiosis. pp. 93–101.

Mark, K., Laanisto, L., Bueno, C.G., Niinemets, Ü., Keller, C., Scheidegger, C., 2020. Contrasting co-occurrence patterns of photobiont and cystobasidiomycete yeast associated with common epiphytic lichen species. New Phytologist 227, 1362–1375. https://doi.org/10.1111/nph.16475

Martos, F., Munoz, F., Pailler, T., Kottke, I., Gonneau, C., Selosse, M.A., 2012. The role of epiphytism in architecture and evolutionary constraint within mycorrhizal networks of tropical orchids. Molecular Ecology 21, 5098–5109. https://doi.org/10.1111/j.1365-294X.2012.05692.x

McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE 8, e61217. https://doi.org/10.1371/journal.pone.0061217

Millanes, A.M., Diederich, P., Ekman, S., Wedin, M., 2011. Phylogeny and character evolution in the jelly fungi (Tremellomycetes, Basidiomycota, Fungi). Molecular Phylogenetics and Evolution 61, 12–28. https://doi.org/10.1016/j.ympev.2011.05.014

Millanes, A.M., Diederich, P., Wedin, M., 2016. *Cyphobasidium* gen. nov., a new lichen-inhabiting lineage in the Cystobasidiomycetes (Pucciniomycotina, Basidiomycota, Fungi). Fungal Biology 120, 1468–1477. https://doi.org/10.1016/j.funbio.2015.12.003

Millanes, A.M., Diederich, P., Westberg, M., Wedin, M., 2021. *Crittendenia* gen. nov., a new lichenicolous lineage in the Agaricostilbomycetes (Pucciniomycotina), and a review of the biology, phylogeny and classification of lichenicolous heterobasidiomycetes. The Lichenologist 53, 103–116. https://doi.org/10.1017/S002428292000033X

Millanes, A.M., Truong, C., Westberg, M., Diederich, P., Wedin, M., 2014. Host switching promotes diversity in host-specialized mycoparasitic fungi: uncoupled evolution in the *Biatoropsis-Usnea* system. Evolution 68, 1576–1593. https://doi.org/10.1111/evo.12374

Miller, M.A., Pfeiffer, W., Schwartz, T., 2011. The CIPRES science gateway: a community resource for phylogenetic analyses. In: Proceedings of the TeraGrid 2011 Conference: Extreme Digital Discovery, p. 41. https://doi.org/10.1145/2016741.2016785

Molins, A., Moya, P., García-Breijo, F.J., Reig-Armiñana, J., Barreno, E., 2018. A multi-tool approach to assess microalgal diversity in lichens: isolation, Sanger sequencing, HTS and ultrastructural correlations. The Lichenologist 50, 123–138. https://doi.org/10.1017/S0024282917000664

Morillas, L., Roales, J., Cruz, C., Munzi, S., 2022. Lichen as multipartner symbiotic relationships. Encyclopedia 2, 1421–1431. https://doi.org/10.3390/encyclopedia2030096

Mouhamadou, B., Molitor, C., Baptist, F., Sage, L., Clément, J.C., Lavorel, S., Monier, A., Geremia, R.A., 2011. Differences in fungal communities associated to *Festuca paniculata* roots in subalpine grasslands. Fungal Diversity 47, 55–63. https://doi.org/10.1007/s13225-011-0091-3

Moya, P., Molins, A., Martinez-Alberola, F., Muggia, L., Barreno, E., 2017. Unexpected associated microalgal diversity in the lichen *Ramalina farinacea* is uncovered by pyrosequencing analyses. PLoS ONE 12, e0175091. https://doi.org/10.1371/journal.pone.0175091

Muggia, L., Ametrano, C.G., Sterflinger, K., Tesei, D., 2020. An overview of genomics, phylogenomics and proteomics approaches in ascomycota. Life 10, 356. https://doi.org/10.3390/life10120356

Muggia, L., Fleischhacker, A., Kopun, T., Grube, M., 2016. Extremotolerant fungi from alpine rock lichens and their phylogenetic relationships. Fungal Diversity 76, 119–142. https://doi.org/10.1007/s13225-015-0343-8

Muggia, L., Grube, M., 2018. Fungal diversity in lichens: from extremotolerance to interactions with algae. Life 8, 15. https://doi.org/10.3390/life8020015

Muggia, L., Grube, M., Tretiach, M., 2008. Genetic diversity and photobiont associations in selected taxa of the *Tephromela atra* group (Lecanorales, lichenised Ascomycota). Mycological Progress 7, 147–160. https://doi.org/10.1007/s11557-008-0560-6

Muggia, L., Kopun, T., Ertz, D., 2015. Phylogenetic placement of the lichenicolous, anamorphic genus *Lichenodiplis* and its connection to *Muellerella*-like teleomorphs. Fungal Biology 119, 1115–1128. https://doi.org/10.1016/j.funbio.2015.08.011

Muggia, L., Kopun, T., Grube, M., 2017. Effects of growth media on the diversity of culturable fungi from lichens. Molecules 22, 824. https://doi.org/10.3390/molecules22050824

Muggia, L., Pérez-Ortega, S., Ertz, D., 2019. *Muellerella*, a lichenicolous fungal genus recovered as polyphyletic within Chaetothyriomycetidae (Eurotiomycetes, Ascomycota). Plant and Fungal Systematics 64, 367–381. https://doi.org/10.2478/pfs-2019-0024

Muggia, L., Pérez-Ortega, S., Fryday, A., Spribille, T., Grube, M., 2014a. Global assessment of genetic variation and phenotypic plasticity in the lichen-forming species *Tephromela atra*. Fungal Diversity 64, 233–251. https://doi.org/10.1007/s13225-013-0271-4

Muggia, L., Pérez-Ortega, S., Kopun, T., Zellnig, G., Grube, M., 2014b. Photobiont selectivity leads to ecological tolerance and evolutionary divergence in a polymorphic complex of lichenized fungi. Annals of Botany 114, 463–475. https://doi.org/10.1093/aob/mcu146

Muggia, L., Quan, Y., Gueidan, C., Al-Hatmi, A.M.S., Grube, M., De Hoog, S.G., 2021. Sequence data from isolated lichen-associated melanized fungi enhance delimitation of two new lineages within Chaetothyriomycetidae. Mycological Progress 20, 911–927. https://doi.org/10.1007/s11557-021-01706-8

Muggia, L., Zellnig, G., Rabensteiner, J., Grube, M., 2010. Morphological and phylogenetic study of algal partners associated with the lichen-forming fungus *Tephromela atra* from the Mediterranean region. Symbiosis 51, 149–160. https://doi.org/10.1007/s13199-010-0060-8

Mundra, S., Halvorsen, R., Kauserud, H., Bahram, M., Tedersoo, L., Elberling, B., Cooper, E.J., Eidesen, P.B., 2016. Ectomycorrhizal and saprotrophic fungi respond differently to long-term experimentally increased snow depth in the High Arctic. MicrobiologyOpen 5, 856–869.

Muriel, S., Olariaga, I., Prieto, M., 2022. Gypsum content influences culturable soil fungal communities in semi-arid steppes from central Spain. Journal of Arid Environments 202, 104756. https://doi.org/10.1016/j.jaridenv.2022.104756

Nai, C., Wong, H.Y., Pannenbecker, A., Broughton, W.J., Benoit, I., Vries, R.P. de, Gueidan, C., Gorbushina, A.A., 2013. Nutritional physiology of a rock-inhabiting, model microcolonial fungus from an ancestral lineage of the Chaetothyriales (Ascomycetes). Fungal Genetics and Biology 56, 54–66. https://doi.org/10.1016/j.fgb.2013.04.001

Nash, T.H., 2008. Lichen Biology. Second edition, Cambridge: Cambridge University Press. https://doi.org/10.1017/CBO9780511790478

Nemergut, D.R., Townsend, A.R., Sattin, S.R., Freeman, K.R., Fierer, N., Neff, J.C., Bowman, W.D., Schadt, C.W., Weintraub, M.N., Schmidt, S.K., 2008. The effects of chronic nitrogen fertilization on alpine tundra soil microbial communities: implications for carbon and nitrogen cycling. Environmental Microbiology 10, 467–478. https://doi.org/10.1111/j.1462-2920.2008.01735.x

Nilsson, R.H., Anslan, S., Bahram, M., Wurzbacher, C., Baldrian, P., Tedersoo, L., 2019. Mycobiome diversity: High-Throughput sequencing and identification of fungi. Nature Reviews Microbiology 17, 95–109. https://doi.org/10.1038/s41579-018-0116-y

Oberwinkler, F., 2017. Yeasts in pucciniomycotina. Mycological Progress 16, 831–856.

Oberwinkler, F., 1987. Heterobasidiomycetes with ontogenetic yeast stages-systematic and phylogenetic aspects. Studies in Mycology 30, 61–74.

Oberwinkler, F., Bauer, R., Bandoni, R.J., 1990. *Colacogloea*: a new genus in the auricularioid heterobasidiomycetes. Canadian Journal of Botany 68, 2531–2536.

Ogaki, M.B., Pinto, O.H.B., Vieira, R., Neto, A.A., Convey, P., Carvalho-Silva, M., Rosa, C.A., Câmara, P.E.A.S., Rosa, L.H., 2021. Fungi present in Antarctic deep-sea sediments assessed using DNA metabarcoding. Microbial Ecology 82, 157–164. https://doi.org/10.1007/s00248-020-01658-8

Olivier, H., 1907. Les principaux parasites de nos lichens français: Premier supplément. Chez l'Auteur.

Ondov, B.D., Bergman, N.H., Phillippy, A.M., 2011. Interactive metagenomic visualization in a Web browser. BMC Bioinformatics 12, 1–10. https://doi.org/10.1186/1471-2105-12-385

Ørum, H., Nielsen, P.E., Egholm, M., et al., 1993. Single-base pair mutation analysis by PNA directed PCR clamping. Nucleic Acids Research 21, 5332–5336.

Pagano, J., Levin, J.D., Trejo, W., 1958. Diagnostic medium for differentiation of species of *Candida*. Antibiotics Annual 5, 137–143.

Page, R.D.M., 2003. Tangled trees: phylogeny, cospeciation, and coevolution. University of Chicago Press.

Page, R.D.M., 1996. Tree View: An application to display phylogenetic trees on personal computers. Bioinformatics 12, 357–358.

Pang, K.L., Guo, S.Y., Chen, I.A., Burgaud, G., Luo, Z.H., Dahms, H.U., Hwang, J.S., Lin, Y.L., Huang, J.S., Ho, T.W., Tsang, L.M., Chiang, M.W.L., Cha, H.J., 2019. Insights into fungal diversity of a shallowwater hydrothermal vent field at Kueishan Island, Taiwan by culture-based and metabarcoding analyses. PLoS ONE 14, e0226616. https://doi.org/10.1371/journal.pone.0226616

Pankratov, T.A., Kachalkin, A.V., Korchikov, E.S., Dobrovol'skaya, T.G., 2017. Microbial communities of lichens. Microbiology 86, 293–309. https://doi.org/10.1134/S0026261717030134

Pavlova, K., Grigorova, D., Hristozova, T., Angelov, A., 2001. Yeast strains from Livingston Island, Antarctica. Folia Microbiologica 46, 397–401. https://doi.org/10.1007/BF02814428

Peršoh, D., Rambold, G., 2012. Lichen-associated fungi of the *Letharietum vulpinae*. Mycological Progress 11, 753–760. https://doi.org/10.1007/s11557-011-0786-6

Petrini, O., Hake, U., Dreyfuss, M.M., 1990. An analysis of fungal communities isolated from fruticose lichens. Mycologia 82, 444–451. https://doi.org/10.2307/3760015

Petrzik, K., Koloniuk, I., Sehadová, H., Sarkisova, T., 2019. *Chrysoviruses* inhabited symbiotic fungi of lichens. Viruses 11, 1120. https://doi.org/10.3390/v11121120

Piñol, J., Mir, G., Gomez-Polo, P., Agustí, N., 2015. Universal and blocking primer mismatches limit the use of High-Throughput DNA sequencing for the quantitative metabarcoding of arthropods. Molecular Ecology Resources 15, 819–830. https://doi.org/10.1111/1755-0998.12355

Poelt, J., Mayrhofer, H., 1988. Systematics and evolution uber Cyanotrophie bei Flechten*. Plant Systematics and Evolution 82, 265–281.

Pohl, C.H., Smit, M.S., Albertyn, J., 2011. *Rhodotorula bloemfonteinensis* sp. nov., *Rhodotorula eucalyptica* sp. nov., *Rhodotorula orientis* sp. nov. and *Rhodotorula pini* sp. nov., yeasts isolated from monoterpene-rich environments. International Journal of Systematic and Evolutionary Microbiology 61, 2320–2327.

Prenafeta-Boldú, F.X., Summerbell, R., De Hoog, S.G., 2006. Fungi growing on aromatic hydrocarbons: biotechnology's unexpected encounter with biohazard? FEMS Microbiology Reviews 30:109–130.

Prillinger, H., Kraepelin, G., Lopandic, K., Schweigkofler, W., Molnár, O., Weigang, F., Dreyfuss, M.M., 1997. New species of *Fellomyces* isolated from epiphytic lichen species. Systematic and Applied Microbiology 20, 572–584. https://doi.org/10.1016/S0723-2020(97)80029-X

Qin, J., Zhang, W., Ge, Z.W., Zhang, S.B., 2019. Molecular identifications uncover diverse fungal symbionts of *Pleione* (Orchidaceae). Fungal Ecology 37, 19–29. https://doi.org/10.1016/j.funeco.2018.10.003

Quan, Y., Muggia, L., Moreno, L.F., Wang, M., Al-Hatmi, A.M.S., Menezes, N. da S., Shi, D., Deng, S., Ahmed, S., Hyde, K.D., Vicente, V.A., Kang, Y., Stielow, J.B., De Hoog, S.G., 2020. A re-evaluation of the Chaetothyriales using criteria of comparative biology. Fungal Diversity 103, 47–85. https://doi.org/10.1007/s13225-020-00452-8

Rafferty, N.E., Caradonna, P.J., Bronstein, J.L., 2015. Phenological shifts and the fate of mutualisms. Oikos 124, 14–21. https://doi.org/10.1111/oik.01523

Rambaut, A., Drummond, A.J., 2007. Tracer v1. 4. Available from http://www.beast.bio.ed.ac.uk. Tracer.

Rambaut, A., Drummond, A.J., Xie, D., Baele, G., Suchard, M.A., 2018. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. Systematic Biology 67, 901–904. https://doi.org/10.1093/sysbio/syy032

Rambold, G., Triebel, D., 1992. The inter lecanoralean-associations. Bibliotheca Lichenologica 48, 201. Cramer J, Berlin, Stuttgart.

Ray, M.K., Shivaji, S., Rao, N.S., Bhargava, P.M., 1989. Yeast strains from the Schirmacher Oasis, Antarctica. Polar Biology 9, 305–309.

Richardson, D.H.S., Hill, D.J., Smith, D.C., 1968. Lichen physiology: XI. The role of the alga in determining the pattern of carbohydrate movement between lichen symbionts. New Phytologist 67, 469–486.

Roberts, P., 1997. New heterobasidiomycetes from Great Britain. Mycotaxon 63, 195–216.

Ronquist, F., Teslenko, M., Mark, P.V.D., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61, 539–542. https://doi.org/10.1093/sysbio/sys029

Ronquist, F.R., Huelsenbeck, J., Teslenko, M., 2011. Draft MrBayes version 3.2 manual: tutorials and model summaries. Distributed with the software from https://nbisweden.github.io/MrBayes/manual.

Rosenberg, E., Sharon, G., Atad, I., Zilber-Rosenberg, I., 2010. The evolution of animals and plants via symbiosis with microorganisms. Environmental Microbiology Reports 2, 500–506. https://doi.org/10.1111/j.1758-2229.2010.00177.x

Rosenberg, E., Zilber-Rosenberg, I., 2011. Symbiosis and development: the hologenome concept. Birth Defects Research Part C: Embryo Today: Reviews 93, 56–66. https://doi.org/10.1002/bdrc.20196

Ruibal, C., Gueidan, C., Selbmann, L., Gorbushina, A.A., Crous, P.W., Groenewald, J.Z., Muggia, L., Grube, M., Isola, D., Schoch, C.L., Staley, J.T., Lutzoni, F., De Hoog, S.G., 2009. Phylogeny of rock-inhabiting fungi related to Dothideomycetes. Studies in Mycology 64, 123–133. https://doi.org/10.3114/sim.2009.64.06

Ruibal, C., Millanes, A.M., Hawksworth, D.L., 2011. Molecular phylogenetic studies on the lichenicolous *Xanthoriicola physciae* reveal Antarctic rock-inhabiting fungi and *Piedraia* species among closest relatives in the Teratosphaeriaceae. IMA Fungus 2, 97–103. https://doi.org/10.5598/imafungus.2011.02.01.13

Sampaio, J.P., 2004. Diversity, phylogeny and classification of basidiomycetous yeasts. In Agerer, R., Piepenbring, M., Blanz, P. (eds), Frontiers in Basidiomycote Mycology, 49–80. IHW-Verlag, Eching, Germany.

Sampaio, J.P., Fell, J.W., Gadanho, M., Bauer, R., 1999. *Kurtzmanomyces insolitus* sp. nov., a new anamorphic heterobasidiomycetous yeast species. Systematic and Applied Microbiology 22, 619–625.

Sanders, W.B., Lücking, R., 2002. Reproductive strategies, relichenization and thallus development observed in situ in leaf-dwelling lichen communities. New Phytologist 155, 425–435.

Santiago, I.F., Soares, M.A., Rosa, C.A., Luiz, ·, Rosa, H., 2015. Lichensphere: a protected natural microhabitat of the non-lichenised fungal communities living in extreme environments of Antarctica. Extremophiles 19, 1087–1097. https://doi.org/10.1007/s00792-015-0781-y

Schoch, C.L., Crous, P.W., Groenewald, J.Z., Boehm, E.W.A., Burgess, T.I., et al., 2009. A class-wide phylogenetic assessment of Dothideomycetes. Studies in Mycology 64, 1–15. https://doi.org/10.3114/sim.2009.64.01

Scorzetti, G., Fell, J.W., Fonseca, A., Statzell-Tallman, A., 2002. Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. FEMS Yeast Research 2, 495–517. https://doi.org/10.1111/j.1567-1364.2002.tb00117.x

Scorzetti, G., Petrescu, I., Yarrow, D., Fell, J.W., 2000. *Cryptococcus adeliensis* sp. nov., a xylanase producing basidiomycetous yeast from Antarctica. Antonie van Leeuwenhoek 77, 153–157. https://doi.org/10.1023/A:1002124504936

Selbmann, L., De Hoog, G.S., Mazzaglia, A., Friedmann, E.I., Onofri, S., 2005. Fungi at the edge of life: cryptoendolithic black fungi from Antarctic desert. Studies in Mycology 51, 1–32.

Selbmann, L., De Hoog, G.S., Zucconi, L., Isola, D., Ruisi, S., Ende, A.H.G.G. van den, Ruibal, C., Leo, F.D., Urzì, C., Onofri, S., 2008. Drought meets acid: three new genera in a dothidealean clade of extremotolerant fungi. Studies in Mycology 61, 1–20. https://doi.org/10.3114/sim.2008.61.01

Selbmann, L., Grube, M., Onofri, S., Isola, D., Zucconi, L., 2013. Antarctic epilithic lichens as niches for black meristematic fungi. Biology 2, 784–797. https://doi.org/10.3390/biology2020784

Selbmann, L., Isola, D., Egidi, E., Zucconi, L., Gueidan, C., De Hoog, G.S., Onofri, S., 2014. Mountain tips as reservoirs for new rock-fungal entities: *Saxomyces* gen. nov. and four new species from the Alps. Fungal Diversity 65, 67–182. https://doi.org/10.1007/s13225-013-0234-9

Shivaji, S., Prasad, G.S., 2009. Antarctic yeasts: biodiversity and potential applications. In: Yeast biotechnology: diversity and applications, pp. 3–18. Springer, Dordrecht.

Sidransky, D., 1997. Nucleic acid-based methods for the detection of cancer. Science 278, 1054–1058.

Simon, J.C., Marchesi, J.R., Mougel, C., Selosse, M.-A., 2019. Host-microbiota interactions: from holobiont theory to analysis. Microbiome 7, 1–5. https://doi.org/10.1186/s40168-019-0619-4

Smith, H.B., Grande, F.D., Muggia, L., Keuler, R., Divakar, P.K., Grewe, F., Schmitt, I., Lumbsch, H.T., Leavitt, S.D., 2020. Metagenomic data reveal diverse fungal and algal communities associated with the lichen symbiosis. Symbiosis 82, 133–147. https://doi.org/10.1007/s13199-020-00699-4

Spatafora, J.W., Aime, M.C., Grigoriev, M.F., Stajich, J.E., Blackwell, M., 2017. The fungal tree of life: from molecular systematics to genome-scale phylogenies. The Fungal Kingdom 5, 1–34. https://doi.org/10.1128/9781555819583.ch1

Spribille, T., 2018. Relative symbiont input and the lichen symbiotic outcome. Current opinion in plant biology 44, 57–63. https://doi.org/10.1016/j.pbi.2018.02.007

Spribille, T., Tagirdzhanova, G., Goyette, S., Tuovinen, V., Case, R., Zandberg, W.F., 2020. 3D biofilms: in search of the polysaccharides holding together lichen symbioses. FEMS Microbiology Letters 367, fnaa023. https://doi.org/10.1093/femsle/fnaa023

Spribille, T., Tuovinen, V., Resl, P., Vanderpool, D., Wolinski, H., Aime, M.C., Schneider, K., Stabentheiner, E., Toome-Heller, M., Thor, G., Mayrhofer, H., Johannesson, H., McCutcheon, J.P., 2016. Basidiomycete yeasts in the cortex of ascomycete macrolichens. Science 353, 488–492. https://doi.org/10.1126/science.aaf8287

Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Sterflinger, K., Krumbein, W.E., 1995. Multiple stress factors affecting growth of rock-inhabiting black fungi. Botanica Acta 108, 1438–8677. https://doi.org/10.1111/j.1438-8677.1995.tb00526.x

Sun, J.Z., Liu, X.Z., McKenzie, E.H.C., et al., 2019. Fungicolous fungi: terminology, diversity, distribution, evolution, and species checklist. Fungal Diversity 95, 337–430. https://doi.org/10.1007/s13225-019-00422-9

Sun, W., Su, L., Yang, S., Sun, J., Liu, B., Fu, R., Wu, B., Liu, X., Cai, L., Guo, L., 2020. Unveiling the hidden diversity of rock-inhabiting fungi: Chaetothyriales from China. Journal of Fungi 6, 187. https://doi.org/10.3390/jof6040187

Suryanarayanan, T.S., Thirunavukkarasu, N., 2017. Endolichenic fungi: the lesser known fungal associates of lichens. Mycology 8, 189–196. https://doi.org/10.1080/21501203.2017.1352048

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

Tagirdzhanova, G., Saary, P., Tingley, J.P., Díaz-Escandón, D., Abbott, D.W., Finn, R.D., Spribille, T., 2021. Predicted input of uncultured fungal symbionts to a lichen symbiosis from metagenome-assembled genomes. Genome Biology and Evolution 13, evab047. https://doi.org/10.1093/gbe/evab047

Teixeira, M.M., Moreno, L.F., Stielow, B.J., Muszewska, A., Hainaut, M., Gonzaga, L., Abouelleil, A., Patané, J.S.L., Priest, M., Souza, R., Young, S., et al., 2017. Exploring the genomic diversity of black yeasts and relatives (Chaetothyriales, Ascomycota). Studies in Mycology 86, 1–28. https://doi.org/10.1016/j.simyco.2017.01.001

Thanh, V.N., Smit, M.S., Moleleki, N., Fell, J.W., 2004. *Rhodotorula cycloclastica* sp. nov., *Rhodotorula retinophila* sp. nov., and *Rhodotorula terpenoidalis* sp. nov., three limonene-utilizing yeasts isolated from soil. FEMS Yeast Research 4, 857–863. https://doi.org/10.1016/j.femsyr.2004.05.002

Timling, I., Walker, D.A., Nusbaum, C., Lennon, N.J., Taylor, D.L., 2014. Rich and cold: diversity, distribution and drivers of fungal communities in patterned-ground ecosystems of the North American Arctic. Molecular Ecology 23, 3258–3272. https://doi.org/10.1111/mec.12743

Travadon, R., Lawrence, D., Vallance, J., Rey, P., 2015. Influence of pruning systems on trunk pathogens and other fungi colonizing grapevine wood. American Phytopahtological Society Abstracts, 68-O.

Travadon, R., Lecomte, P., Diarra, B., Lawrence, D.P., Renault, D., Ojeda, H., Rey, P., Baumgartner, K., 2016. Grapevine pruning systems and cultivars influence the diversity of wood-colonizing fungi. Fungal Ecology 24, 82–93. https://doi.org/10.1016/j.funeco.2016.09.003

Tsuji, M., 2018. Genetic diversity of yeasts from East Ongul Island, East Antarctica and their extracellular enzymes secretion. Polar Biology 41, 249–258. https://doi.org/10.1007/s00300-017-2185-1

Tuovinen, V., Ekman, S., Thor, G., Vanderpool, D., Spribille, T., Johannesson, H., 2019. Two Basidiomycete fungi in the cortex of wolf lichens. Current Biology 29, 476–483. https://doi.org/10.1016/j.cub.2018.12.022

Tuovinen, V., Millanes, A.M., Freire-Rallo, S., Rosling, A., Wedin, M., 2021. *Tremella macrobasidiata* and *Tremella variae* have abundant and widespread yeast stages in *Lecanora* lichens. Environmental Microbiology 23, 2484–2498. https://doi.org/10.1111/1462-2920.15455

U'Ren, J.M., Lutzoni, F., Miadlikowska, J., Arnold, A.E., 2010. Community analysis reveals close affinities between endophytic and endolichenic fungi in mosses and lichens. Microbial Ecology 60, 340–353. https://doi.org/10.1007/s00248-010-9698-2

U'Ren, J.M., Lutzoni, F., Miadlikowska, J., Laetsch, A.D., Arnold, A.E., 2012. Host and geographic structure of endophytic and endolichenic fungi at a continental scale. American Journal of Botany 99, 898–914. https://doi.org/10.3732/ajb.1100459

U'Ren, J.M., Riddle, J.M., Monacell, J.T., Carbone, I., Miadlikowska, J., Arnold, A.E., 2014. Tissue storage and primer selection influence pyrosequencing-based inferences of diversity and community composition of endolichenic and endophytic fungi. Molecular Ecology Resources 14, 1032–1048. https://doi.org/10.1111/1755-0998.12252

Urzı, C., De Leo, F., 2001. Sampling with adhesive tape strips: an easy and rapid method to monitor microbial colonization on monument surfaces. Journal of Microbiological Methods 44, 1–11. https://doi.org/10.1016/S0167-7012(00)00227-X

Vaidya, G., Lohman, D.J., Meier, R., 2011. SequenceMatrix: concatenation software for the fast assembly of multi-gene datasets with character set and codon information. Cladistics 27, 171–180. https://doi.org/10.1111/j.1096-0031.2010.00329.x

Vánky, K., 2011. Smut fungi of the world. APS press, St. Paul, MN, USA.

Vasse, M., Voglmayr, H., Mayer, V., Gueidan, C., Nepel, M., Moreno, L., De Hoog, S.G., Selosse, M.A., Mckey, D., Blatrix, R.R., 2017. A phylogenetic perspective on the association between ants (*Hymenoptera*: Formicidae) and black yeasts (Ascomycota: Chaetothyriales). Proceedings of the Royal Society B: Biological Sciences 284, 20162519. https://doi.org/10.1098/rspb.2016.2519

Vázquez-Nion, D., Rodríguez-Castro, J., López-Rodríguez, M.C., Fernández-Silva, I., Prieto, B., 2016. Subaerial biofilms on granitic historic buildings: microbial diversity and development of phototrophic multi-species cultures. Biofouling 32, 657–669. https://doi.org/10.1080/08927014.2016.1183121

Vestheim, H., Jarman, S.N., 2008. Blocking primers to enhance PCR amplification of rare sequences in mixed samples - a case study on prey DNA in Antarctic krill stomachs. Frontiers in Zoology 5, 1–11. https://doi.org/10.1186/1742-9994-5-12

Vilgalys, R., Hester, M., 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. Journal of Bacteriology 172, 4238–4246. https://doi.org/10.1128/jb.172.8.4238-4246.1990

Vishniac, H.S., Kurtzman, C.P., 1992. *Cryptococcus antarcticus* sp. nov. and *Cryptococcus albidosimilis* sp. nov., basidioblastomycetes from Antarctic soils. International Journal of Systematic Bacteriology 42, 547–553. https://doi.org/10.1099/00207713-42-4-547

Vouaux, L., 1912. Synopsis des champignons, parasites des lichens. Au siège de la société.

Wang, Q.M., Begerow, D., Groenewald, M., Liu, X.Z., Theelen, B., Bai, F.Y., Boekhout, T., 2015a. Multigene phylogeny and taxonomic revision of yeasts and related fungi in the Ustilaginomycotina. Studies in Mycology 81, 149–189. https://doi.org/10.1016/j.simyco.2015.10.004

Wang, Q.M., Yurkov, A.M., Göker, M., Lumbsch, H.T., Leavitt, S.D., Groenewald, M., Theelen, B., Liu, X.Z., Boekhout, T., Bai, F.Y., 2015b. Phylogenetic classification of yeasts and related taxa within Pucciniomycotina. Studies in Mycology 81, 55–83. https://doi.org/10.1016/j.simyco.2015.12.002

Wang, Y., Zheng, Y., Wang, X., Wei, X., Wei, J., 2016. Lichen-associated fungal community in *Hypogymnia hypotrypa* (Parmeliaceae, Ascomycota) affected by geographic distribution and altitude. Frontiers in Microbiology 7, 1231. https://doi.org/10.3389/fmicb.2016.01231

Wang, Y.-Y., Liu, B., Zhang, X.-Y., Zhou, Q.-M., Zhang, T., Li, H., Yu, Y.-F., Zhang, X.-L., Hao, X.-Y., Wang, M., 2014. Genome characteristics reveal the impact of lichenization on lichen-forming fungus *Endocarpon pusillum* Hedwig (Verrucariales, Ascomycota). BMC Genomics 15, 1–18. https://doi.org/10.1186/1471-2164-15-34

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications 8, 315–322.

Wijayawardene, N.N., Bahram, M., Sánchez-Castro, I., et al., 2021. Current insight into culture-dependent and culture-independent methods in discovering ascomycetous taxa. Journal of Fungi 7, 703. https://doi.org/10.3390/jof7090703

Wijayawardene, N.N., Crous, P.W., Kirk, P.M., Hawksworth, D.L., Boonmee, S., Braun, U., Dai, D.-Q., D, M.J., Diederich, P., Dissanayake, A., Doilom, M., Hongsanan, S., et al., 2014. Naming and outline of Dothideomycetes-2014 including proposals for the protection or suppression of generic names. Fungal Diversity 69, 1–55. https://doi.org/10.1007/s13225-014-0309-2

Wijayawardene, N.N., Hyde, K.D., Al-Ani, L.K.T., Tedersoo, L., Haelewaters, D., Becerra, A.G., Schnittler, M., Shchepin, O.N., Novozhilov, Y.K., Silva-Filho, A.G.S., 2020. Outline of Fungi and fungus-like taxa. Mycosphaere 11, 1060–1456.

Wilkinson, D.M., Creevy, A.L., Kalu, C.L., Schwartzman, D.W., 2015. Are heterotrophic and silica-rich eukaryotic microbes an important part of the lichen symbiosis? Mycology 6, 4–7. https://doi.org/10.1080/21501203.2014.974084

Wintzingerode, F., Göbel, U. B., Stackebrandt, E., 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiology Reviews 21, 213–229. https://doi.org/10.1111/j.1574-6976.1997.tb00351.x

Williams, L., Colesie, C., Ullmann, A., Westberg, M., Wedin, M., Büdel, B., 2017. Lichen acclimation to changing environments: photobiont switching vs. climate-specific uniqueness in *Psora decipiens*. Ecology and Evolution 7, 2560–2574.

Wollenzien, U., De Hoog, S.G., Krumbein, W.E., Urzí, C., 1995. On the isolation of microcolonial fungi occurring on and in marble and other calcareous rocks. Science of the Total Environment 167, 287–294. https://doi.org/10.1016/0048-9697(95)04589-S

Yamamoto, Y., Kinoshita, Y., Yoshimura, I., 2002. Culture of thallus fragments and redifferentiation of lichens. In: Kranner, I.C., Beckett, R.P., Varma, A.K. (Eds.), Protocols in Lichenology, pp 34–46. Springer Lab Manuals. Springer, Berlin, Heidelberg.

Yang, J.H., Oh, S.Y., Kim, W., Hur, J.S., 2022. Endolichenic fungal community analysis by pure culture isolation and metabarcoding: a case study of *Parmotrema tinctorum*. Mycobiology 50, 55–65. https://doi.org/10.1080/12298093.2022.2040112

Yeh, Y.-C., Needham, D.M., Sieradzki, E.T., Fuhrman, J.A., 2018. Taxon disappearance from microbiome analysis reinforces the value of mock communities as a standard in every sequencing run. mSystems 3, e00023-18. https://doi.org/10.1128/msystems.00023-18

Yoshino, K., Yamamoto, K., Masumoto, H., Degawa, Y., Yoshikawa, H., Harada, H., Sakamoto, K., 2020. Polyol-assimilation capacities of lichen-inhabiting fungi. The Lichenologist 52, 49–59. https://doi.org/10.1017/S0024282919000483

Yu, N.H., Park, S.Y., Kim, J.A., Park, C.H., Jeong, M.H., Oh, S.O., Hong, S.G., Talavera, M., Divakar, P.K., Hur, J.S., 2018. Endophytic and endolichenic fungal diversity in maritime Antarctica based on cultured material and their evolutionary position among Dikarya. Fungal Systematics and Evolution 2, 263–272. https://doi.org/10.3114/fuse.2018.02.07

Yurkov, A.M., Wehde, T., Federici, J., Schäfer, A.M., Ebinghaus, M., Lotze-Engelhard, S., Mittelbach, M., Prior, R., Richter, C., Röhl, O., Begerow, D., 2016. Yeast diversity and species recovery rates from beech forest soils. Mycological Progress 15, 845–859. https://doi.org/10.1007/s11557-016-1206-8

Zamora, J.C., Millanes, A.M., Wedin, M., Rico, V.J., Pérez-Ortega, S., 2016. Understanding lichenicolous heterobasidiomycetes: new taxa and reproductive innovations in *Tremella* s.l. Mycologia 108, 381–396. https://doi.org/10.3852/15-090

Zamora, J.C., Pérez-Ortega, S., Rico, V.J., 2011. *Tremella macrobasidiata* (Basidiomycota, Tremellales), a new lichenicolous fungus from the Iberian Peninsula. The Lichenologist 43, 407–415. https://doi.org/10.1017/S0024282911000405

Zhang, T., Jia, R.L., Zhang, Y.Q., Liu, H.Y., Su, J., Yu, L.Y., 2013. *Kurtzmanomyces shapotouensis* sp. nov., an anamorphic, basidiomycetous yeast isolated from desert soil crusts. International Journal of Systematic and Evolutionary Microbiology 63, 3892–3895. https://doi.org/10.1099/ijs.0.053058-0

Zhang, T., Wie, X.L., Zhang, Y.Q., Liu, H.Y., Yu, L.Y., 2015. Diversity and distribution of lichen-associated fungi in the Ny-Ålesund Region (Svalbard, High Arctic) as revealed by 454 pyrosequencing. Scientific Reports 5, 14850.

Zhang, T., Wei, X.L., Wei, Y.Z., Liu, H.Y., Yu, L.Y., 2016. Diversity and distribution of cultured endolichenic fungi in the Ny-Ålesund Region, Svalbard (High Arctic). Extremophiles 20, 461–470. https://doi.org/10.1007/s00792-016-0836-8

APPENDIX 1

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

Agnese Cometto[1*], Claudio G. Ametrano[1] & Lucia Muggia[1]

[1]Department of Life Sciences, University of Trieste, via L. Giorgieri 10, 34127, Trieste, Italy

* Corresponding author: Agnese Cometto. Address: University of Trieste, Department of Life Sciences, via Giorgieri 10, 34127 Trieste, Italy. E-mail: agnese.cometto@phd.units.it

Abstract

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

Keywords

Chaetothyriales, Dothideomycetes, *Myrmecia*, symbiosis, *Trebouxia*.

Introduction

Rocks represent one of the oldest terrestrial niches hosting a multiplicity of life forms, whose diversity has been only partially uncovered to date. The presence of rock-associated microorganisms was documented by Diels (1914) for the first time, but it was only in the 1970s that

Friedmann and Galun (1974) highlighted the existence of bacteria and microalgae on rock surfaces and within rocks, giving shape to the modern research field of geobiology. In the evident scenario of climate change, geo(micro)biologists nowadays suggest that rocks are cradle of habitats where microorganisms find protection from the continuous fluctuation of temperatures, UV radiation, humidity, salinity and deposition of inorganic and organic nutrients (Gorbushina 2007; Onofri et al. 2007a; De Los Ríos et al. 2014; Selbmann et al. 2015; Coleine et al. 2021).

Rock-associated organisms are recognized into the two main categories of epiliths (those colonizing the exposed, external rock surfaces) and endoliths (those colonizing the rock matrices inside; Golubic et al. 1981). The endoliths further differentiate into cryptoendolithic (those hiding into the rocks, i.e., colonizing natural empty pores and fissures within the rock but connected indirectly to the rock surface), chasmoendolithic (those which grow in fissures and cracks connected to the rock surface), and hypoendolithic (those which colonize the underside of rocks in contact with the underlying soil (Wierzchos et al. 2011, 2012). Particularly in extreme environments, climate conditions play a key role in determining the type of endolithic growth the microbes can adopt. Epilithic organisms can build extensive and massive covers over rocks and are usually dominated by lichens (e.g. Carter and Viles 2003; Baur et al. 2007; Bjelland et al. 2011; Selbmann et al. 2013) and mosses (e.g. Spitale and Nascimbene 2012; Jackson 2015; Nelson et al. 2020). Lichens are successful colonizers of rocks also in extreme environments: such when conditions become harsher, even lichens can enter the first millimeters of rocks, and become endolithic (Friedmann 1982; Nienow et al. 1988; Onofri et al. 2007a), further enriching the community of other microfungi, microalgae and bacteria. This phenomenon is extremely evident and well documented for high mountain peaks and crests, and Antarctica, where other organisms cannot cope with high solar radiation, long snow cover, very low temperature or strong winds (Boustie et al. 2011; Zucconi et al. 2016; Armstrong 2017). In such cases, it seems like there would be a loss of biodiversity, which is partially compensated by the diversity of endolithic microorganisms. Rock pits and crevices represent for them more protected niches: they adapt their body structures to become more and more inconspicuous, optimizing the surface/volume ratio and becoming difficult to be detected (Zucconi et al. 2016). The dominance of endoliths is indicative of harsh environmental conditions, which select a relatively low phylogenetic diversity of microorganisms in which many closely related species diversify into own lineages (Walker and Pace 2007). Walker and Pace (2007) described the endolithic metacommunities formed by bacteria, cyanobacteria, microalgae and microfungi as true ecosystems which can be specifically adapted to different rock types. In fact, endolithic organisms usually colonize at first orthoquartzite rocks, but they are also able to settle on granite (De Los Ríos et al. 2005; Selbmann et al. 2017), halite

(Gómez-Silva 2018), gypsum (Wierzchos et al. 2015) and carbonatic rocks (Di Ruggiero et al. 2013; Crits-Christoph et al. 2016). Indeed, the different 'rock architectures', given by the type of the rock and its porosity (Wierzchos et al. 2015), influence the possibility to colonize the rocks only superficially or deeper into the first millimetres. At the same time, the endolithic microorganisms play an important role in the biogeochemical degradation processes of the lithological substrate by the extracellular release of secondary metabolites (acids), which contribute to the transformation of mineral and metals according to the chemical and structural features of the rocks (Gadd 2007).

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

Fungi indeed are the organisms best adapted to endolithic growth, especially in habitats where conditions are at the edge of life. These rock-inhabiting fungi (RIF; Ruibal et al. 2009) usually are heavily melanised and present a microcolonial type of growth (Wollenzien et al. 1995). Their peculiar morphology and physiological traits allow them to thrive under prolonged desiccation, high solar/UV irradiation, osmotic stress, extreme temperatures and limited nutrient availability (Sterflinger 1998; Ruibal et al. 2005; Onofri et al. 2007b; Gorbushina et al. 2008). The strong melanisation of their cell walls (Dadachova and Casadevall 2008) confers them a polyextremotolerance towards a multiplicity of abiotic stress factors (Gostinčar et al. 2011, 2012b;

Pacelli et al. 2020), while the typical isodiametrical (meristematic) growth allow them to reach the optimal volume/surface ratio to cope with extreme temperatures and desiccation (Wollenzien et al. 1995), but also to thrive in the tiniest spaces of the rocks. RIF belong to two main classes of fungi of Eurotiomycetes (mostly Chaetothyriales) and Dothideomycetes (mostly Capnodiales, Pleosporales, Myriangiales and Dothideales; Gueidan et al. 2008, 2011; Ruibal et al. 2009; Muggia et al. 2016, 2020; Coleine et al. 2020; Gostinčar 2020). Previous evolutionary studies inferred a rock-inhabiting ancestor for either classes (Gueidan et al. 2011), hypothesizing the evolution of Dothideomycetes RIF in cold and dry conditions, whereas Eurotiomycetes RIF would have specialized to hot, semi-arid but also humid environments (Gueidan et al. 2008, 2011).

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

Material and methods

Sampling

Small rocks were collected on the top of the two mountains Cerro Mercedario and Muztagh Ata (Fig. 1a,b), both 6700 m above sea level (a.s.l.). Both mountain summits represent extreme environments at the edge of life, as they remain completely covered by snow for most of the year; the very strong winds can blow snow away and let the high solar radiation hitting the rock surfaces. This, together with the very low precipitation regimes, makes the environment to be extremely dry and either summit is completely devoid of vegetation.

Cerro Mercedario is the highest peak of the Cordillera de la Ramada range and the eighth highest mountain of the Cordillera de los Andes (Argentina, province of San Juan, 31°58′45″ S/ 70°06′45″ W; Fig. 1a). It is 6720 m a.s.l. high and made of volcanic acidic rocks with feldspar and pyroxene/amphibolite (Figs. 1c-e). Temperature minimum can reach below -40 °C, though when no

wind blows, at the rock surface it can reach several degrees above 0 °C. The nearest meteorological station, about 80 km south, is Cristo Redentor, set at 3109 m a.s.l.: here the mean annual temperature measured are about -1.6 °C and the total annual precipitation is 360 mm (De Jong et al. 2013; Kinnard et al. 2020).

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

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

Culture isolation

Rock pieces were fragmented from three different rocks coming from each mountain summit. The pieces were washed 10 minutes in a sterile water, followed by 15 minutes of washing with Tween80 (0.1%) and rinsed four times for 10 minutes with sterile water to remove the detergent. The rock pieces were dried in sterile conditions under the biological hood, wrapped into sterilized aluminum foil, and fragmented by hammering them. The small fragments were taken with a sterile forceps and placed on solid Bold Basal Medium (BBM; Bischoff and Bold 1963) in Petri dishes. Six plates were prepared for each locality, and each plate was inoculated with five rock fragments. The plates were incubated under three different conditions: a) at 17 °C with a light/dark cycle of 14/10 h and 20 μmol $\times$ photons m^{-2} $\times$ s $^{-1}$; b) 20 °C with a light/dark cycle of 14/10 h and 20 μmol $\times$ photons m^{-2} $\times$ s $^{-1}$; c) at 3 °C in complete darkness in the fridge. The Petri dishes were inspected every month until colonies of fungi or algae appeared. After about 10 months we observed the first development of a few fungal and algal colonies which started to grow out of the rock fragments. After about 16

months, these colonies have grown to a sufficient biomass allowing sub-cultivation, and were transferred individually on new BBM, malt yeast (MY, for fungi) and *Trebouxia* media (TM, for algae; Ahmadjian 1987). After about further six months, the subcultures have grown sufficiently to allow molecular analyses and morphological identification.
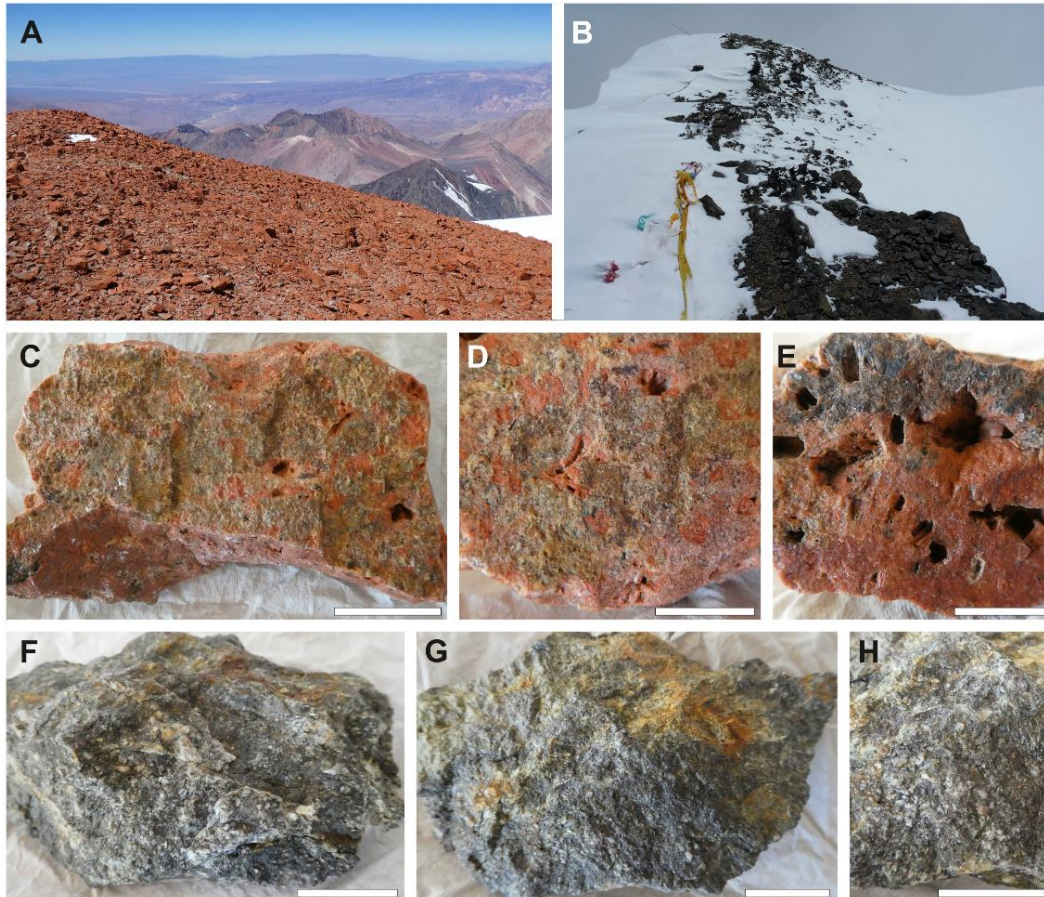
Figure 1. Original environments and rock samples from which the fungal and algal strains were isolated: Cerro Mercedario summit 6720 m a.s.l (A).; Muztagh Ata summit 7534 m a.s.l. (B); volcanic acidic rock with feldspar and pyroxene/amphibolite from Cerro Mercedario (C-E); low degree metamorphic, pelitic rock with sedimentary mica-schist from Muztagh Ata (F-H). Scale bars: 2 cm (C, D, F-H); 1 cm (E).

Molecular analyses: DNA extraction, PCR amplification and sequencing

Small parts of the cultured fungal and algal colonies were taken with a sterile inoculation loop and transferred into a 1.5 ml reaction tubes, containing three sterile tungsten beads for homogenization, frozen and ground using a TissueLyserII (Retsch). The DNA extractions were performed following the CTAB protocol of Cubero et al. (1999), with minor adjustments. The identity of fungal strains was checked with sequences of the nuclear internal transcribed spacers (nucITS) and 5.8S rDNA ribosomal gene, amplified with the primers ITS1F (Bruns and Gardes 1993) and ITS4 (White et al. 1990), and the D1/D2 domain of the 28S nuclear large ribosomal subunit (nucLSU), amplified with

the primers LR0R and LR5 (Vilgalys and Hester 1990; http://www.biology.duke.edu/fungi/mycolab/primers.htm). The identity of the algal strains was checked with the sequences of the nucITS, amplified with the primers ITS1T and ITS4T (Kroken and Taylor 2000) and of the ribulose-1,5- biphosphate carboxylase large subunit (*rbcL*), amplified with the primers rbcL320 and rbcL803 (Nelsen et al. 2011). Polymerase chain reactions (PCR) were prepared for a 25 µl final volume containing 5 µl DNA, 12.5 µl of AccuStart II PCR ToughMix, 0.4 µl for each of the 10 µM primers. PCR amplifications were performed under the following conditions: one initial heating step of 3 minutes at 94 °C linked to 35 cycles of 45 seconds at 94 °C, 45 seconds at 55 °C, 1 minute at 72 °C, and one final extension step of 5 minutes at 72 °C after which the samples were kept at 4 °C. A negative control reaction was always used to check for contamination. All the amplicons were checked for their quality and size by 1% agarose gel electrophoresis stained with Green Safe Gel (Sigma-Aldrich) and purified using Mag-Bind® Normalizer Kit (Omega bio-tek). Clean amplicons were sent for Sanger sequencing to Macrogen Europe (The Netherlands).

Phylogenetic analyses

The identity of the newly generated fungal nucITS and nucLSU and the algal nucITS and *rbcL* sequences was checked with BLAST similarity search (Altschul et al. 1990) using sequences available in Genbank (NCBI) database. To delineate the systematic relationships of the isolated taxa, we built a multiple sequence alignment (MSA) for each sequenced locus, and for each of the four major taxonomic group identified, i.e. Chaetothyriales and Dothideomycetes for the fungal strains, and *Trebouxia* s. str. and Trebouxiophyceae s. lat. ‒ including *Myrmecia* spp. ‒ for the algal strains. The MSAs included both the closest Genbank matches that were recovered for our strains and a broader taxon sampling comprising closely related genera and families selected from previous studies (Supplementary Tables S1-S4). In particular, the Chaetothyriales dataset was based on Quan et al. (2020) and Muggia et al. (2021), that of Dothideomycetes was based on Ametrano et al. (2019a), that of *Trebouxia* s. str. on Muggia et al. (2020) and De Carolis et al., (2022), and that of Trebouxiophyceae s. lat. including *Myrmecia* spp. was based on Samolov et al. (2020) and Moya et al. (2018). The MSAs were prepared in Bioedit v.7.2.5 (Hall 1999) and they were aligned with MAFT v.7 (Katoh and Standley 2013) using the g-ins-I alignment strategy. Ambiguously aligned positions and introns were manually removed from the alignments. Single locus phylogenies were inferred with Maximum Likelihood (ML) and Bayesian Inference (BI) approaches, the analyses were run on CIPRES Science Gateway v.3.3 (Miller et al. 2011). RAxML v.8.2 (Stamatakis 2014) was used for the ML analysis applying GTRGAMMA substitution model and 1000 bootstrap pseudoreplicates. The BI analysis was performed with the program MrBayes v.3.2 (Ronquist et al. 2012) running five million generations with six chains and a random starting tree. Tree sampling

was performed every 100 generations, the first 25% of data were discarded as burn-in. After checking the phylogenetic concordance of the two loci, they were concatenated (nucITS and nucLSU for fungi, nucITS and *rbcL* for algae) with MEGA (Kumar et al. 2018) and then analysed with both RAxML and MrBayes with the same settings of the single locus analyses. Normalized Robinson-Fould (nRF; Robinson and Foulds 1981) distance between the ML and BI phylogenies was calculated using ETEtoolkit v3.1.2 (Mutawalli et al. 2019). To visualize the mismatches between the ML and BI topologies we used an R script with the cowplot library (R Development Core Team 2019). The phylogenetic trees were plotted in ITOL (Letunic and Bork 2019).

Morphological analysis

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

Results

Culture isolation

A total of nine fungal and five algal strains were isolated and identified (Figs. 2-5). In particular, six fungal strains (L3140, L3141, L3144, L3156, L3157 and L3159) belonging to the order of Chaetothyriales (Fig. 2), and four algal strains (L3142, L3145, L3147 and L3148) belonging to the genus *Myrmecia* (Fig. 4) were isolated from the rocks collected on Cerro Mercedario, while three fungal strains (L2633, L2634 and L3151) belonging to the class Dothideomycetes (Fig. 3) and one algal strain (L3150) belonging to the genus *Trebouxia* (Fig. 5) were isolated from the rocks collected on Muztagh Ata.

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

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

ID culture	Rocks of origin	Temperature growth chamber	Phylogentic placement	nucITS	nucLSU	*rbcL*
2633	Muztagh Ata	20 °C	*Coniosporium* sp.	ON620069	ON569432	-
L2634	Muztagh Ata	20 °C	*Coniosporium* sp.	ON620070	ON569433	-
L3151	Muztagh Ata	17 °C	Dothideomycetes sp.	ON620071	ON569434	-
L3150	Muztagh Ata	17 °C	*Trebouxia* sp. A15	ON620064	-	ON603529
L3140	Cerro Mercedario	17 °C	Chaetothyriomycetes sp.	ON620072	ON569435	-
L3141	Cerro Mercedario	17 °C	Chaetothyriomycetes sp.	ON620073	ON569436	-
L3144	Cerro Mercedario	17 °C	Chaetothyriomycetes sp.	ON620074	ON569437	-
L3156	Cerro Mercedario	3 °C	Chaetothyriomycetes sp.	ON620075	ON569438	-
L3157	Cerro Mercedario	3 °C	Chaetothyriomycetes sp.	ON620076	ON569439	-
L3159	Cerro Mercedario	3 °C	Chaetothyriomycetes sp.	ON620077	ON569440	-
L3142	Cerro Mercedario	17 °C	*Myrmecia* sp.	ON620065	-	ON603530
L3145	Cerro Mercedario	17 °C	*Myrmecia* sp.	ON620066	-	ON603531
L3147	Cerro Mercedario	17 °C	*Myrmecia* sp.	ON620067	-	ON603532
L3148	Cerro Mercedario	17 °C	*Myrmecia* sp.	ON620068	-	ON603533

Phylogenetic and morphological analysis

We obtained a total of nine new nucITS and nucLSU fungal sequences, and five new nucITS and *rbcL* algal sequences. We report the phylogenetic analyses performed for each taxonomic group – Chaetothyriales, Dothideomycetes, *Myrmecia* and *Trebouxia* – based on the concatenated datasets of fungal nucITS-nucLSU and algal nucITS-rbcL, respectively. The Bayesian and the ML phylogenetic inferences were highly concordant, and clades were well supported and topologically congruent with previously published phylogenies (Moya et al. 2018; Ametrano et al. 2019b; Muggia et al. 2020, 2021; Quan et al. 2020; Samolov et al. 2020). The phylogenetic distance between the ML and BI topologies was 0.23 nRF for Chaetothyriales, 0.24 nRF for Dothideomycetes, 0.30 for *Myrmecia* and 0.17 for *Trebouxia*. At the family level, only one conflicting placement was reported. The family Chaethothyriaceae (Chaetothyriales) was placed closely related to Trichomeriaceae and the lineage formed by our newly sequenced samples in the ML analysis, whereas it was placed closely related to the clade *Melanina* by the Bayesian inference (Supplementary Figs. S1, S2, S3, S4).

Chaetothyriales (Fig. 2) – The strains L3140, L3141, L3144, L3156, L3157 and L3159 isolated from Cerro Mercedario, formed a separate and well supported clade together with to specimens named *Exophiala* sp. HF22 and *Exophiala* sp. 4-11C, and three uncultured fungi (FR682329, ZSH201207 and ZSH201205; Fig. 2a). These strains are characterized by a dense, melanized mycelium, which reached about 2 cm in diameter after growing about six months on MY

medium (Fig. 2b). The hyphae are hyaline to heavily melanized, septate, cells are elongated, almost rectangular in shape (12 × 4 μm; Figs. 2c-h).

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

On MY medium L2633 and L2634 (Fig. 3b) developed a melanized mycelium which reached about 2 cm in diameter after about six months. These strains were characterized by two types of hyphal cells: melanized, rectangular (10 × 5 μm) cells along the hyphae (Figs. 3c, e-g); moniliform, ovoid to round and heavily melanized cells resembling conidia (10 μm) towards the terminal parts of the hyphae (Figs. 3c-f).

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

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

Trebouxia (Fig. 5) – The strain L3150, isolated from Muztagh Ata rocks, was found in the *Trebouxia* clade 'A' (Fig. 5a) and it is closely related to the species level lineage *Trebouxia* 'A15' (*sensu* Leavitt et al. 2015; Muggia et al. 2020). The colony develops three-dimensionally in a coralloid type of growth typical of *Trebouxia* colonies (Fig. 5b). The cells are coccoid, of about 18 μm in diameter; the chloroplast is massively occupying almost the entire volume of the cytoplasm and the nucleus is confined at one side of the cell (Figs. 5c-d). Autospores were not observed in L3150 culture, but several cells seemed to be in the first mitotic division phase.
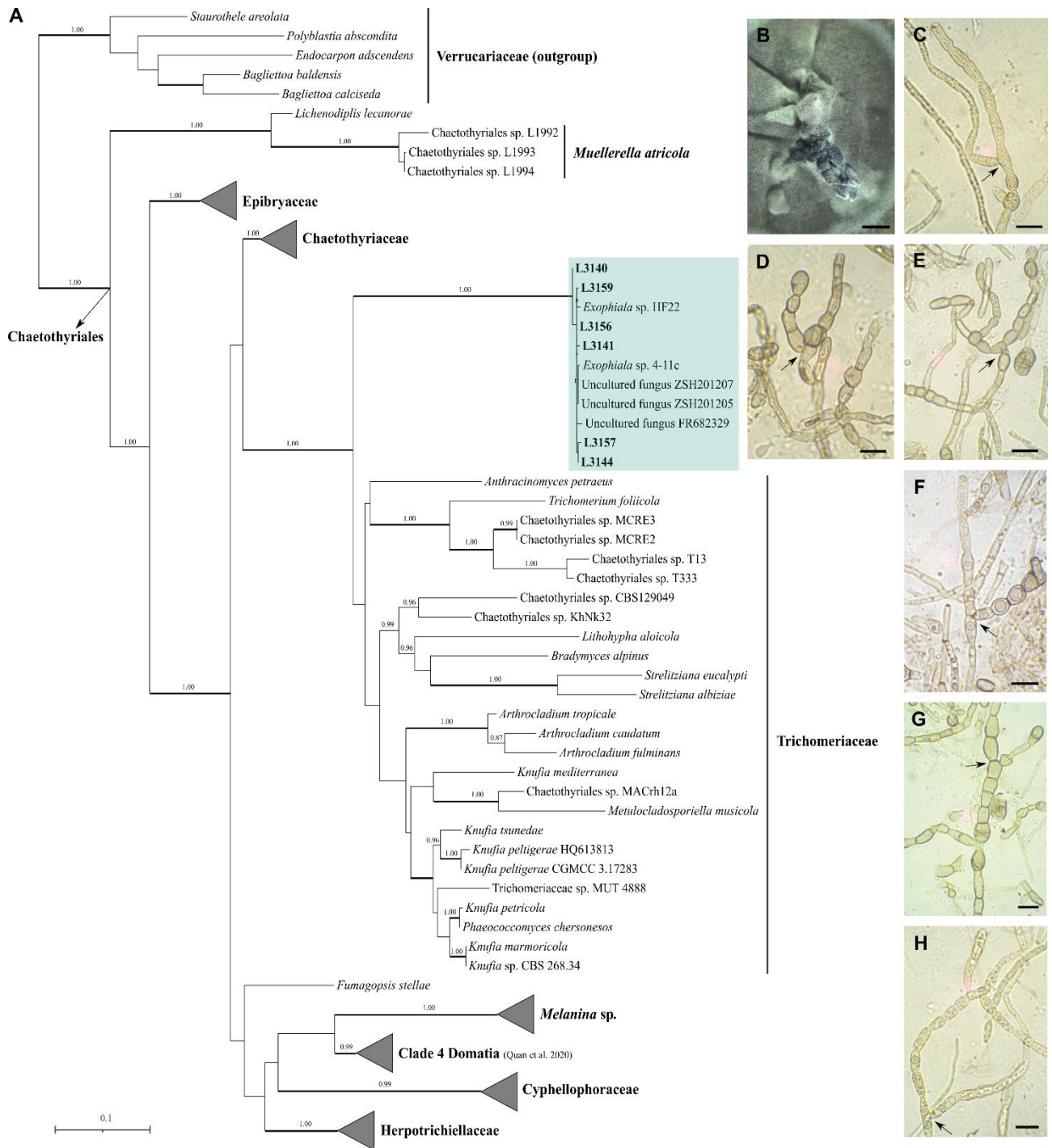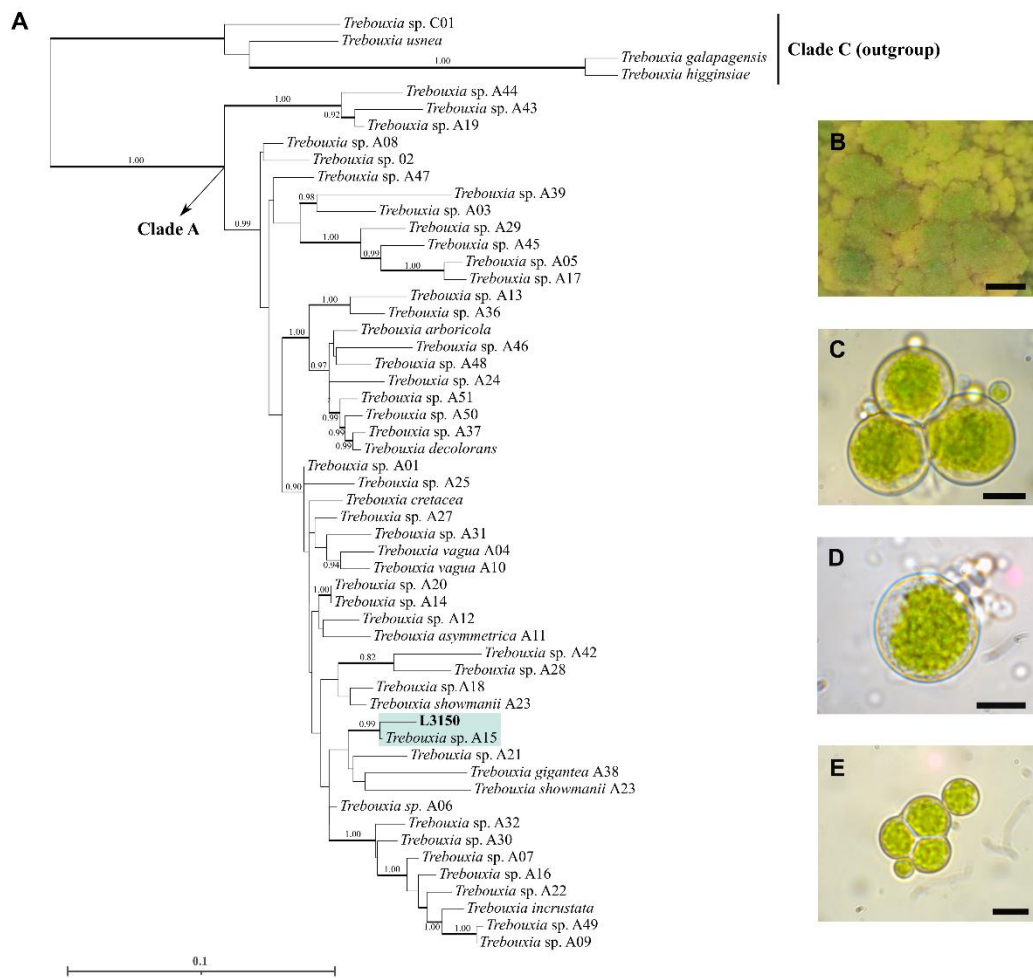
Figure 2. Phylogenetic inference of Chaetothyriales: Maximum Likelihood analysis based on the concatenated nuclear ITS-LSU dataset (A); branches in bold denote RAxML bootstrap support ≥75%; Bayesian posterior probabilities ≥0.8 are reported above branches; newly obtained sequences are in bold and the corresponding clade is highlighted in light blue. Morphology of six-month old representative cultured Chaetothyriales strains (B-H): colony shape on solid malt yeast medium of L3144 (B), filamentous and septate hyphae with branches of L3140 (C), L3159 (D), L3156 (E), L3141 (F), L3157 (G) and L3144 (H). Scale bars: 2 mm (B), 10 μm (C-H).

161
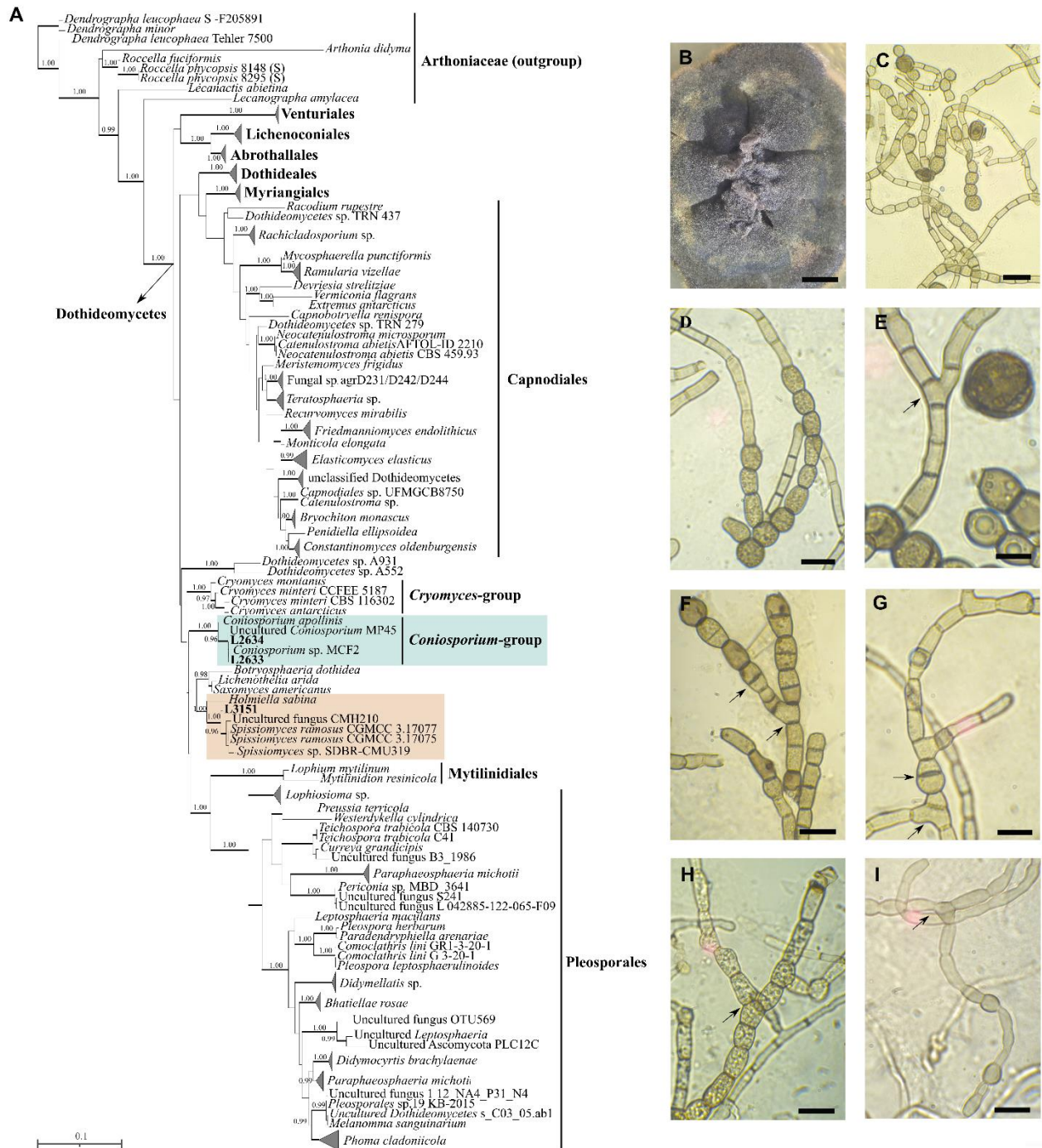
Figure 3. Phylogenetic inference of Dothideomycetes (A): Maximum Likelihood analysis based on the concatenated nuclear ITS-LSU dataset; branches in bold denote RAxML bootstrap support ≥75%; Bayesian posterior probabilities ≥0.8 are reported above branches; newly obtained sequences are in bold and the corresponding clades are highlighted in light blue and orange. Morphology of six-month old representative cultured Dothideomycetes strains (B-I): colony shape on solid malt yeast medium of L3151 (B), filamentous and septate hyphae with branches of L2633 (C-E), L2634 (F, G) and L3151 (H, I). Scale bars: 2 mm (B), 20 μm (C), 10 μm (D-H).
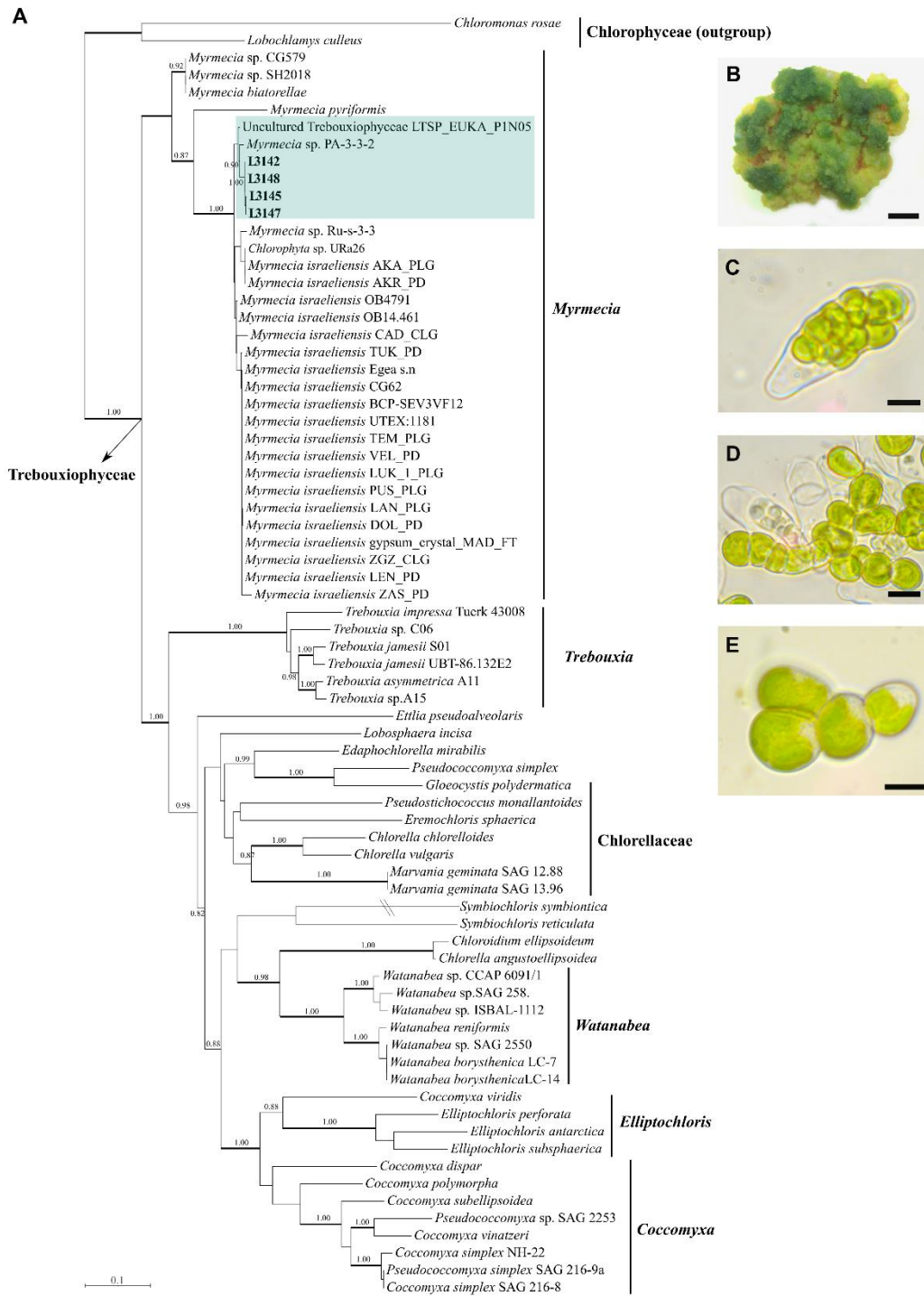
Figure 4. Phylogenetic inference of *Myrmecia* (A): Maximum Likelihood analysis based on the concatenated nuclear ITS-rbcL dataset; branches in bold denote RAxML bootstrap support ≥75%; Bayesian posterior probabilities ≥0.8 are reported above branches; newly obtained sequences are in bold and the corresponding clade is highlighted in light blue. Morphology of six-month old representative cultured *Myrmecia* strains (B-E): colony shape on solid *Trebouxia* medium of strain L3145 (B), autospore (C), mature cells (D, E). Scale bars: 2 mm (B), 10 μm (C-E).

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

Discussion

We have documented here for the first time the isolation and the taxonomic characterization of fungi and algae from altitudes over 6000 m a.s.l.. The fungal and algal strains started to grow in culture after ten months from the rock fragments were inoculated on the media and have developed extremely slowly their mycelia and cell colonies, respectively. This hints to the generally extremely slow growth of these microorganisms, used to cope with harsh conditions (i.e. endolithic and in selective environments) as well as to their need to adapt to *in vitro* culture. The isolation of microorganisms in axenic culture is crucial to perform the morphological characterization of taxa, especially for species new to science, which otherwise could not be characterized if only environmental DNA (eDNA) was analyzed. In the present study, the cultural approach was

preferred, the samples were indeed characterized by an extremely low amount of mixed biomass embedded in the rock, which was often unsuitable for direct extraction, amplification and Sanger sequencing. The culture-dependent approach is flawed by the fact that only a fraction of the microorganisms can grow in culture, leaving the rest of the diversity undetected. This certainly leads to an underestimation of the entire biodiversity of the rock communities (Wijayawardene et al. 2021). Moreover, cultures may be affected by contaminant microorganisms which can be erroneously included in the original community. These contaminants can even overgrow the native species from extreme environments, which often need longer time to develop in culture. Nevertheless, the low success rate of culture isolation, the low number of isolated strains and their extremely slow growth rate is reassuring about the fact that we detected part of the actual diversity of these extreme rock environments. The algal cultures also showed a certain preference for lower temperatures, as all the strains grew at 17 °C. In contrast, fungal cultures did not show any preference to the temperature, as Chaetothyriomycetes sp. grew at 17 and 3 °C, *Coniosporium* sp. at 20 °C and Dothideomycetes sp. L3151 at 17 °C. However, the optimal growth temperature should be properly tested with *ad hoc* experiments, potentially considering a larger temperature gradient, which was, though, behind the scope of this study.

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

The six fungal strains belonging to the order Chaetothyriales (isolated from Cerro Mercedario: L3140, L3141, L3144, L3156, L3157 and L3159) segregated into a fully supported clade basal to the family Trichomeriaceae, which includes epiphytic (Chomnunti et al. 2012) and rock inhabiting species (Isola et al. 2016). This clade comprises additional black fungi, i.e. two sample named *Exophiala* sp. HF22 and *Exophiala* sp. 4-11C, and three uncultured taxa (FR682329, ZSH201207 and ZSH201205; Fig. 2a). The sequence diversity of both nuclear LSU and ITS markers is very low among our strains and between these and the two "*Exophiala"* samples and the three uncultured taxa. As this new clade is supported by a long branch, it seems to have diversified significantly from the rest of Tricomeriaceae. This phylogenetic placement may suggest the clade as a potential new taxon deserving formal description, however this goes beyond the scope of the present study and additional data are needed. Notwithstanding the phylogenetic position of the here

isolated strains, the lineage comprises two samples named *Exophiala* which may represent misidentified samples. Indeed *Exophiala* is a genus confirmed to belong to Herpotrichiellaceae (Quan et al. 2020; Muggia et al. 2021) and this has been strengthen by our results. The present dataset, indeed, includes 13 species of *Exophiala* (Supplementary Table S1) which, among others, represent the collapsed Herpotrichiellaceae clade in the phylogenetic analyses (Fig. 2). All these black fungi share rather peculiar and selective environments of origin, as they come either from saline, rock and contaminated substrate, which may support their monophyletic lineage, likely hinting to the recognition of a new taxon. In fact, our strains were isolated from volcanic acidic rock with feldspar and pyroxene/amphibolite, whereas the sample named *Exophiala* sp. HF22 was isolated from 3100-year-old staircase in salt mine of Hallstatt (Austria; Piñar et al. 2016), and that named *Exophiala* sp. 4-11c from cadmium contaminated soil (Long and Zhu unpublished work). The uncultured fungus FR682329 was detected from building material (Pitkäranta et al. 2011), whereas the other two uncultured fungi were sequenced from rainwater samples (Du et al., direct submission to NCBI). It is well known that black melanised fungi have adapted to halophythic and endolithic growth (Kogej et al. 2005; Gunde-Cimerman et al. 2011), but they also have been detected in waters and rainwater (Babič et al. 2017), where melanisation protect them from the high UV radiation in the atmosphere, thus making rainwater an extreme environment as well. The identification of this clade comprising potentially polyextremotolerant fungi (Gunde-Cimerman et al. 2011; Gostinčar et al. 2012a) within Chaetothyriales further strengthen the renown of this order as one of the fungal lineages in which the greatest diversity of lifestyle and a complex ecological versatility has evolved (De Hoog 2014; Teixeira et al. 2017, Zhang et. al 2020).

Interestingly, from the Muztagh Ata rocks three dothideomycetous strains were instead isolated. These are identified in two different clades, both formed by rock inhabiting fungi (RIF) and closely related to other RIF clades, here represented by *Cryomyces* spp., *Saxomyces* and *Lichenothelia*. The two strain L2633 and L2634 are nested with two *Coniosporium* specimens isolated from limestones and one endophytic *Coniosporium* (uncultured *Coniosporium* MP45). Dothideomycetes sp. L3151 is basal to a clade formed by two samples of *Spissiomyces ramosus* isolated form rocks (Su et al. 2015) and one from plant (*Spissiomyces* sp. SDBR-CMU319); the epiphytic fungus *Holmiella sabina* is basal to these samples (Fig. 3a). The phylogenetic position of the RIF *Coniosporium* species was originally discussed by Ruibal et al. (2009), who demonstrated that *Coniosporium apollinis* and *C. uncinatum* belong to Dothideomycetes. Later, Selbmann et al. (2005, 2011) described two new species of Antarctic rocks-inhabiting meristematic fungi able to form cryptoendolithic community, i.e. *Cryomyces antarticus* and *C. minteri*, they showed that they were closely related to the *Coniosporium* clade. Our present results further support these

evolutionary relationships, as the strains we identified seem to enrich the diversity of already known lineages. Also, the close phylogenetic placement of the two RIF genera *Lichenothelia* with *Saxomyces* inferred by our analysis was previously presented by Ametrano et al. (2019a, b). Interestingly, these two genera do come from environments similar to that of Muztagh Ata, which is compatible with their close relationships with our strain.

It is of particular interest that the isolated algal strains correspond to *Trebouxia* and *Myrmecia*, as both genera can occur either free living (Bubrick et al. 1984; Yung et al. 2014) or are more notably known to be photobionts in lichen symbioses (Rambold et al. 1998; Tschermak-Woess 2019). However, we could neither detect any sign of lichen thalli on the rocks, nor it was possible to spot any algal colony by inspecting the original rock fragments in stereo-microscopy. It is likely that these algae would reside in the tiniest and most hidden rock crevices, being invisible to the eyes. More specifically, the genus *Trebouxia* is one of the most common and best studied lichen photobionts (see Muggia et al. 2020; Bordenave et al. 2021; De Carolis et al. 2022), for which the genetic and morphological diversities were recently clarified by pursuing an integrative taxonomic approach. The strain L3150 here isolated is sister to the species-level lineage *Trebouxia* 'A15', included in the clade 'A' (Beck et al. 2002; Leavitt et al. 2015; Muggia et al. 2020; De Carolis et al. 2022) and it is closely related to the species-level lineages recognized as the '*gigantea*-group' (Muggia et al. 2020). So far, this strain could be only genetically identified, but future analyses should address its morphological characterization by investigating the ultrastructure of its chloroplast (pyrenoid included), likely confirming its affiliation to the 'gigantea-group' (Bordenave et al. 2021).

The *Myrmecia* sp. strains (L3142, L3145, L3147 and L3148) form a clade together with a *Myrmecia* sp. and an uncultured Trebouxiophyceae samples identified from biological soil crusts and forest soil, respectively (Samolov et al. 2020; Hartmann et al. 2009). This clade is unresolved with *M. israeliensis* (Fig. 4a) which is the primary symbiotic microalga in the lichen genera *Psora* spp., *Placidium* spp. and *Clavascidium* spp. (Moya et al. 2018). Lichen species of these three genera grow on soil and often form conspicuous biological soil crusts in either cold or warm, arid and desert habitats (Lewis and Lewis 2005; Flechtner et al. 2013; Fučíková et al. 2014; Samolov et al. 2020). As on Cerro Mercedario the rocks were laying on soil, it is reasonable that *Myrmecia* cells/colonies, isolated from there, could have reside in the rock crevices, and thus has grown in culture. As we neither detect any layer of melanised fungi that could form a protective layer above the algae (as often documented for cryptoendolithic microbial communities; Selbmann et al. 2013, Gorbushina et al. 2005), nor any lichen mycobiont was isolated, we assume that these algae were growing free-living in/on the rock (Yung et al. 2014). They would likely receive protection from

high solar UV radiation by the rock matrix itself, colonizing the inner pits. Indeed, Wong et al. (2010) identified even free-living *Trebouxia* in hypolithic communities of the Tibetan tundra, supporting the hypothesis of a general phenomenon for extreme cold-arid landscapes.

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

The altitude factor may also play an important role in the specie selections. Stevens (1989, 1992) introduced the term Rapoport's elevation (RE) gradients to indicate the broader distribution range of organisms living at a higher altitude than of those distributed at lower elevation. This concept is only partially in accordance with what we found in these two high-elevation, far apart, environments. The high selective pressure possibly led to few species able to adapt to this extreme, but rather homogeneous, ecological niches; we indeed detected a very low diversity of algae and fungi. However, we have no evidence that the same algal or fungal species are present in both sites. Fungal diversity of mountain regions is only partially known, as most of the works focused on specific fungal groups such as arbuscular mycorrhizal (AM) fungi, macromycetes, ectomycorrhizal (EcM; Liu et al. 2011; Velázquez et al. 2016). Also, microbial composition of soil at low-mid elevations is usually dominated by Basidiomycota in comparison to Ascomycota and Zygomycota (Praeg et al. 2020; see James et al. 2020 for the new reappraisal of the Phylum Zygomycota). At high elevations, instead, the abundance of Ascomycota, Chytridiomycota, and Glomeromycota tend to increase and the dominant classes have been shown to be Agaricomycetes, Sordariomycetes, Dothideomycetes and Leotiomycetes and Zygomycota (Ogwu et al. 2019), supporting our finding on the fungal diversity in rocks collected at high elevation. The culturable fungi we detected were indeed all ascomycetes.

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

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

Acknowledgments

Funding

References

Ahmadjian, V., 1987. Coevolution in Lichens. Annals of the New York Academy of sciences 503, 307–315. https://doi.org/10.1111/j.1749-6632.1987.tb40617.x

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. Journal of Molecular Biology 215, 403–410. doi.org/10.1016/S0022-2836(05)80360-2.

Alves, I.M.S., Gonçalves, V.N., Oliveira, F.S., Schaefer, C.E.G.R., Rosa, C.A., Rosa, L.H., 2019. The diversity, distribution, and pathogenic potential of cultivable fungi present in rocks from the South Shetlands archipelago, Maritime Antarctica. Extremophiles. 23, 327–336 https://doi.org/10.1007/s00792-019-01086-8

Ametrano, C.G., Grewe, F., Crous, P.W., Goodwin, S.B., Liang, C., Selbmann, L., Lumbsch, T.H., Leavitt, S.D., Muggia, L., 2019a. Genome-scale data resolve ancestral rock-inhabiting lifestyle in Dothideomycetes (Ascomycota). IMA Fungus 10, 1–12. https://doi.org/10.1186/s43008-019-0018-2

Ametrano, C.G., Knudsen, K., Kocourková, J., Grube, M., Selbmann, L., Muggia, L., 2019b. Phylogenetic relationships of rock-inhabiting black fungi belonging to the widespread genera *Lichenothelia* and *Saxomyces*. *Mycologia* 111, 127–160. https://doi.org/10.1080/00275514.2018.1543510

Armstrong, R.A., 2017. Adaptation of lichens to extreme conditions. In Shukla, V., Kumar, S., Kumar, N., (eds). Plant adaptation strategies in changing environment, pp. 1–27. Springer, Singapore. https://doi.org/10.1007/978-981-10-6744-0_1

Babič, M.N., Gunde-Cimerman, N., Vargha, M., Tischner, Z., Magyar, D., Veríssimo, C., Sabino, R., Viegas, C., Meyer, W., Brandão, J., 2017. Fungal contaminants in drinking water regulation? A tale of ecology, exposure, purification and clinical relevance. International Journal of Environmental Research and Public Health 14, 636. https://doi.org/10.3390/ijerph14060636

Baur B., Fröberg, L., Müller, S.W., 2007. Effect of rock climbing on the calcicolous lichen community of limestone cliffs in the northern Swiss Jura Mountains. Nova Hedwigia 85, 429–444. https://doi.org/10.1127/0029-5035/2007/0085-0429

Beck, A., Rambold, G., Kasalicky, T., 2002. Myco-photobiontal selection in a Mediterranean cryptogam community with *Fulgensia fulgida*. New Phytologist 153, 317–326. https://doi.org/10.1046/j.0028-646X.2001.00315.x

Bischoff, H.W., Bold, H.C., 1963. Some soil algae from enchanted rock and related algae species. Phycological Studies 44, 1–95.

Bjelland, T., Grube, M., Home, S., Jorgensen, S.L., Daae, F.L., Thorseth, I.H., Øvreås, L., 2011. Microbial metacommunities in the lichen-rock habitat. Environmental Microbiology Reports 3, 434–442. https://doi.org/10.1111/j.1758-2229.2010.00206.x

Bordenave, C.D., Muggia, L., Chiva, S., Leavitt, S.D., Carrasco, P., Barreno, E., 2021. Chloroplast morphology and pyrenoid ultrastructural analyses reappraise the diversity of the lichen phycobiont genus *Trebouxia* (Chlorophyta). Algal Research 61, 102561. https://doi.org/10.1016/j.algal.2021.102561

Boustie, J., Tomasi, S., Grube, M., 2011. Bioactive lichen metabolites: alpine habitats as an untapped source. Phytochemistry Reviews 10, 287–307. https://doi.org/10.1007/s11101-010-9201-1

Bruns, T.D., Gardes, M., 1993. Molecular tools for the identification of ectomycorrhizal fungi-taxon-specific oligonucleotide probes for suilloid fungi. Molecular Ecology 2, 233–242.

Burford, E.P., Fomina, M., Gadd, G.M., 2003. Fungal involvement in bioweathering and biotransformation of rocks and minerals. Mineralogical Magazine 67, 1127–1155. https://doi.org/10.1180/0026461036760154

Bubrick, P., Galun, M., Frensdorff, A., 1984. Observation of free-living *Trebuxia* De Puymalyand *Pseudotrebouxia* Archibald, and evidence that boths symbionts from *Xanthoria Parietina* (L.) Th. Fr. can be found free-living in nature. New Phytologist 97, 455–462. https://doi.org/10.1111/j.1469-8137.1984.tb03611.x

Carter, N.E.A., Viles, H.A., 2003. Experimental investigations into the interactions between moisture, rock surface temperatures and an epilithic lichen cover in the bioprotection of limestone. Building and Environment 38, 1225–1234. https://doi.org/10.1016/S0360-1323(03)00079-9

Chomnunti, P., Bhat, D.J., Jones, E.B.G., Chukeatirote, E., Bahkali, A.H., Hyde, K.D., 2012. *Trichomeriaceae*, a new sooty mould family of Chaetothyriales. Fungal Diversity 56, 63–76. https://doi.org/10.1007/s13225-012-0197-2

Cockell, C., Rettberg, P., Horneck, G., Scherer, K., Stokes, D.M., 2003. Measurements of microbial protection from ultraviolet radiation in polar terrestrial microhabitats. Polar Biology 26, 62–69. https://doi.org/10.1007/s00300-002-0438-z

Coleine, C., Pombubpa, N., Zucconi, L., Onofri, S., Stajich, J.E., Selbmann, L., 2020. Endolithic fungal species markers for harshest conditions in the McMurdo Dry Valleys, Antarctica. Life 10, 13. https://doi.org/10.3390/life10020013

Coleine, C., Stajich, J. E., de Los Ríos, A., Selbmann, L., 2021. Beyond the extremes: rocks as ultimate refuge for fungi in drylands. Mycologia 113, 108–133.

Coleine, C., Delgado-Baquerizo, M., Albanese, D., Singh, B.K., Stajich, J.E., Selbmann, L., Egidi, E., 2022. Rocks support a distinctive and consistent mycobiome across contrasting dry regions of Earth. FEMS Microbiology Ecology 98, fiac030. https://doi.org/10.1093/femsec/fiac030

Crits-Christoph, A., Gelsinger, D.R., Ma, B., Wierzchos, J., Ravel, J., Davila, A., Casero, M.C., DiRuggiero, J., 2016. Functional interactions of archaea, bacteria and viruses in a hypersaline endolithic community. Environmental Microbiology 18, 2064–2077. https://doi.org/10.1111/1462-2920.13259

Cubero, O.F., Crespo, A., Fatehi, J., Bridge, P.D., 1999. DNA extraction and PCR amplification method suitable for fresh, herbarium-stored, lichenized, and other fungi. Plant Systematics and Evolution 216, 243–249. doi.org/10.1007/BF01084401.

Cutler, N.A., Chaput, D.L., Oliver, A.E., Viles, H.A., 2015. The spatial organization and microbial community structure of an epilithic biofilm. FEMS Microbiology Ecology 91, fiu027. https://doi.org/10.1093/femsec/fiu027

Dadachova, E., Casadevall, A., 2008. Ionizing radiation: how fungi cope, adapt, and exploit with the help of melanin. Current Opinion in Microbiology 1, 525–531.

De Carolis, R., Cometto, A., Moya, P., Barreno, E., Grube, M., Tretiach, M., Leavitt, S.D., Muggia, L., 2022. Photobiont diversity in lichen symbioses from extreme environments. Frontiers in Microbiology 13, 809804. https://doi.org/10.3389/fmicb.2022.809804

De Hoog, G.S., Guarro, J., Gené, J., Ahmed, S., Al-Hatmi, A.M.S., Figueras, M.J., Vitale, R.G., 2019. Atlas of Clinical Fungi, 3rd e-edition. *Utrecht/Reus*. De Hoog, G. S. 2014. Ecology and phylogeny of black yeast-like fungi: diversity in unexplored habitats. Fungal Diversity 65, 1–2. https://doi.org/10.1007/s13225-014-0284-7

De Jong, R., Von Gunten, L., Maldonado, A., Grosjean, M., 2013. Late Holocene summer temperatures in the central Andes reconstructed from the sediments of high-elevation Laguna Chepical, Chile (32 S). Climate of the Past 9, 19211–1932. https://doi.org/10.5194/cp-9-1921-2013

De la Torre, J.R., Goebel, B.M., Friedmann, E.I., Pace, N.R., 2003. Microbial diversity of cryptoendolithic communities from the McMurdo Dry Valleys, Antarctica. Applied and Environmental Microbiology 69, 3858–3867. https://doi.org/10.1128/AEM.69.7.3858-3867.2003

De Los Ríos, A., Grube, M., Sancho, L.G., Ascaso, C., 2007. Ultrastructural and genetic characteristics of endolithic cyanobacterial biofilms colonizing Antarctic granite rocks. FEMS Microbiology Ecology 59, 386–395. https://doi.org/10.1111/j.1574–6941.2006.00256.x

De Los Ríos, A., Wierzchos, J., Ascaso, C., 2014. The lithic microbial ecosystems of Antarctica's McMurdo Dry Valleys. Antarctic Science 25, 459–477. https://doi.org/10.1017/S0954102014000194

De Los Ríos, A., Wierzchos, J., Sancho, L. G., Green, T. A., Ascaso, C., 2005. Ecology of endolithic lichens colonizing granite in continental Antarctica. The Lichenologist 37, 383–395. https://doi.org/10.1017/S0024282905014969

De Menezes, G.C.A., Câmara, P.E.A.S., Pinto, O.H.B., Carvalho-Silva, M., Oliveira, F.S., Souza, C.D., Reynaud Schaefer C.E.G., Convey, P., Rosa, C.A., Rosa, L.H., 2021. Fungal diversity present on rocks from a polar desert in continental Antarctica assessed using DNA metabarcoding. Extremophiles 25, 193–202. https://doi.org/10.1007/s00792-021-01221-4

Diels, L., 1914. Die Algen-Vegetation der Südtiroler Dolomitriffe: Ein Beitrag zur Ökologie der Lithophyten. Borntraeger.

DiRuggiero, J., Wierzchos, J., Robinson, C. K., Souterre, T., Ravel, J., Artieda, O., Souza-Egipsy, V., Ascaso, C., 2013. Microbial colonisation of chasmoendolithic habitats in the hyper-arid zone of the Atacama Desert. Biogeosciences 10, 2439–2450. https://doi.org/10.5194/bg-10-2439-2013

Farrell, R.L., Arenz, B.E., Duncan, S.M., Held, B.W., Jurgens, J.A., Blanchette, R.A., 2011. Introduced and indigenous fungi of the Ross Island historic huts and pristine areas of Antarctica. Polar Biology 34, 1669–1677. https://doi.org/10.1007/s00300-011-1060-8

Flechtner, V.R., Pietrasiak, N., Lewis, L.A., 2013. Newly revealed diversity of green microalgae from wilderness areas of Joshua Tree National Park (JTNP). Monographs of the Western North American Naturalist 6, 43–63. https://doi.org/10.3398/042.006.0103

Frenot, Y., Chown, S.L., Whinam, J., Selkirk, P.M., Convey, P., Skotnicki, M., Bergstrom, D.M., 2005. Biological invasions in the Antarctic: extent, impacts and implications. Biological Reviews of the Cambridge Philosophical Society 80, 45–72. https://doi.org/10.1017/S1464793104006542

Friedmann, E.I., 1982. Endolithic microorganisms in the Antarctic cold desert. Science 215, 1045–1053. https://doi.org/10.1126/science.215.4536.1045

Friedmann, E.I., Galun, M., 1974. Desert biology. Academic Press, New York.

Friedmann, E.I., Ocampo-Friedmann, R., 1984. The antarctic cryptoendolithic ecosystem: relevance to exobiology. Origins of Life 14, 771–776. https://doi.org/10.1007/BF00933732

Fučíková, K., Lewis, P.O., Lewis, L.A., 2014. Widespread desert affiliation of trebouxiophycean algae (Trebouxiophyceae, Chlorophyta) including discovery of three new desert genera. Phycological Research 62, 294–305. https://doi.org/10.1111/pre.12062

Gadd, G.M., 2007. Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. Mycological Research 111, 3–49.

Gaylarde, P., Englert, G., Ortega-Morales, O., Gaylarde, C., 2006. Lichen-like colonies of pure *Trentepohlia* on limestone monuments. International Biodeterioration and Biodegradation 58, 119–123. https://doi.org/10.1016/j.ibiod.2006.05.005

Golubic, S., Friedmann, I., Schneider, J., 1981. The lithobiontic ecological niche, with special reference to microorganisms. Journal of Sedimentary Petrology 51, 475–478. https://doi.org/10.1306/212f7cb6-2b24-11d7-8648000102c1865d

Gómez-Alarcón, G., Muñoz, M., Ariño, X., Ortega-Calvo, J.J., 1995. Microbial communities in weathered sandstones: the case of Carrascosa del Campo church, Spain. Science of the Total Environment 167, 249–254. https://doi.org/10.1016/0048-9697(95)04586-P

Gómez-Silva, B., 2018. Lithobiontic life: "Atacama rocks are well and alive." Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology 111, 1333–1343. https://doi.org/10.1007/s10482-018-1033-9

Gorbushina, A.A., 2007. Life on the rocks. Environmental Microbiology 9, 1613–1631.

Gorbushina, A.A., Beck, A., Schulte, A., 2005. Microcolonial rock inhabiting fungi and lichen photobionts: evidence for mutualistic interactions. Mycological Research 109, 1288–1296. https://doi.org/10.1017/S0953756205003631

Gorbushina, A.A., Kotlova, E.R., Sherstneva, O.A., 2008. Cellular responses of microcolonial rock fungi to long-term desiccation and subsequent rehydration. Studies in Mycology 61, 91–97. https://doi.org/10.3114/sim.2008.61.09

Gorbushina, A.A., Petersen, K., 2000. Distribution of microorganisms on ancient wall paintings as related to associated faunal elements. International Biodeterioration and Biodegradation 46, 277–284. https://doi.org/10.1016/S0964-8305(00)00103-7

Gostinčar, C. 2020. Towards genomic criteria for delineating fungal species. Journal of Fungi 6, 246. https://doi.org/10.3390/jof6040246

Gostinčar, C., Gunde-Cimerman, N., Turk, M., 2012a. Genetic resources of extremotolerant fungi: a method for identification of genes conferring stress tolerance. Bioresource Technology 111, 246. https://doi.org/10.1016/j.biortech.2012.02.039

Gostinčar, C., Lenassi, M., Gunde-Cimerman, N., Plemenitaš, A., 2011. Fungal adaptation to extremely high salt concentrations. In Advances in applied microbiology (Vol. 77, pp. 71–96). Academic Press.

Gostinčar, C., Muggia, L., Grube, M. 2012b. Polyextremotolerant black fungi: oligotrophism, adaptive potential, and a link to lichen symbioses. Frontiers in Microbiology 3, 390. https://doi.org/10.3389/fmicb.2012.00390

Gueidan, C., Ruibal, C., De Hoog, G.S., Schneider, H., 2011. Rock-inhabiting fungi originated during periods of dry climate in the late Devonian and middle Triassic. Fungal Biology 115, 987–996. https://doi.org/10.1016/j.funbio.2011.04.002

Gueidan, C., Villaseñor, C.R., De Hoog, G.S., Gorbushina, A.A., Untereiner, W.A., Lutzoni, F., 2008. A rock-inhabiting ancestor for mutualistic and pathogen-rich fungal lineages. Studies in Mycology 61, 111–119. https://doi.org/10.3114/sim.2008.61.11

Gunde-Cimerman. N., Grube, M., De Hoog, G.S., 2011. The emerging potential of melanized fungi: black yeast between beauty and the beast. Fungal Biology 115, 935–936.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ NT. Nucleic Acids Symposium Series [London]: Information Retrieval Ltd., c1979-c2000., pp. 95–98.

Hartmann, M., Lee, S., Hallam, S.J., Mohn, W.W., 2009. Bacterial, archaeal and eukaryal community structures throughout soil horizons of harvested and naturally disturbed forest stands. Environmental Microbiology 11, 3045–3062. https://doi.org/10.1111/j.1462-2920.2009.02008.x

Harutyunyan, S., Muggia, L., Grube, M., 2008. Black fungi in lichens from seasonally arid habitats. Studies in Mycology 6, 83–90. https://doi.org/10.3114/sim.2008.61.08

Hirsch, P., Eckhardt, F.E.W., Palmer, R.J., 1995. Methods for the study of rock-inhabiting microorganisms-A mini review. Journal of Microbiological Methods 23, 143–167.

Hoffmann, L. 1989. Algae of terrestrial habitats. The Botanical Review 55, 77–105. https://doi.org/10.1007/BF02858529

Isola, D., Zucconi, L., Onofri, S., Caneva, G., De Hoog, G.S., Selbmann, L., 2016 Extremotolerant rock inhabiting black fungi from Italian monumental sites. Fungal Diversity 76, 75–96. https://doi.org/10.1007/s13225-015-0342-9

Jackson, T.A., 2015. Weathering, secondary mineral genesis, and soil formation caused by lichens and mosses growing on granitic gneiss in a boreal forest environment. Geoderma 251, 79–91. https://doi.org/10.1016/j.geoderma.2015.03.012

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular Biology and Evolution 30, 772–780. https://doi.org/10.1093/molbev/mst010

Khan, A., Kong, W., Ji, M., Yue, L., Xie, Y., Liu, J., Xu, B., 2020. Disparity in soil bacterial community succession along a short time-scale deglaciation chronosequence on the Tibetan Plateau. Soil Ecology Letters 2, 83–92. https://doi.org/10.1007/s42832-020-0027-5

Kinnard, C., Ginot, P., Surazakov, A., MacDonell, S., Nicholson, L., Patris, N., Rabatel, A., Rivera, A., Squeo, F.A., 2020. Mass balance and climate history of a high-altitude glacier, Desert Andes of Chile. Frontiers in Earth Science 8, 40. https://doi.org/10.3389/feart.2020.00040

Kogej, T., Ramos, J., Plemenitaš, A., Gunde-Cimerman, N., 2005. The halophilic fungus *Hortaea werneckii* and the halotolerant fungus *Aureobasidium pullulans* maintain low intracellular cation concentrations in hypersaline environments. Applied and Environmental Microbiology 71, 6600–6605. https://doi.org/10.1128/AEM.71.11.6600-6605.2005

Kroken, S., Taylor, J.W., 2000. Phylogenetic species, reproductive mode, and specificity of the green alga *Trebouxia* forming lichens with the fungal genus *Letharia*. Bryologist 103, 645–660. https://doi.org/10.1639/0007-2745(2000)103[0645:PSRMAS]2.0.CO;2

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution 35, 1547–1549. https://doi.org/10.1093/molbev/msy096

Kumar, S., Suyal, D.C., Yadav, A., Shouche, Y., Goel, R., 2019. Microbial diversity and soil physiochemical characteristic of higher altitude. *PLoS ONE* 14, e0213844. https://doi.org/10.1371/journal.pone.0213844

Leavitt, S. D., Kraichak, E., Nelsen, M. P., Altermann, S., Divakar, P. K., Alors, D., Esslinger, T. L., Crespo, A., Lumbsch, T., 2015. Fungal specificity and selectivity for algae play a major role in determining lichen partnerships across diverse ecogeographic regions in the lichen-forming family Parmeliaceae (Ascomycota). Molecular Ecology *24*, 3779–3797.

Letunic, I., Bork, P., 2019. Interactive Tree of Life (iTOL) v4: Recent updates and new developments. Nucleic Acids Research 47, 256–259. https://doi.org/10.1093/nar/gkz239

Lewis, L.A., Lewis, P.O., 2005. Unearthing the molecular phylodiversity of desert soil green algae (Chlorophyta). Systematic Biology 54, 936–947. https://doi.org/10.1080/10635150500354852

Liu, Y., He, J., Shi, G., An, L., Öpik, M., Feng, H., 2011. Diverse communities of arbuscular mycorrhizal fungi inhabit sites with very high altitude in Tibet Plateau. FEMS Microbiology Ecology 78, 355–365. https://doi.org/10.1111/j.1574-6941.2011.01163.x

Matthes, U., Turner, S. J., Larson, D.W., 2001. Light attenuation by limestone rock and its constraint on the depth distribution of endolithic algae and cyanobacteria. International Journal of Plant Sciences 162, 263–270. https://doi.org/10.1086/319570

Miller, M.A., Pfeiffer, W., Schwartz, T., 2011. The CIPRES science gateway: a community resource for phylogenetic analyses. In: Proceedings of the TeraGrid 2011 Conference: Extreme Digital Discovery, pp. 1–8.

Moya, P., Chiva, S., Molins, A., Jadrná, I., Škaloud, P., Peksa, O., Barreno, E., 2018. *Myrmecia israeliensis* as the primary symbiotic microalga in squamulose lichens growing in European and Canary Island terricolous communities. Fottea 18, 72–85. https://doi.org/10.5507/fot.2017.022

Muggia, L., Fleischhacker, A., Kopun, T., Grube, M., 2016. Extremotolerant fungi from alpine rock lichens and their phylogenetic relationships. Fungal Diversity 76, 119–142. https://doi.org/10.1007/s13225-015-0343-8.

Muggia, L., Nelsen, M.P., Kirika, P.M., Barreno, E., Beck, A., Lindgren, H., Lumbsch, T.H., Leavitt, S.D., Trebouxia working group 2020. Formally described species woefully underrepresent phylogenetic diversity in the common lichen photobiont genus *Trebouxia* (Trebouxiophyceae, Chlorophyta): an impetus for developing an integrated taxonomy. Molecular Phylogenetics and Evolution 149, 106821. https://doi.org/10.1016/j.ympev.2020.106821

Muggia, L., Quan, Y., Gueidan, C., Al-Hatmi, A., Grube, M., De Hoog, S.G, 2021. Sequence data from isolated lichen-associated melanized fungi enhance delimitation of two new lineages within Chaetothyriomycetidae. Mycological Progress 20, 911–927. https://doi.org/10.1007/s11557-021-01706-8

Mutawalli, L., Syaifur Rizal, M.R., Artama, W.T., Fajriyah, R., Muhimmah, I., Rujito, L. 2019. Analisa visual menggunakan etetoolkit framework terhadap penyakit beta-thalassemia di jawa tengah begian selatan. Jurnal Informatika dan Rekayasa Elektronik 2, 16–27. https://doi.org/10.36595/jire.v2i1.75

Nelsen, M.P., Plata, E.R., Andrew, C.J., Lücking, R., Lumbsch, H.T., 2011. Phylogenetic diversity of trentepohlialean algae associated with lichen-forming fungi. Journal of Phycology 47, 282–290. https://doi.org/10.1111/j.1529-8817.2011.00962.x

Nelson, D.R., Bartels, P.J., Fegley, S.R., 2020. Environmental correlates of tardigrade community structure in mosses and lichens in the Great Smoky Mountains National Park (Tennessee and North Carolina, USA). Zoological Journal of the Linnean Society 188, 913–924. https://doi.org/10.1093/zoolinnean/zlz043

Nienow, J.A., McKay, C.P., Friedmann, E.I., 1988. The cryptoendolithic microbial environment in the Ross Desert of Antarctica: mathematical models of the thermal regime. Microbial Ecology 16, 253–270. https://doi.org/10.1007/BF02011699

Ogwu, M.C., Takahashi, K., Dong, K., Song, H.K., Moroenyane, I., Waldman, B., Adams, J.M., 2019. Fungal elevational rapoport pattern from a high mountain in Japan. Scientific Reports 9, 1–10. https://doi.org/10.1038/s41598-019-43025-9

Olech, M., Chwedorzewska, K.J., 2011. Short Note: the first appearance and establishment of an alien vascular plant in natural habitats on the forefield of a retreating glacier in Antarctica. Antarctic Science 23, 153–154.

Omelon, C. R. 2008. Endolithic microbial communities in polar desert habitats. Geomicrobiology Journal 25, 404–414. https://doi.org/10.1080/01490450802403057

Onofri, S., Selbmann, L., De Hoog, S.G, Grube, M., Barreca, D., Ruisi, S., Zucconi, L., 2007a. Evolution and adaptation of fungi at boundaries of life. Advances in Space Research 40, 1657–1664. https://doi.org/10.1016/j.asr.2007.06.004

Onofri, S., Zucconi, L., Isola, D., Selbmann, L. 2014. Rock-inhabiting fungi and their role in deterioration of stone monuments in the Mediterranean area. Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology 148, 384–391. https://doi.org/10.1080/11263504.2013.877533

Onofri, S., Zucconi, L., Selbmann, L., De Hoog, S.G., Ríos, D.A.D.L., Ruisi, S., Grube, M., 2007b. Fungal associations at the cold edge of life. In Algae and cyanobacteria in extreme environments pp. 735–757. Springer, Dordrecht.

Ortega-Morales, O., Guezennec, J., Hernandez-Duque, G., Gaylarde, C.C., Gaylarde, P.M., 2000. Phototrophic biofilms on ancient Mayan buildings in Yucatan, Mexico. Current Microbiology 40, 81–85. https://doi.org/10.1007/s002849910015

Pacelli, C., Cassaro, A., Maturilli, A., Timperio, A.M., Gevi, F., Cavalazzi, B., Stefan, M., Ghica, D., Onofri, S., 2020. Multidisciplinary characterization of melanin pigments from the black fungus *Cryomyces antarcticus*. Applied Microbiology and Biotechnology 104, 6385–6395. https://doi.org/10.1007/s00253-020-10666-0

Peel, M.C., Finlayson, B.L., McMahon, T.A., 2007. Updated world map of the Köppen-Geiger climate classification. Hydrology and Earth System Sciences 11, 1633–1644. https://doi.org/10.5194/hess-11-1633-2007

Peraza Zurita, Y., Cultrone, G., Sánchez Castillo, P., Sebastián E., Bolívar, F.C., 2005. Microalgae associated with deteriorated stonework of the fountain of Bibatauín in Granada, Spain. International Biodeterioration & Biodegradation 55, 55–61. https://doi.org/10.1016/j.ibiod.2004.05.006

Piñar, G., Dalnodar, D., Voitl, C., Reschreiter, H., Sterflinger, K., 2016. Biodeterioration risk threatens the 3100 year old staircase of hallstatt (Austria): possible involvement of halophilic microorganisms. PLoS ONE 11. https://doi.org/10.1371/journal.pone.0148279

Pitkäranta, M., Meklin, T., Hyvärinen, A., Nevalainen, A., Paulin, L., Auvinen, P., Lignell, U., Rintala, H., 2011. Molecular profiling of fungal communities in moisture damaged buildings before and after remediation-a comparison of culture-dependent and culture-independent methods. BMC Microbiology 11, 1–16. https://doi.org/10.1186/1471-2180-11-235

Pointing, S.B., Chan, Y., Lacap, D.C., Lau, M.C., Jurgens, J.A. and Farrell, R.L., 2009. Highly specialized microbial diversity in hyper-arid polar desert. Proceedings of the National Academy of Sciences 106, 19964–19969. https://doi.org/10.1073/pnas.0908274106

Praeg, N., Seeber, J., Leitinger, G., Tasser, E., Newesely, C., Tappeiner, U., Illmer, P., 2020. The role of land management and elevation in shaping soil microbial communities: insights from the Central European Alps. Soil Biology and Biochemistry 150, 107951. https://doi.org/10.1016/j.soilbio.2020.107951

Quan, Y., Muggia, L., Moreno, L. F., Wang, M., Al-Hatmi, A., da Silva Menezes, N., Shi, D., Deng, S., Ahmed, S., Hyde, K.D., Vicente, V.A., Kang, Y., Stielow, J.B., De Hoog, S.G., 2020. A re-evaluation of the Chaetothyriales using criteria of comparative biology. Fungal Diversity 103, 47–85. https://doi.org/10.1007/s13225-020-00452-8

R Development Core Team (2019) R Core Team (2020). R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/. R Foundation for Statistical Computing 2

Rambold, G., Friedl, T., Beck, A.,1998. Photobionts in Lichens: possible indicators of phylogenetic relationships? Bryologist 101, 392–397. https://doi.org/10.1639/0007-2745(1998)101[392:pilpio]2.0.co;2

Robinson, C.K., Wierzchos, J., Black, C., Crits-Christoph, A., Ma, B., Ravel, J., Ascaso, A., Artieda, O., Valea, S., Roldán, M., Gómez-Silva, B., DiRuggiero, J., 2015. Microbial diversity and the presence of algae in halite endolithic communities are correlated to atmospheric moisture in the hyper-arid zone of the Atacama Desert. Environmental microbiology 17, 299–315. https://doi.org/10.1111/1462-2920.12364

Robinson, D.F., Foulds, L.R., 1981. Comparison of phylogenetic trees. Mathematical Biosciences 53, 131–147. https://doi.org/10.1016/0025-5564(81)90043-2

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic biology 61, 539–542. https://doi.org/10.1093/sysbio/sys029

Ruibal, C., Gueidan, C., Selbmann, L., Gorbushina, A.A., Crous, P.W., Groenewald, J.Z., Muggia, L., Grube, M., Isola, D., Schoch, C.L., Staley, J.T., Lutzoni, F., De Hoog, G. S. 2009. Phylogeny of rock-inhabiting fungi related to Dothideomycetes. Studies in Mycology 64, 131–136. https://doi.org/10.3114/sim.2009.64.06

Ruibal, C., Platas, G., Bills, G.F., 2005. Isolation and characterization of melanized fungi from limestone formations in Mallorca. Mycological Progress 4, 23–38. https://doi.org/10.1007/s11557-006-0107-7

Samolov, E., Baumann, K., Büdel, B., Jung, P., Leinweber, P., Mikhailyuk, T., Karsten, U., Glaser, K., 2020. Biodiversity of algae and cyanobacteria in biological soil crusts collected along a climatic gradient in Chile using an integrative approach. Microorganisms 8, 1047. https://doi.org/10.3390/microorganisms8071047

Samsonoff, W.A., MacColl, R., 2001. Biliproteins and phycobilisomes from cyanobacteria and red algae at the extremes of habitat. Archives of Microbiology 176, 400–405.

Selbmann, L., Grube, M., Onofri, S., Isola, D., Zucconi, L., 2013. Antarctic epilithic lichens as niches for black meristematic fungi. Biology 2, 784–797. https://doi.org/10.3390/biology2020784

Selbmann, L., Isola, D., Zucconi, L., Onofri, S., 2011. Resistance to UV-B induced DNA damage in extreme-tolerant cryptoendolithic Antarctic fungi: detection by PCR assays. Fungal Biology 115, 937–944. https://doi.org/10.1016/j.funbio.2011.02.016

Selbmann, L., Onofri, S., Coleine, C., Buzzini, P., Canini, F., Zucconi, L., 2017. Effect of environmental parameters on biodiversity of the fungal component in lithic Antarctic communities. Extremophiles 21, 1069–1080. https://doi.org/10.1007/s00792-017-0967-6

Selbmann, L., Stoppiello, G.A., Onofri, S., Stajich, J.E., Coleine, C., 2021. Culture-dependent and amplicon sequencing approaches reveal diversity and distribution of black fungi in Antarctic cryptoendolithic communities. Journal of Fungi 7, 213. https://doi.org/10.3390/jof7030213

Selbmann, L., Zucconi, L., Isola, D., Onofri, S., 2015. Rock black fungi: excellence in the extremes, from the Antarctic to space. Current Genetics 61, 335–345. https://doi.org/10.1007/s00294-014-0457-7

Seong, Y.B., Owen, L.A., Yi, C., Finkel, R.C., 2009. Quaternary glaciation of Muztag Ata and Kongur Shan: evidence for glacier response to rapid climate changes throughout the late glacial

and holocene in westernmost Tibet. Bulletin of the Geological Society of America 121, 346–365. https://doi.org/10.1130/B26339.1

Shangguan, D., Liu, S., Ding, Y., Ding, L., Xiong, L., Cai, D., Li, G., Lu, A., Zhang, S., Zhang, Y., 2006. Monitoring the glacier changes in the Muztag Ata and Konggur mountains, east Pamirs, based on Chinese Glacier Inventory and recent satellite imagery. Annals of Glaciology 43, 79–85.

Souza-Egipsy, V., Wierzchos, J., Sancho, C., Belmonte, A., Ascaso, C., 2004. Role of biological soil crust cover in bioweathering and protection of sandstones in a semi-arid landscape (Torrollones de Gabarda, Huesca, Spain). Earth Surface Processes and Landforms: The Journal of the British Geomorphological Research Group 29, 1651–1661. https://doi.org/10.1002/esp.1118

Spitale, D., Nascimbene, J., 2012. Spatial structure, rock type, and local environmental conditions drive moss and lichen distribution on calcareous boulders. Ecological Research 27, 633–638. https://doi.org/10.1007/s11284-012-0935-7

Stamatakis, A. 2014. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Sterflinger, K., 1998. Temperature and NaCl- tolerance of rock-inhabiting meristematic fungi. Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology 74, 271–281. https://doi.org/10.1023/A:1001753131034

Stevens, G.C., 1992. The elevational gradient in altitudinal range: an extension of rapoport's latitudinal rule to altitude. American Naturalist 140, 893–911. https://doi.org/10.1086/285447

Stevens, G C. 1989. The latitudinal gradient in geographical range: how so many species coexist in the tropics. American Naturalist 133, 240–256. https://doi.org/10.1086/284913

Su, L., Guo, L., Hao, Y., Xiang, M., Cai, L., Liu, X., 2015. *Rupestriomyces* and *Spissiomyces*, two new genera of rock-inhabiting fungi from China. Mycologia 107, 831–844. https://doi.org/10.3852/14-305

Tanaka, D., Sato, K., Goto, M., Fujiyoshi, S., Maruyama, F., Takato, S., Shimada, T., Sakatoku, A., Aoki, K., Nakamura, S. 2019. Airborne microbial communities at high-altitude and suburban sites in Toyama, Japan suggest a new perspective for bioprospecting. Frontiers in bioengineering and biotechnology 7, 12.

Tang, M., Li, L., Wang, X., You, J., Li, J., Chen, X., 2020. Elevational is the main factor controlling the soil microbial community structure in alpine tundra of the Changbai Mountain. Scientific Reports 10, 1–15. https://doi.org/10.1038/s41598-020-69441-w

Teixeira, M.D.M., Moreno, L.F., Stielow, B.J., Muszewska, A., Hainaut, M., Gonzaga, L., Abouelleil, A., Patané, J.S.L., Priest, M., Souza, R., Young, S., et al., 2017. Exploring the genomic diversity of black yeasts and relatives (Chaetothyriales, Ascomycota). Studies in mycology 86, 1–28. https://doi.org/10.1016/j.simyco.2017.01.001

Tschermak-Woess, E., 2019. The Algal Partner. In Handbook of Lichenology. https://doi.org/10.1201/9780429291784-3

Velázquez, M.S., Stürmer, S.L., Bruzone, C., Fontenla, S., Barrera, M., Cabello, M., 2016. Occurrence of arbuscular mycorrhizal fungi in high altitude sites of the Patagonian Altoandina Region in Nahuel Huapi National Park (Argentina). Acta Botanica Brasilica 30. https://doi.org/10.1590/0102-33062016abb0223

Vilgalys, R., Hester, M., 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. Journal of Bacteriology 172, 4238–4246. https://doi.org/10.1128/jb.172.8.4238-4246.1990

Vincent, W.F., 1988. Microbial ecosystems of Antarctica. Microbial ecosystems of Antarctica. Cambridge University Press. https://doi.org/10.2307/1551307

Walker, J.J., Pace, N.R., 2007. Phylogenetic composition of rocky mountain endolithic microbial ecosystems. Applied and Environmental Microbiology 73, 3497–3504. https://doi.org/10.1128/AEM.02656-06

Wei, S.T., Lacap-Bugler, D.C., Lau, M.C., Caruso, T., Rao, S., De Los Rios, A., Archer, S.K., Chiu, J.M.Y., Higgins, C., Van Nostrand, J.D., Zhou, J., Hopkins, D.W., Pointing, S.B., 2016. Taxonomic and functional diversity of soil and hypolithic microbial communities in Miers Valley, McMurdo Dry Valleys, Antarctica. Frontiers in Microbiology 7, 1642. https://doi.org/10.3389/fmicb.2016.01642

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications* 8: 315–322.

Wierzchos, J., Ascaso, C., McKay, C.P., 2006. Endolithic cyanobacteria in halite rocks from the hyperarid core of the Atacama Desert. Astrobiology 6, 415–422. https://doi.org/10.1089/ast.2006.6.415

Wierzchos, J., Cámara, B., De Los Ríos, A., Davila, A.F., Sánchez Almazo, I.M., Artieda, O., Wierzchos, K., Gómez-silva, B., Mckay, C., Ascaso, C., 2011. Microbial colonization of Ca-sulfate crusts in the hyperarid core of the Atacama Desert: implications for the search for life on Mars. Geobiology 9, 44–60. https://doi.org/10.1111/j.1472-4669.2010.00254.x

Wierzchos, J., De Los Ríos, A., Ascaso, C., 2012. Microorganisms in desert rocks: the edge of life on Earth. In International Microbiology 15, 171–181 https://doi.org/10.2436/20.1501.01.170

Wierzchos, J., DiRuggiero, J., Vítek, P., Artieda, O., Souza-Egipsy, V., Skaloud, P., Tisza, M., Davila, A., Vílchez, C., Garbayo, I., Ascaso, C., 2015. Adaptation strategies of endolithic chlorophototrophs to survive the hyperarid and extreme solar radiation environment of the Atacama Desert. Frontiers in Microbiology 6, 934. https://doi.org/10.3389/fmicb.2015.00934

Wijayawardene, N.N., Bahram, M., Sánchez-Castro, I., Dai, D.Q., Ariyawansa, K.G., Jayalal, U., Suwannarach, N., Tedersoo, L., 2021. Current insight into culture-dependent and culture-independent methods in discovering Ascomycetous Taxa. Journal of Fungi 7, 703. https://doi.org/10.3390/jof7090703

Wollenzien, U., De Hoog, G.S., Krumbein, W.E., Urzí, C., 1995. On the isolation of microcolonial fungi occurring on and in marble and other calcareous rocks. Science of the Total Environment 167, 287–294. https://doi.org/10.1016/0048-9697(95)04589-S

Wong, F.K., Lacap, D.C., Lau, M.C., Aitchison, J.C., Cowan, D.A., Pointing, S.B., 2010. Hypolithic microbial community of quartz pavement in the high-altitude tundra of central Tibet. Microbial ecology 60, 730–739. https://doi.org/10.1007/s00248-010-9653-2

Xiang, S., Yao, T., An, L., Xu, B., Wang, J., 2005. 16S rRNA sequences and differences in bacteria isolated from the Muztag Ata glacier at increasing depths. Applied and Environmental Microbiology 71, 4619–4627. https://doi.org/10.1128/AEM.71.8.4619-4627.2005

Yamamoto, N., Bibby, K., Qian, J., Hospodsky, D., Rismani-Yazdi, H., Nazaroff, W.W., Peccia, J. 2012. Particle-size distributions and seasonal diversity of allergenic and pathogenic fungi in outdoor air. The ISME journal 6, 1801–1811. https://doi.org/10.1038/ismej.2012.30

Yan, S., Guo, H., Liu, G., Ruan, Z., 2013. Mountain glacier displacement estimation using a DEM-assisted offset tracking method with ALOS/PALSAR data. Remote Sensing Letters 4, 494–503. https://doi.org/10.1080/2150704X.2012.754561

Yang, J., Warnow, T., 2011. Fast and accurate methods for phylogenomic analyses. BMC Bioinformatics 12, 4. https://doi.org/10.1186/1471-2105-12-S9-S4

Yung, C., Chan, Y., Lacap, D.C., Pérez-Ortega, S., De Los Rios-Murillo, A., Lee, C. K., Cary, S. C., Pointing, S.B., 2014. Characterization of chasmoendolithic community in Miers valley, McMurdo dry valleys, Antarctica. Microbial Ecology 68, 351–359. https://doi.org/10.1007/s00248-014-0412-7

Zhou, J., Li, Z., Guo, W., 2014. Estimation and analysis of the surface velocity field of mountain glaciers in Muztag Ata using satellite SAR data. Environmental Earth Sciences 71, 3581–3592. https://doi.org/10.1007/s12665-013-2749-5

Zucconi, L., Onofri, S., Cecchini, C., Isola, D., Ripa, C., Fenice, M., Madonna, S., Reboleiro-Rivas, P., Selbmann, L., 2016. Mapping the lithic colonization at the boundaries of life in Northern Victoria Land, Antarctica. Polar Biology 39, 91–102. https://doi.org/10.1007/s00300-014-1624-5
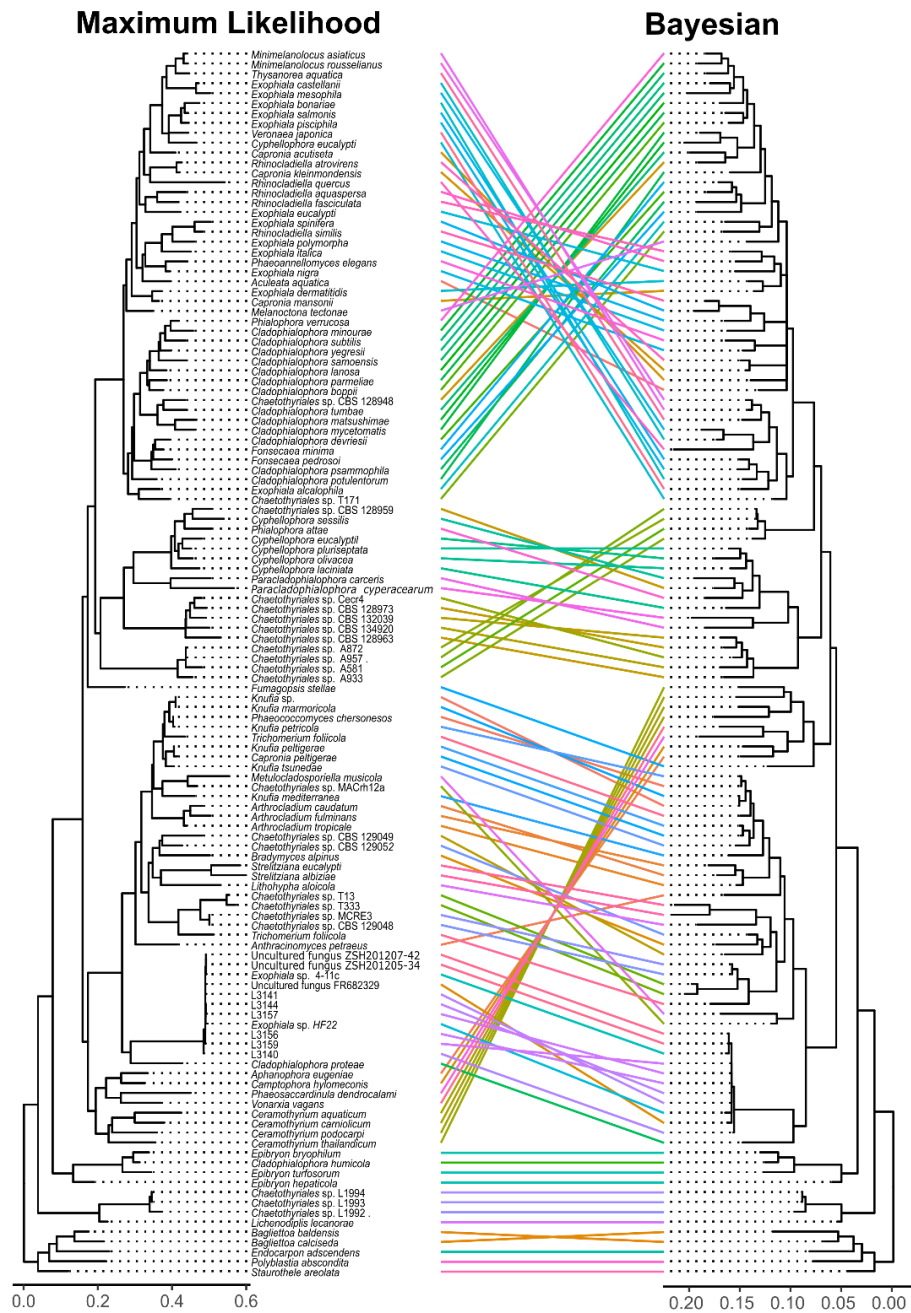
Supplementary information

Figure S1. Tanglegram of ML and BI phylogenies (Chaetothyriales).
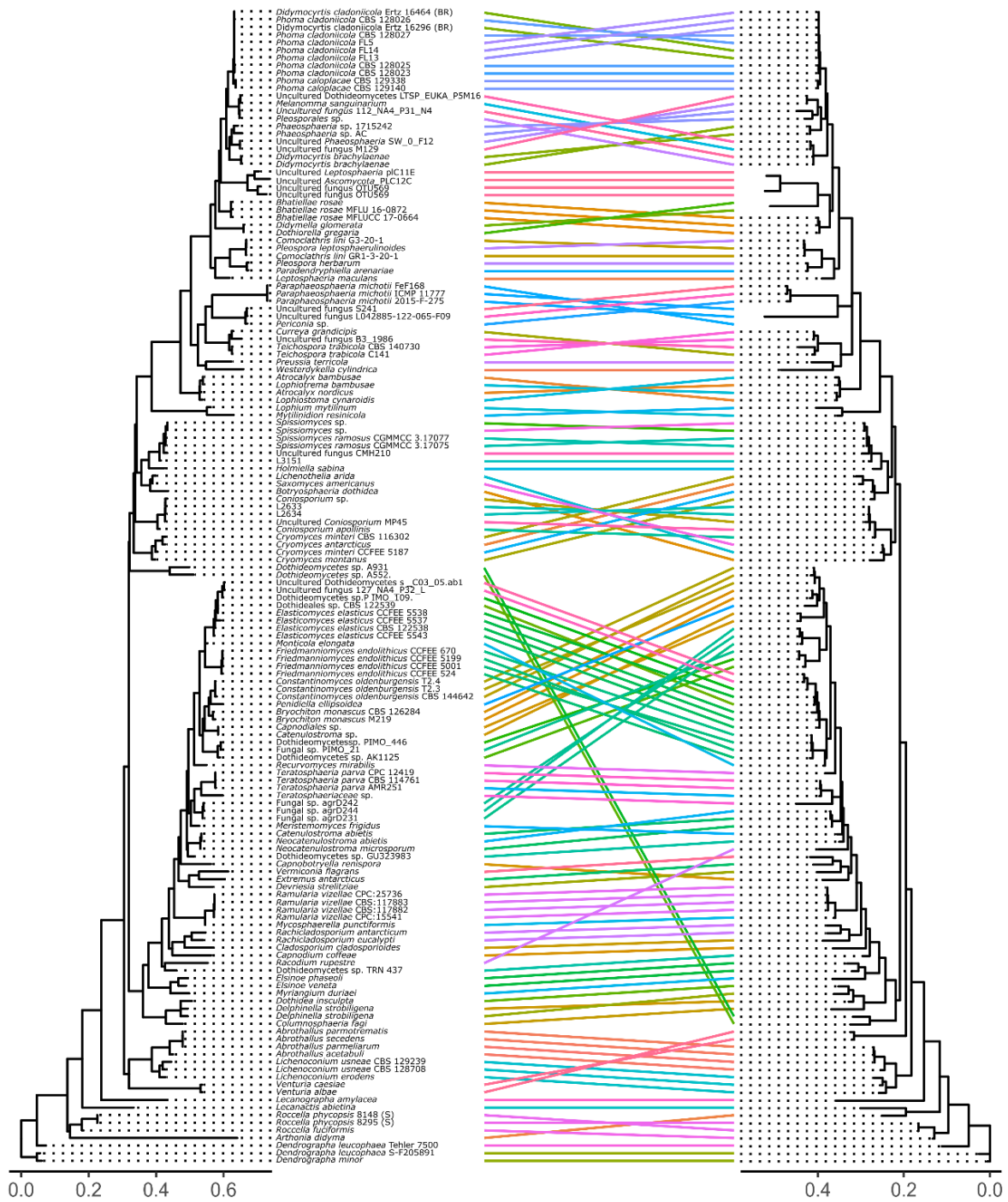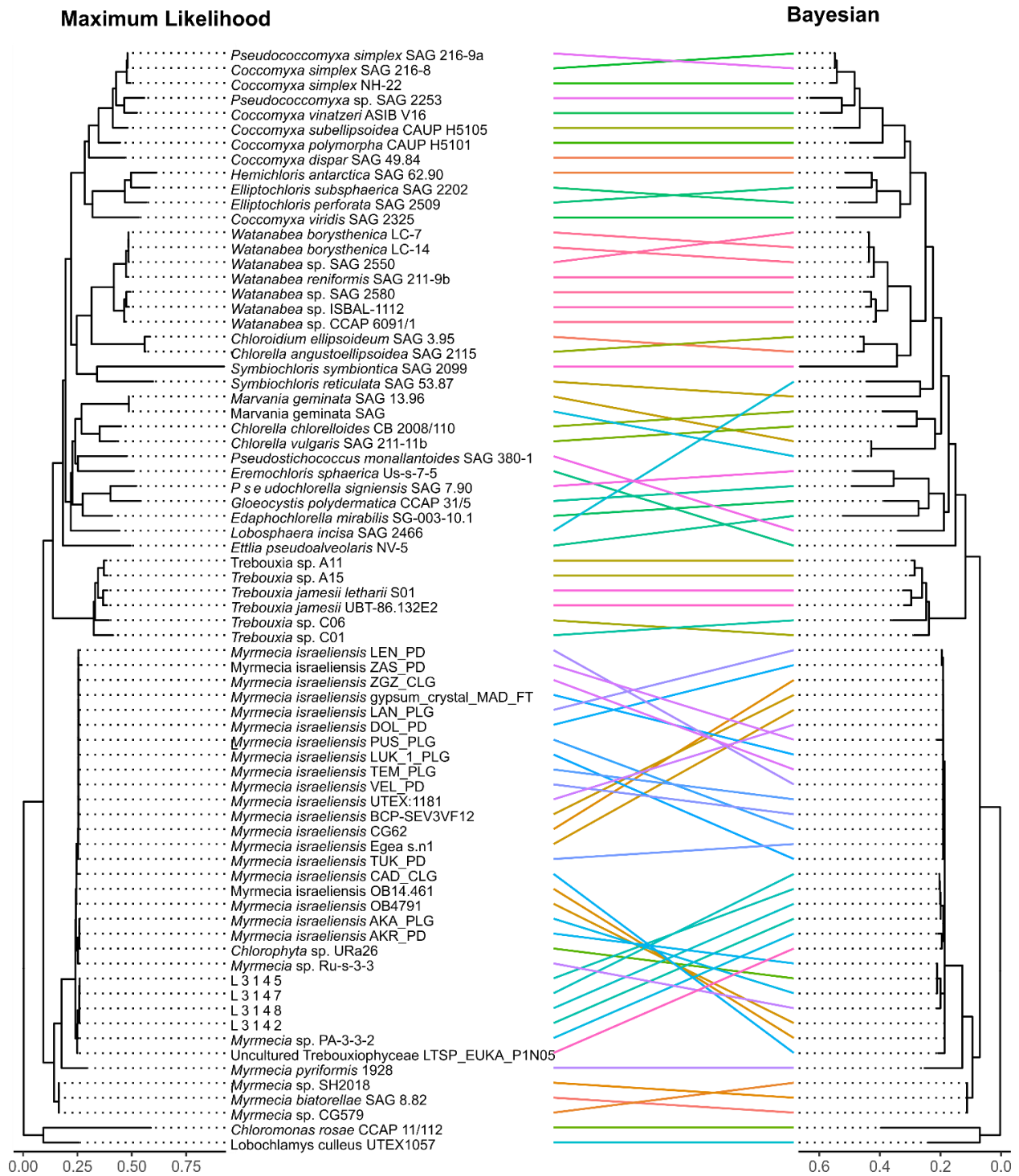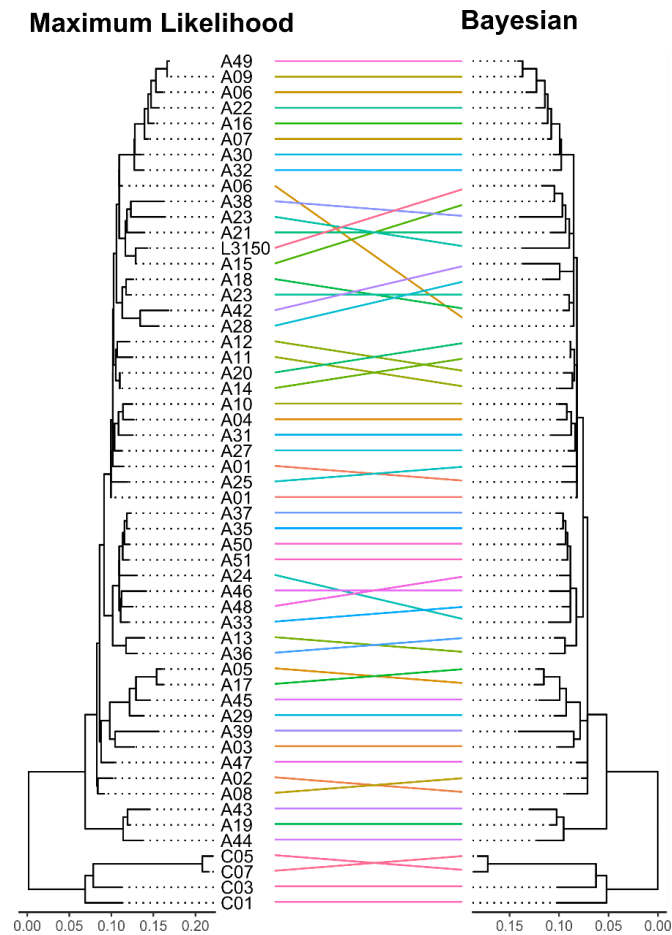
Figure S2. Tanglegram of ML and BI phylogenies (Dothideomycetes).

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

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

Table S1. List of taxa included in the phylogenetic analysis of Chaetothyriales and their NCBI accessions (see separate external Excel file: https://doi.org/10.5281/zenodo.7261320).

Table S2. List of taxa included in the phylogenetic analysis of Dothideomycetes and their NCBI accessions (see separate external Excel file: https://doi.org/10.5281/zenodo.7261320).

Table S3. List of taxa included in the phylogenetic analysis of *Myrmecia* and their NCBI accessions (see separate external Excel file: https://doi.org/10.5281/zenodo.7261320).

Table S4. List of taxa, reported as ID of the species level lineage according to Muggia et al. (2020), included in the phylogenetic analysis of *Trebouxia* and their NCBI accessions (see separate external Excel file: https://doi.org/10.5281/zenodo.7261320).

APPENDIX 2

The origin of human pathogenicity and biological interactions in Chaetothyriales

Yu Quan[1,2], Shuwen Deng[3], Francesc X. Prenafeta-Boldǔ[4], Veronika Mayer[5], Lucia Muggia[6], Agnese Cometto[6], Vania Vicente[7], Nickolas Menezes da Silva[8], Maria Eduarda Grisolia[8], Yinggai Song[1,9], Sarah Ahmed[1,10], Xueke Niu[1,2], Bruna Jacomel Favoreto de Souza Lima[7], Peiying Feng[11], Roxana Vitale[12], Marcus Teixeira[13], Montarop Sudhadham[14], Conceicao Pedrozo e Silva de Azevedo[15], Anamelia Bocca[16], Gerhard Haase[17], Laura Selbmann[18],·Dongmei Shi[19], Yingqian Kang[2], Sybren de Hoog[1,2,4,7,9*]

[1]Centre of Expertise in Mycology of Radboud University Medical Centre / Canisius Wilhelmina Hospital, Nijmegen, The Netherlands

[2]Key Laboratory of Environmental Pollution Monitoring and Disease Control, Ministry of Education of Guizhou & Key Laboratory of Medical Microbiology and Parasitology, School of Basic Medical Sciences, Guizhou Medical University, Guiyang, China

[3]Department of Medical Microbiology, People's Hospital of Suzhou National New & Hi-Tech Industrial Development Zone, Suzhou, China

[4]Program of Sustainability in Biosystems, Institute of Agrifood Research and Technology, Caldes de Montbui, Spain

[5]Department of Botany and Biodiversity Research, Faculty of Life Sciences, University of Vienna, Vienna, Austria

[6]Department of Life Sciences, University of Trieste, Trieste, Italy

[7]Postgraduate Program in Microbiology, Parasitology and Pathology, Biological Sciences, Department of Basic Pathology, Federal University of Paranà, Curitiba, Brazil

[8]Engineering Bioprocess and Biotechnology Post-graduation Program, Department of Bioprocess Engineering and Biotechnology, Federal University of Parana, Curitiba, Brazil

[9]Research Center for Medical Mycology, Peking University, Beijing, China

[10]Foundation Atlas of Clinical Fungi, Hilversum, The Netherlands

[11]Third Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

[12]Consejo Nacional de Investigaciones Científicas y Tecnológicas / Hospital JM Ramos Mejía, Sector Micología, Buenos Aires, Argentina

[13]Faculdade de Medicina, Universidade de Brasília, Brasília, Brazil

[14]Department of Biology, Suan Sunandha Rajabhat University, Bangkok, Thailand

[15]Nucleus of Tropical Pathology and Social Medicine, Department of Pathology, Federal University of Maranhao, Maranhao, Brazil

[16]Biological Sciences Institute, Universidade de Brasília, Brasília, DF, Brazil

[17]Labordiagnostisches Zentrum, Uniklinik RWTH, Aachen, Germany

[18]Department of Ecological and Biological Sciences, University of Tuscia, Viterbo, Italy

[19]Department of Dermatology & Laboratory of Medical Mycology, Jining No. 1 People's Hospital, Jining, Shandong, China

* Corresponding author: Sybren de Hoog. E-mail: sybren.dehoog@radboudumc.nl

Abstract

Fungi in the order Chaetothyriales are renowned for their ability to cause human infections. Nevertheless, they are not regarded as primary pathogens, but rather as opportunists with a natural habitat in the environment. Extremotolerance is a major trend in the order, but quite different from black yeasts in Capnodiales which focus on endurance, an important additional parameter is advancing toxin management. In the ancestral ecology of rock colonization, the association with metabolite-producing lichens is significant. Ant-association, dealing with pheromones and repellents, is another mainstay in the order. The most advanced family, Herpotrichiellaceae, shows dual ecology in monoaromatic hydrocarbon assimilation and ability to cause disease in humans and cold-blooded vertebrates. Since most species have to be traumatically inoculated in order to cause disease, their invasive potential is categorized as opportunism. However, in chromoblastomycosis, with agents having endophytic life styles, virulence factors join with microaerophily, all properties enhancing survival in living tissue. If agents of vertebrate disease are able to escape from the host transmitting adapted genotypes to next generations, we may witness development towards pathogenicity. In this study, data on ecology, phylogeny, and genomics were collected and analyzed in order to support this hypothesis on the evolutionary route of the species of Chaetothyriales.

Keywords

Chaetothyriales, ecology, phylogeny, genomics, evolution, pathogenicity.