



ORIGINAL ARTICLE

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

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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 microfungi diversity has been studied. Metabarcoding analyses have shown high diversity of ascomycetous lichen-associated fungi in the two cosmopolitan rock-inhabiting lichens – *Rhizoplaca melanophthalma* and *Tephromela atra* – and many of these taxa were successfully isolated in culture. Based on DNA sequence data and morphological analyses, two new lineages within *Chaetothyriales* are here recognized. Both occur in lichens from dry habitats and are described here as the new species *Cladophialophora endolichena* Cometto, de Hoog, Muggia and *Paracladophialophora lichenicola* Cometto, de Hoog, Muggia. 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 · *Paracladophialophora* · *Sordariomycetes*

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 nineteenth century and have been the

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focus of a wide range of studies. They are commonly known as “lichenicolous fungi” for over a century, and more than 2300 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). Lichenicolous fungi usually develop symptoms of infections on their host lichen thalli, but many 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). Most of the symptomatically occurring lichenicolous fungi show some level of host-specificity – and sometimes even dependency, on their lichen host (Lawrey and Diederich 2003; Hafellner 2018). However, the specificity observed for the lichenicolous fungi, has been shown in only a few cases for some of the 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; Cometto et al. 2022).

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 and Škaloud 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 (Spribille 2018; 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 and Škaloud 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 cryptic 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 *R. melanophthalma* and *T. atra*, as these lichens revealed to be important sources for lichenicolous fungi (Muggia et al. 2016; Smith et al. 2020; Cometto et al. 2022). 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 taxa, 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 *R. melanophthalma* aggregate (Leavitt et al. 2011) have an umbilicate thallus (attached at a single point), whereas lichens representing the *T. atra* group (Muggia et al. 2014) 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, 2014; 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 (i.e., schist-arenaria, 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 *R. melanophthalma* and *T. atra* following the protocol of Yamamoto et al. (2002). Approximately 2 mm² 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). If 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 – for only a single

strain the D1/D2 domain of the 28S nuclear large ribosomal subunit (nucLSU) was further amplified 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 MAFFT 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 analysed 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 recognized within *Chaetothyriales* we performed a further analysis based on a more extended taxon sampling of *Herpotrichiellaceae* and *Paracladophialophoraceae* (Supplementary Table S5), selecting six species of *Cyphellophoraceae* as outgroups (*Cyphellophora eucalypti*, *C. laciniata*, *C. olivacea*, *C. pluriseptata*, *C. sessilis* and *Phialophora attae*), 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: form of growth, 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 Inkscape (www.inkscape.org).

Results

Culture isolation

A total of 131 fungal strains were isolated and characterized from 80 *R. melanophthalma* and 20 *T. atra* samples (Fig. 1a, b and Table 1; Supplementary Table S1): 65 strains belong to *Eurotiomycetes* (order *Chaetothyriales*), 53 strains to *Dothideomycetes* and 13 strains to *Sordariomycetes*. The *Eurotiomycetes* (order *Chaetothyriales*) strains were isolated from 20 samples of *R. melanophthalma* collected in 10 localities across South America (Argentina), North America (Utah) and Europe (Spain) and from eight samples *T. atra* collected in five localities across South America (Chile), Tasmania, Mauritius and Europe (Spain). The *Dothideomycetes* strains were isolated from 20 samples of *R. melanophthalma* collected in 13 localities across South America (Argentina and Chile), North America (Utah, Idaho and Nevada) and from

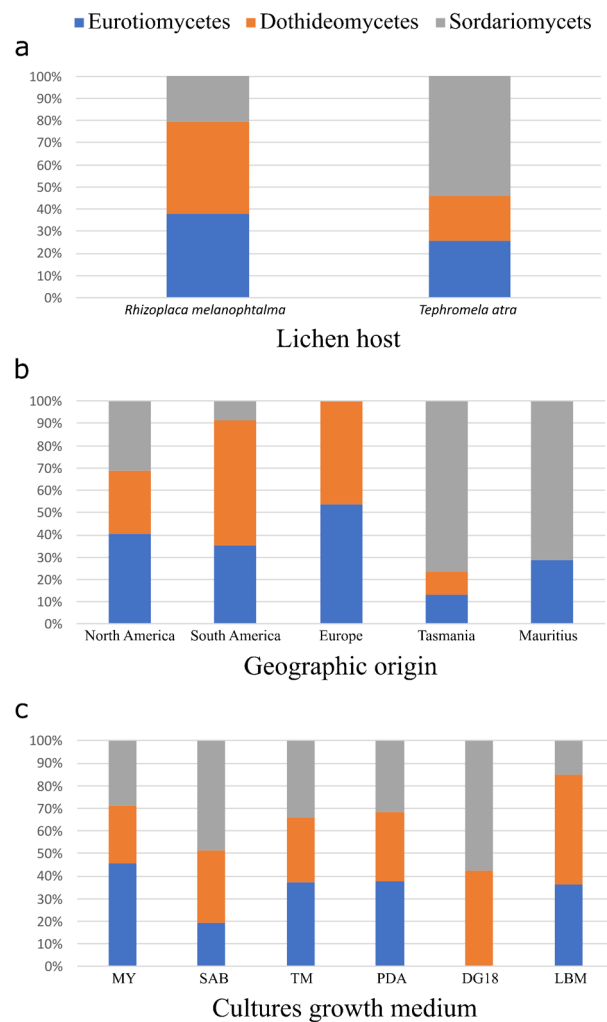


Fig. 1 **a** Lichen host, **b** geographic distribution, **c** cultures growth percentage on the six growth culture media of *Eurotiomycetes* (blue), *Dothideomycetes* (orange) and *Sordariomycetes* (grey)

seven samples *T. atra* collected in three localities in South America (Chile) and Europe (Spain). The *Sordariomycetes* strains were isolated from four samples of *R. melanophthalma* collected in two localities in South America (Argentina) and North America (Utah) and from two samples of *T. atra* collected in two localities in Tasmania and Mauritius. No fungal strains were obtained from *R. melanophthalma* thalli coming from three populations in Argentina above 3600 m a.s.l. and from one population 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*.

Table 1 Origin data and sequence accession numbers of *Eurotiomyces*, *Dothideomyces* and *Sordariomyces* 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 | <i>R. melanophthalma</i> L2803 | LBM | 24 | <i>Eurotiomyces</i> , incertae sedis, <i>Pleostigmataceae</i> | OQ921052 | OQ955706 |
| L3810 | <i>R. melanophthalma</i> L2803 | MY | 24 | <i>Eurotiomyces</i> , incertae sedis, <i>Pleostigmataceae</i> | OQ920988 | – |
| L3258 | <i>T. atra</i> L2583 | LBM | 13 | <i>Eurotiomyces</i> , incertae sedis, <i>Pleostigmataceae</i> | OQ920989 | OQ955703 |
| L3819 | <i>T. atra</i> L2599 | MY | 15 | <i>Eurotiomyces</i> , incertae sedis, <i>Pleostigmataceae</i> | OQ920991 | OQ955707 |
| L3774 | <i>T. atra</i> L2596 | MY | 15 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Muellerella</i> | OQ920990 | OQ955711 |
| L2881 | <i>R. melanophthalma</i> L2387 | LBM | 1 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Trichomeriaceae</i> , <i>Neophaeococcomyces</i> | OQ920995 | – |
| L2606 | <i>R. melanophthalma</i> L2390 | TM | 1 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Trichomeriaceae</i> , <i>Neophaeococcomyces</i> | OQ920994 | OQ955691 |
| L3238 | <i>R. melanophthalma</i> L2668 | TM | 17 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Trichomeriaceae</i> , <i>Neophaeococcomyces</i> | OQ920993 | OQ955702 |
| L3112 | <i>R. melanophthalma</i> L2669 | MY | 17 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Trichomeriaceae</i> , <i>Neophaeococcomyces</i> | OQ920992 | OQ955716 |
| L3099 | <i>R. melanophthalma</i> L2670 | SAB | 17 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Trichomeriaceae</i> , <i>Knufia</i> | OQ920996 | OQ955714 |
| L3064 | <i>R. melanophthalma</i> L2786 | LBM | 23 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Trichomeriaceae</i> , <i>Knufia</i> | OQ920998 | OQ955713 |
| L3065 | <i>R. melanophthalma</i> L2786 | MY | 23 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Trichomeriaceae</i> , <i>Knufia</i> | OQ920999 | OQ955720 |
| L3103 | <i>R. melanophthalma</i> L2786 | MY | 23 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Trichomeriaceae</i> , <i>Knufia</i> | OQ921000 | – |
| L3105 | <i>R. melanophthalma</i> L2802 | MY | 24 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Trichomeriaceae</i> , <i>Knufia</i> | OQ921001 | OQ955715 |
| L3252 | <i>R. melanophthalma</i> L2825 | TM | 25 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Trichomeriaceae</i> , <i>Knufia</i> | OQ920997 | – |
| L2876 | <i>R. melanophthalma</i> L2387 | MY | 1 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Melanina gunde-cimermaniae</i> | OQ921004 | OQ955696 |
| L2604 | <i>R. melanophthalma</i> L2390 | TM | 1 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Melanina gunde-cimermaniae</i> | OQ921008 | OQ955692 |
| L2605 | <i>R. melanophthalma</i> L2390 | MY | 1 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Melanina gunde-cimermaniae</i> | OQ921003 | – |
| L2618 | <i>R. melanophthalma</i> L2394 | LBM | 2 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Melanina gunde-cimermaniae</i> | OQ921005 | – |
| L2628 | <i>R. melanophthalma</i> L2398 | SAB | 2 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Melanina gunde-cimermaniae</i> | OQ921006 | OQ955712 |
| L3067 | <i>T. atra</i> L2572 | TM | 12 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Melanina gunde-cimermaniae</i> | OQ921007 | OQ955698 |
| L3068 | <i>T. atra</i> L2572 | PDA | 12 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Melanina gunde-cimermaniae</i> | OQ921002 | – |
| L3233 | <i>T. atra</i> L2753 | MY | 22 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> sp. | OQ921010 | OQ955701 |
| L3784 | <i>T. atra</i> L2755 | PDA | 22 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> sp. | OQ921009 | OQ955705 |
| L3260 | <i>R. melanophthalma</i> L2636 | PDA | 16 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Paracladophialophoraceae</i> , <i>Paracladophialophora lichenicola</i> sp. nov. | OQ921012 | – |
| L3261 | <i>R. melanophthalma</i> L2636 | SAB | 16 | <i>Eurotiomyces</i> , <i>Chaetothyriales</i> , <i>Paracladophialophoraceae</i> , <i>Paracladophialophora lichenicola</i> sp. nov. | OQ921022 | – |

Table 1 (continued)

| Culture ID | Lichen of origin | Culture medium | Population ID | Phylogenetic placement | ITS | nucLSU |
|------------|--------------------------------|----------------|---------------|---|-----------------|-----------------|
| L3262 | <i>R. melanophthalma</i> L2636 | SAB | 16 | <i>Eurotiomycetes, Chaetothyriales, Paracladophialophoraceae, Paracladophialophora lichenicola</i> sp. nov. | OQ921011 | OQ955717 |
| L3763 | <i>R. melanophthalma</i> L2638 | PDA | 16 | <i>Eurotiomycetes, Chaetothyriales, Paracladophialophoraceae, Paracladophialophora lichenicola</i> sp. nov. | OQ921013 | – |
| L3764 | <i>R. melanophthalma</i> L2638 | MY | 16 | <i>Eurotiomycetes, Chaetothyriales, Paracladophialophoraceae, Paracladophialophora lichenicola</i> sp. nov. | OQ921014 | – |
| L3765 | <i>R. melanophthalma</i> L2638 | MY | 16 | <i>Eurotiomycetes, Chaetothyriales, Paracladophialophoraceae, Paracladophialophora lichenicola</i> sp. nov. | OQ921015 | OQ955719 |
| L3771 | <i>R. melanophthalma</i> L2638 | MY | 16 | <i>Eurotiomycetes, Chaetothyriales, Paracladophialophoraceae, Paracladophialophora lichenicola</i> sp. nov. | OQ921017 | – |
| L3779 | <i>T. atra</i> L2570 | MY | 12 | <i>Eurotiomycetes, Chaetothyriales, Paracladophialophoraceae, Paracladophialophora lichenicola</i> sp. nov. | OQ921019 | – |
| L3780 | <i>T. atra</i> L2570 | MY | 12 | <i>Eurotiomycetes, Chaetothyriales, Paracladophialophoraceae, Paracladophialophora lichenicola</i> sp. nov. | OQ921020 | – |
| L3781 | <i>T. atra</i> L2570 | TM | 12 | <i>Eurotiomycetes, Chaetothyriales, Paracladophialophoraceae, Paracladophialophora lichenicola</i> sp. nov. | OQ921023 | – |
| L3782* | <i>T. atra</i> L2570 | PDA | 12 | <i>Eurotiomycetes, Chaetothyriales, Paracladophialophoraceae, Paracladophialophora lichenicola</i> sp. nov. | OQ921021 | OQ955704 |
| L3783 | <i>T. atra</i> L2570 | PDA | 12 | <i>Eurotiomycetes, Chaetothyriales, Paracladophialophoraceae, Paracladophialophora lichenicola</i> sp. nov. | OQ921016 | – |
| L3776 | <i>T. atra</i> L2572 | MY | 12 | <i>Eurotiomycetes, Chaetothyriales, Paracladophialophoraceae, Paracladophialophora lichenicola</i> sp. nov. | OQ921018 | OQ955718 |
| L3096 | <i>R. melanophthalma</i> L2734 | TM | 21 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae</i> | OQ921024 | OQ955699 |
| L2863 | <i>T. atra</i> L2596 | TM | 15 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae</i> | OQ921025 | OQ955695 |
| L3110 | <i>R. melanophthalma</i> L2421 | MY | 3 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora</i> sp. | OQ921047 | OQ955725 |
| L2865 | <i>R. melanophthalma</i> L2568 | PDA | 14 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora</i> sp. | OQ921026 | OQ955708 |
| L3111 | <i>R. melanophthalma</i> L2669 | LBM | 17 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora</i> sp. | OQ921048 | OQ955724 |
| L3057 | <i>T. atra</i> L2561 | LBM | 10 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora</i> sp. | OQ921050 | – |
| L3058 | <i>T. atra</i> L2561 | LBM | 10 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora</i> sp. | OQ921049 | OQ955710 |
| L3087 | <i>T. atra</i> L2598 | PDA | 15 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora</i> sp. | OQ921027 | OQ955723 |
| L2871 | <i>R. melanophthalma</i> L2389 | MY | 1 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921046 | – |
| L2612 | <i>R. melanophthalma</i> L2390 | LBM | 1 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921028 | – |

Table 1 (continued)

| Culture ID | Lichen of origin | Culture medium | Population ID | Phylogenetic placement | ITS | nucLSU |
|---------------|--------------------------------|----------------|---------------|--|-----------------|-----------------|
| L2614 | <i>R. melanophthalma</i> L2390 | SAB | 1 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921029 | OQ955693 |
| L2619 | <i>R. melanophthalma</i> L2394 | LBM | 2 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921030 | OQ955694 |
| L3059 | <i>R. melanophthalma</i> L2635 | SAB | 16 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921033 | – |
| L3060 | <i>R. melanophthalma</i> L2635 | TM | 16 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921034 | OQ955722 |
| L3061 | <i>R. melanophthalma</i> L2635 | MY | 16 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921035 | – |
| L3251 | <i>R. melanophthalma</i> L2637 | MY | 16 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921044 | – |
| L3072 | <i>R. melanophthalma</i> L2638 | LBM | 16 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921040 | OQ955697 |
| L3073 | <i>R. melanophthalma</i> L2638 | MY | 16 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921041 | – |
| <u>L3074*</u> | <i>R. melanophthalma</i> L2638 | PDA | 16 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921042 | – |
| L3766 | <i>R. melanophthalma</i> L2638 | MY | 16 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921045 | – |
| L3770 | <i>R. melanophthalma</i> L2638 | PDA | 16 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921043 | – |
| L3106 | <i>R. melanophthalma</i> L2668 | PDA | 17 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921051 | – |
| L3240 | <i>R. melanophthalma</i> L2668 | TM | 17 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921036 | – |
| L3098 | <i>R. melanophthalma</i> L2670 | TM | 17 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921038 | OQ955700 |
| L3100 | <i>R. melanophthalma</i> L2670 | PDA | 17 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921039 | OQ955721 |
| L3264 | <i>R. melanophthalma</i> L2670 | MY | 17 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921037 | – |
| L2880 | <i>T. atra</i> L2596 | LBM | 15 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921032 | – |
| L2870 | <i>T. atra</i> L2598 | MY | 15 | <i>Eurotiomycetes, Chaetothyriales, Herpotrichiellaceae, Cladophialophora endolichena</i> sp. nov. | OQ921031 | OQ955709 |
| L3077 | <i>R. melanophthalma</i> L2724 | LBM | 20 | <i>Dothideomycetes, incertae sedis</i> | OQ924457 | – |
| L2869 | <i>R. melanophthalma</i> L2387 | PDA | 1 | <i>Dothideomycetes, Pleosporales</i> | OQ924455 | OQ938956 |

Table 1 (continued)

| Culture ID | Lichen of origin | Culture medium | Population ID | Phylogenetic placement | ITS | nucLSU |
|------------|--------------------------------|----------------|---------------|---|-----------------|-----------------|
| L3091 | <i>T. atra</i> L2597 | TM | 15 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Teichosporaceae</i> | OQ924449 | OQ938944 |
| L2868 | <i>T. atra</i> L2598 | PDA | 15 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Massariaceae</i> , <i>Paraphaeosphaeria michotii</i> | OQ924429 | OQ938957 |
| L3036 | <i>R. melanophthalma</i> L2704 | DG18 | 19 | <i>Dothideomycetes</i> , <i>Pleosporales</i> | OQ924430 | OQ938951 |
| L3021 | <i>T. atra</i> L2599 | LBM | 15 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Didymellaceae</i> | OQ924428 | OQ938953 |
| L3078 | <i>R. melanophthalma</i> L2724 | MY | 20 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Pleosporaceae</i> | OQ924427 | OQ938946 |
| L2888 | <i>T. atra</i> L2545 | LBM | 10 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Phaeosphaeriaceae</i> | OQ924445 | – |
| L3056 | <i>T. atra</i> L2569 | TM | 12 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Phaeosphaeriaceae</i> | OQ924446 | OQ938960 |
| L3076 | <i>R. melanophthalma</i> L2724 | MY | 20 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Phaeosphaeriaceae</i> | OQ924451 | OQ938947 |
| L3042 | <i>R. melanophthalma</i> L2734 | SAB | 21 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Phaeosphaeriaceae</i> | OQ924414 | OQ938950 |
| L3030 | <i>R. melanophthalma</i> L2827 | TM | 25 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Phaeosphaeriaceae</i> | OQ924426 | OQ938952 |
| L3094 | <i>T. atra</i> L2596 | TM | 15 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Phaeosphaeriaceae</i> | OQ924415 | OQ938943 |
| L3037 | <i>R. melanophthalma</i> L2827 | SAB | 25 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Phaeosphaeriaceae</i> | OQ925920 | – |
| L3090 | <i>R. melanophthalma</i> L2454 | MY | 4 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Phaeosphaeriaceae</i> | OQ924431 | OQ938945 |
| L3108 | <i>R. melanophthalma</i> L2387 | DG18 | 1 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Phaeosphaeriaceae</i> | OQ924421 | OQ938941 |
| L2622 | <i>R. melanophthalma</i> L2398 | TM | 2 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Phaeosphaeriaceae</i> | OQ924422 | – |
| L2624 | <i>R. melanophthalma</i> L2398 | TM | 2 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Phaeosphaeriaceae</i> | OQ924425 | – |
| L2625 | <i>R. melanophthalma</i> L2398 | LBM | 2 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Phaeosphaeriaceae</i> | OQ924424 | – |
| L2627 | <i>R. melanophthalma</i> L2398 | LBM | 2 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Phaeosphaeriaceae</i> | OQ924423 | – |
| L3107 | <i>R. melanophthalma</i> L2668 | MY | 17 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Phaeosphaeriaceae</i> | OQ924420 | OQ938942 |
| L3265 | <i>R. melanophthalma</i> L2670 | MY | 17 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Phaeosphaeriaceae</i> , <i>Phoma</i> | OQ924419 | OQ938961 |
| L2897 | <i>R. melanophthalma</i> L2670 | SAB | 17 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Phaeosphaeriaceae</i> , <i>Phoma</i> | OQ924418 | – |
| L3048 | <i>R. melanophthalma</i> L2689 | DG18 | 18 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Phaeosphaeriaceae</i> , <i>Phoma</i> | OQ924416 | OQ938949 |
| L3054 | <i>R. melanophthalma</i> L2706 | PDA | 19 | <i>Dothideomycetes</i> , <i>Pleosporales</i> , <i>Phaeosphaeriaceae</i> , <i>Phoma</i> | OQ924417 | OQ938948 |
| L2856 | <i>T. atra</i> L2572 | SAB | 12 | <i>Dothideomycetes</i> , <i>incertae sedis</i> | OQ924458 | – |
| L2857 | <i>T. atra</i> L2572 | SAB | 12 | <i>Dothideomycetes</i> , <i>incertae sedis</i> | OQ924459 | – |
| L2890 | <i>T. atra</i> L2572 | MY | 12 | <i>Dothideomycetes</i> , <i>incertae sedis</i> | OQ924447 | OQ938954 |
| L3049 | <i>T. atra</i> L2572 | MY | 12 | <i>Dothideomycetes</i> , <i>incertae sedis</i> | OQ924456 | – |
| L3776 | <i>T. atra</i> L2572 | DG18 | 12 | <i>Dothideomycetes</i> , <i>incertae sedis</i> | OQ924448 | – |
| L3817 | <i>T. atra</i> L2572 | DG18 | 12 | <i>Dothideomycetes</i> , <i>incertae sedis</i> | OQ924454 | – |
| L3747 | <i>R. melanophthalma</i> L2385 | MY | 1 | <i>Dothideomycetes</i> , <i>Mycosphaerellales</i> , <i>Mycosphaerellaceae</i> , <i>Ramularia</i> | OQ924453 | OQ938938 |

Table 1 (continued)

| Culture ID | Lichen of origin | Culture medium | Population ID | Phylogenetic placement | ITS | nucLSU |
|------------|--------------------------------|----------------|---------------|--|----------|----------|
| L2879 | <i>R. melanophthalma</i> L2389 | LBM | 1 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae</i> | OQ924465 | OQ938955 |
| L3239 | <i>R. melanophthalma</i> L2668 | LBM | 17 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae</i> | OQ924450 | OQ938940 |
| L3270 | <i>R. melanophthalma</i> L2787 | PDA | 23 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae</i> | OQ924452 | OQ938939 |
| L2773 | <i>R. melanophthalma</i> L2469 | MY | 5 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae, Elasticomyces</i> | OQ924460 | OQ938958 |
| L2775 | <i>R. melanophthalma</i> L2469 | SAB | 5 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae, Elasticomyces</i> | OQ924461 | – |
| L2777 | <i>R. melanophthalma</i> L2469 | LBM | 5 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae, Elasticomyces</i> | OQ924462 | – |
| L2784 | <i>R. melanophthalma</i> L2544 | LBM | 9 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae, Elasticomyces</i> | OQ924443 | – |
| L2785 | <i>R. melanophthalma</i> L2544 | LBM | 9 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae, Elasticomyces</i> | OQ924463 | OQ938959 |
| L2886 | <i>R. melanophthalma</i> L2528 | SAB | 9 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae, Elasticomyces</i> | OQ924464 | – |
| L3079 | <i>R. melanophthalma</i> L2687 | LBM | 18 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae, Elasticomyces</i> | OQ924444 | – |
| L3081 | <i>R. melanophthalma</i> L2687 | LBM | 18 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae, Elasticomyces</i> | OQ924432 | – |
| L3082 | <i>R. melanophthalma</i> L2687 | PDA | 18 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae, Elasticomyces</i> | OQ924433 | OQ938937 |
| L3237 | <i>R. melanophthalma</i> L2724 | PDA | 20 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae, Elasticomyces</i> | OQ924441 | OQ938936 |
| L3104 | <i>R. melanophthalma</i> L2802 | TM | 24 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae, Elasticomyces</i> | OQ924434 | OQ938934 |
| L3115 | <i>R. melanophthalma</i> L2824 | MY | 25 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae, Elasticomyces</i> | OQ924435 | – |
| L3116 | <i>R. melanophthalma</i> L2825 | LBM | 25 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae, Elasticomyces</i> | OQ924436 | – |
| L3117 | <i>R. melanophthalma</i> L2825 | MY | 25 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae, Elasticomyces</i> | OQ924439 | – |
| L3118 | <i>R. melanophthalma</i> L2825 | DG18 | 25 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae, Elasticomyces</i> | OQ924438 | OQ938935 |
| L3119 | <i>R. melanophthalma</i> L2825 | PDA | 25 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae, Elasticomyces</i> | OQ924437 | – |
| L3120 | <i>R. melanophthalma</i> L2825 | PDA | 25 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae, Elasticomyces</i> | OQ924440 | OQ938933 |
| L3253 | <i>R. melanophthalma</i> L2825 | SAB | 25 | <i>Dothideomycetes, Mycosphaerellales, Teratosphaeriaceae, Elasticomyces</i> | OQ924442 | – |
| L3814 | <i>R. melanophthalma</i> L2635 | PDA | 16 | <i>Sordariomycetes, Xylariales, Cryptosphaeria</i> | OQ930026 | OQ931227 |
| L3815 | <i>R. melanophthalma</i> L2635 | PDA | 16 | <i>Sordariomycetes, Xylariales, Cryptosphaeria</i> | OQ930024 | OQ931228 |
| L3028 | <i>R. melanophthalma</i> L2827 | DG18 | 25 | <i>Sordariomycetes, Xylariales, Hyalotiella</i> | OQ930025 | OQ931229 |
| L3093 | <i>T. atra</i> L2596 | MY | 15 | <i>Sordariomycetes, Choniochaetales, Coniochaeta</i> | OQ930016 | – |
| L2858 | <i>T. atra</i> L2598 | SAB | 15 | <i>Sordariomycetes, Choniochaetales, Coniochaeta</i> | OQ930014 | OQ931230 |
| L2877 | <i>T. atra</i> L2598 | LBM | 15 | <i>Sordariomycetes, Choniochaetales, Coniochaeta</i> | OQ930015 | – |
| L2859 | <i>T. atra</i> L2598 | SAB | 15 | <i>Sordariomycetes, Hypocreales, Tolypocladium</i> | OQ930020 | – |

Table 1 (continued)

| Culture ID | Lichen of origin | Culture medium | Population ID | Phylogenetic placement | ITS | nucLSU |
|------------|--------------------------------|----------------|---------------|--|-----------------|-----------------|
| L2883 | <i>T. atra</i> L2598 | DG18 | 15 | <i>Sordariomycetes</i> , <i>Hypocreales</i> , <i>Tolypocladium</i> | OQ930019 | – |
| L3086 | <i>T. atra</i> L2598 | TM | 15 | <i>Sordariomycetes</i> , <i>Hypocreales</i> , <i>Tolypocladium</i> | OQ930017 | OQ931232 |
| L3089 | <i>T. atra</i> L2598 | MY | 15 | <i>Sordariomycetes</i> , <i>Hypocreales</i> , <i>Tolypocladium</i> | OQ930018 | – |
| L3114 | <i>T. atra</i> L2754 | TM | 22 | <i>Sordariomycetes</i> , <i>Hypocreales</i> , <i>Tolypocladium</i> | OQ930021 | OQ931231 |
| L2896 | <i>R. melanophthalma</i> L2636 | SAB | 16 | <i>Sordariomycetes</i> , <i>Hypocreales</i> , <i>Mycrocera</i> | OQ930023 | OQ931233 |
| L3122 | <i>R. melanophthalma</i> L2638 | MY | 16 | <i>Sordariomycetes</i> , <i>Hypocreales</i> , <i>Microcera</i> | OQ930022 | – |

Eurotiomycetes (order *Chaetothyriales*) strains were unable to grow on DG18 media, while all the other fungal strains grew on the six different culture media (Fig. 1c and Table 1). *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 and Table 1).

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 class – *Eurotiomycetes*, *Dothideomycetes* and *Sordariomycetes* – using the concatenated two-locus datasets (Supplementary Material Tables S2–S5). Maximum Likelihood 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 (Figs. 2, 3, 4 and Table 1, Supplementary Material Table S2, S3) – Four strains were placed in *Pleostigmataceae* (here unsupported as in Muggia et al. 2021): two (L3809 and L3810) isolated from *R. melanophthalma* collected in South America (Argentina) and other 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 ($4 \times 14 \mu\text{m}$) and by roundish or elliptical cell (5 up to $17 \mu\text{m}$ 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 $11 \mu\text{m}$ diameter) intercalated by rectangular cells ($2\text{--}4 \times 12 \mu\text{m}$) from which ramifications generated (Figs. 3c, d).

The ‘*Muellerella* + *Lichenodiplis*’ clade (sensu Muggia et al. 2015, 2019) and the family *Epibryaceae* were found as the most basal lineages in *Chaetothyriales*. In this clade we found one strain (L3774) isolated from *T. atra* collected in Tasmania, and the strain *Chaetothyriales* sp. Pet 5a (Vasse et al. 2017). The strain L3774 was characterized by heavy melanized elliptical and elongated cells ($4 \times 8 \mu\text{m}$) often constricted at the septa and with rare ramification (Fig. 3e) and conidia-like cells (4 up to $7 \mu\text{m}$ 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 *Neophaeoocomyces 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 ($4 \times 10 \mu\text{m}$; Figs. 3i, k) and by conidia and chlamydo-spore-like cells (4 up to $10 \mu\text{m}$ 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 ($4 \times 6 \mu\text{m}$ to $11 \times 15 \mu\text{m}$; Figs. 3o–q) intercalated by rectangular cells ($4 \times 10 \mu\text{m}$; 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 $10 \mu\text{m}$ 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 to a monophyletic lineage that was identified here for the first time and we referred to it as the new species “*Paracladophialophora lichenicola*” (see below and Fig. 4). The two isolates L3233 and L3784 were characterized by heavy or slight melanized hyphae in which cylindrical and rectangular cells ($4 \times 10\text{--}15 \mu\text{m}$; Fig. 3ac) were intercalated to spherical cells (5 to 15 μm diameter; Fig. 3ad).

The new lineage of *Paracladophialophora lichenicola* was represented by 13 newly isolated 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 (Figs. 2, 4 4), including many *Chaetothyriales* sp. (S1, h2, Sh9, Sh10, Sh12, Sh25, Sh36, L204, 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).

Twenty eight new isolates (Figs. 2 and 4, Table 1) were placed in *Herpotrichiellaceae*, here the largest represented family. 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*, *Exophiala nigra* and *Exophiala spinifera* (Figs. 2 and 4). 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 ($3 \times 12 \mu\text{m}$) with branches (Fig. 3x). Other six 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 related to three *Cladophialophora* strains isolated from lichens, i.e. *Cladophialophora* sp. S5, L359 from *Rusawskia elegans* and *Gyalolechia fulgida*, respectively, and *C. parmeliae* (Figs. 2, 41). Their 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 ($4 \times 12\text{--}17 \mu\text{m}$) from which branches generate (Fig. 3af) and from laterally budding cells (Fig. 3ag).

The remaining 20 strains, isolated from both *R. melanophthalma* collected in North and South America (Utah and Argentina, respectively) and from *T. atra* collected in

Tasmania, formed a separate monophyletic lineage with further *Cladophialophora* strains (Figs. 2 and 4) isolated from lichens in previous studies (Harutyunyan et al. 2008; Muggia et al. 2016, 2017, 2021), i.e. *Cladophialophora* sp. Sh8, A1044 and A1069. We recognize this second new lineage as the new species *Cladophialophora endolichena* (see below). The phylogenetic position of the two new species *Paracladophialophora lichenicola* and *Cladophialophora endolichena* has been further confirmed by the extended analyses of Fig. 4.

The detailed morphological descriptions of both *Paracladophialophora lichenicola* and *Cladophialophora endolichena* are presented below in the Taxonomy section.

Dothideomycetes (Figs. 5, 6 and Table 1; Supplementary Material Table S4) – Fifty-three new strains were found in *Dothideomycetes*. The strain L3077, isolated from *R. melanophthalma* collected in North America (Idaho), was placed on an own branch basal to *Dothideomycetes*, as well as *Lichenothelia papilliformis* (Ametrano et al. 2019), *Lichenostigmatales* sp. A930 (Muggia et al. 2016) and Fungal sp. TRN529 (Ruibal et al. 2009). L3077 was morphologically similar to *Lichenostigmatales* sp. A930, with a yeast-like black mycelium (Fig. 6a), budding hyphae and melanized cells (3 up to 20 μm diameter; Figs. 6b, 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 ($4 \times 15 \mu\text{m}$; 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 with the *Pleosporales* sp. A1039 isolated from lichens (by Muggia et al. 2016), *Periconia* sp. and two uncultured strains 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*, here represented by *Phoma herbarum*, *Ampelomyces* sp. and *Didymella* spp. This strain was characterized by a white to grey mycelium (Fig. 6d) with slight melanized hyphae ($5 \times 15 \mu\text{m}$; Fig. 6e).

The strain L3078, isolated from *R. melanophthalma* collected in North America was placed in the family

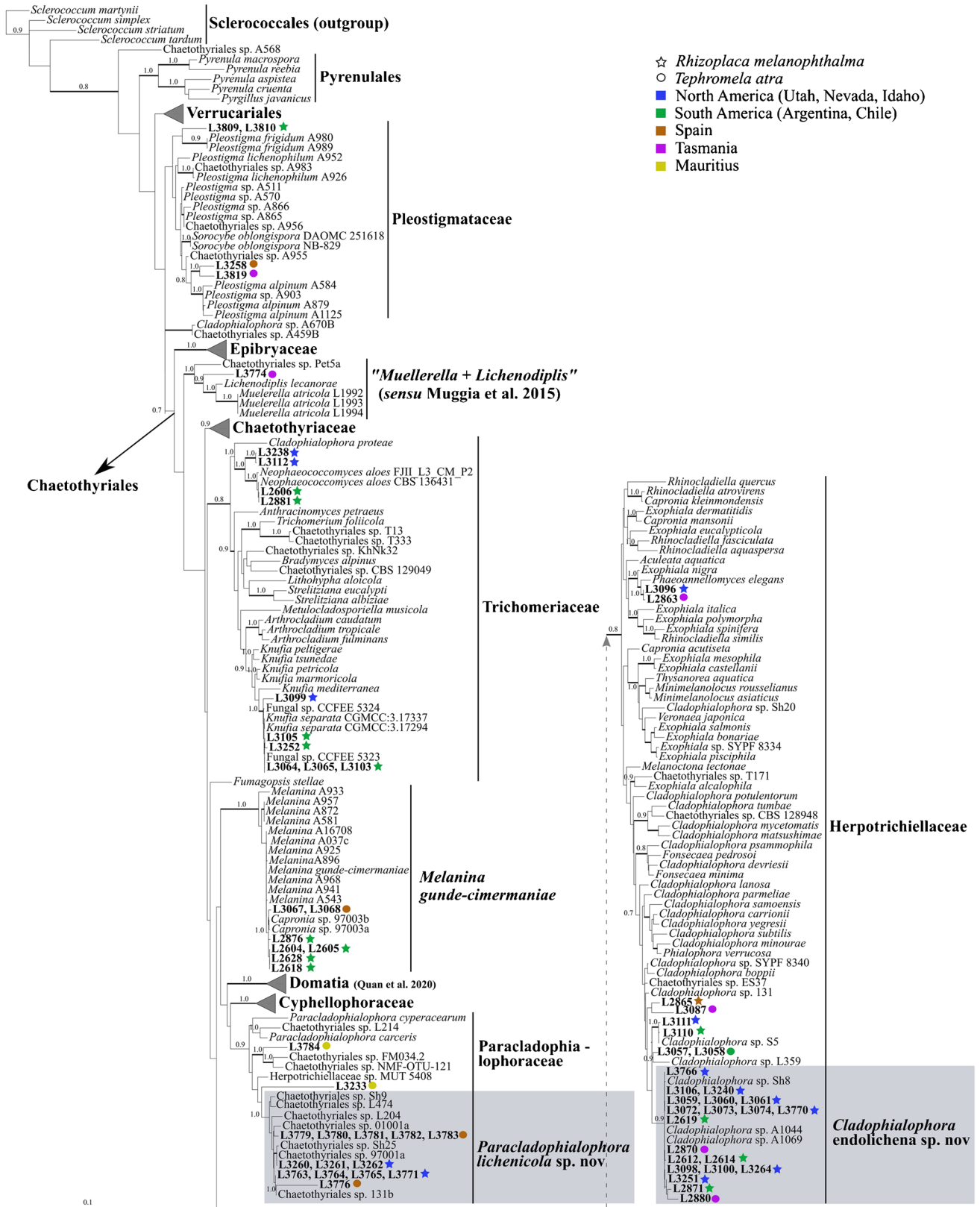


Fig. 2 Phylogenetic inference of *Eurotiomycetes* (*Chaetothyriales*): Maximum Likelihood analysis based on the concatenated nuclear ITS-LSU dataset; branches in bold denote RAxML bootstrap support $\geq 75\%$; Bayesian posterior probabilities ≥ 0.8 are reported above branches. Newly obtained sequences 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 newly identified lineages are highlighted in grey

Pleosporaceae next to *Pleospora* spp. and *Comoclathris lini*. This strain was characterized by a grey mycelium with brown margin and septate hyphae ($4 \times 15 \mu\text{m}$; 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. 6f) and very slightly melanized hyphae composed by cylindrical and rectangular cells ($5 \times 15 \mu\text{m}$) often branching and constricted at the septa (Fig. 6g). Four strains (L3030, L3042, L3076 and L3094), isolated from *R. melanophthalma* collected in North America (Idaho and Nevada) and South America (Argentina) and from *T. atra* thallus collected in Tasmania were closely related to *Ascomycota* PLC12C, *Leptosphaeria* sp. pIC11E (Mouhamadou et al. 2011) and to an uncultured fungus OTU569 (Qin et al. 2019). They were characterized by a black to grey mycelium composed by slight melanized hyphae with rectangular and elliptical cells ($5 \times 15 \mu\text{m}$; Figs. 6h–j) from which the ramifications generate. Conidial cells ($5 \mu\text{m}$ diameter) were observed (Fig. 6j). 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*, *Dothideomyces* LTSP_EUKA_P5M163 (Hartmann et al. 2009), an uncultured fungus 112_NA4_P31_N4 (Timling et al. 2014) and *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 closely related to *Didymocyrtis brachylaenae* and four *Phaeosphaeria* strains [namely *Phaeosphaeria* sp. SW_0_F12, *Phaeosphaeria* sp. AC (Travadon et al. 2016), *Phaeosphaeria* sp. 1715242 and *Phaeosphaeria* sp. M129 (Bérubé and Nicolas 2015)]. These six strains had an ochre to pale pink

mycelium with hyaline hyphae composed by rectangular cells ($4 \times 12 \mu\text{m}$; not shown). Four strains (L2897, L3048, L3054 and L3265), isolated from *R. melanophthalma* collected in North America (Utah), were nested within *Phoma* species described from lichens, i.e., *Phoma caloplacae* and *P. cladoniicola* and likely correspond to these two species. 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 ($5 \times 15 \mu\text{m}$) and conidogenous-like cells ($10 \mu\text{m}$ diameter; Figs. 6k, 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 close related to *Teratosphaeriaceae* sp. CPC 12419 (Crous et al. 2008) and closely related to *Saxomyces peninicus* and *Teratosphaeria parva*. This strain was characterized by a dark black mycelium (Fig. 6p) composed of melanized and hyaline hyphae with rectangular cells ($4 \times 10 \mu\text{m}$) from which ramification started. Filaments of isodiametric, conidia-like cells ($7 \mu\text{m}$ diameter) with apical cell developing into hyphae were observed (Fig. 6q). 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 and Rambold 2012). This strain was characterized by a blackish mycelium composed by heavy melanized hyphae with rectangular cell ($5 \times 15 \mu\text{m}$) from which ramification started (data not shown). The strain L3270, isolated from *R. melanophthalma* collected in South America (Argentina) was close related to three unidentified fungi *Dothideomyces* sp. AK1125 (U'Ren et al. 2012), *Dothideomyces* 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. *Dothideomyces* sp. s_C03_05.ab (Amend et al. 2010), *Dothideomyces* 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 likely correspond to *Elasticomyces elasticus* and were characterized by blackish to greenish mycelia with irregular margin (Fig. 6r) and heavy melanized hyphae composed by cylindrical and rectangular cells ($5 \times 8\text{--}12 \mu\text{m}$) with ramification (Figs. 6s, t).

Six strains (strains L2856, L2857, L2890, L3049, L3776 and L3817), isolated from *T. atra* collected in Europe



Fig. 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 (clade names are in parenthesis). Strains **a** L3819, **b** L3809, **c–d** L3258 (*Pleostigmataceae*); **e–f** L3774 (*Muellerella*+*Lichenodiplis*); **g–i** L2881, **j, k** L3238, **l** L3112, **m, n** L3252; **o, p** L3099, **q, r** L3065 (*Trichomeriaceae*); **s, t** L3067; **u** L2618, **v** L2876 (*Melanina gunde-cimermaniae*); **w** L3096; **x** L2863 (*Herpotrichiellaceae, Phaeoanellomyces* sp.); **y, z** L3262; **aa, ab** L3765; **ac, ad** L3784 (*Paracladophialophora lichenicola* sp. nov.); **ae, af** L2865; **ag** L3058 (*Cladophialophora* sp.); **ah** L3060; **ai, aj** L2870 (*Cladophialophora endolichena* sp. nov.). **a, g, m, s, w, y, ae** Colony appearance on solid medium after six-month to one year of growth. **b, d, p, k, aa, ab, af, ag** Filamentous, septate and melanized hyphae. **c, e, i, o, q, r, x, ac, ai** Branching hyphae. **z, h, j, l, t–v, z, ah, aj** Conidia-like cells. **n** Anastomosis hyphae. Scale bars: **a, g, m, s, w, y, ae** 1 cm; **b, f, h–l, n–r, t–v, x, z–ac, ah** 5 μ m; **c–e, af, ag, ai, aj** 10 μ m

(Spain), were placed together with two *Dothideomycetes* sp., i.e. A931 and A552, isolated from lichens (Muggia et al. 2016) in a clade closely related to *Venturiales*, *Lichenocloniales* and *Abrothallales*. These strains had a pink mycelium with regular margin (Fig. 6m) composed by hyaline hyphae built by rectangular cells ($2 \times 15 \mu$ m) from which branches generated (Figs. 6n, o).

Sordariomycetes (Figs. 7, 8 and Table 1; Supplementary Material Table S5) – Thirteen strains were found belonging to *Xylariales*, *Coniochaetales* and *Hypocreales*. 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 some agglomerations of less melanised hyphae (Fig. 8a), rather thin (3 μ m diameter; Figs. 8b, 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-orange mycelium built by hyaline hyphae (3 μ m diameter; Figs. 8d, 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. from lichens [i.e., A518, A524, A551, A890 and A1007 (Muggia et al. 2016)] and not [1 TKPB-2017 (Kowalski and Bilański 2021), *Sordariomycetes* sp. n165.1 and TS1_1_5i]. These strains were characterized by a white mycelium (Fig. 8f) composed by hyaline hyphae (3 μ m diameter; Fig. 8g).

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 nested in the lineage of *Tolypocladium* sp. (MS217, M1–1-5U and JDF-2013 g; 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. 8h, k) composed of hyaline hyphae (3 μ m diameter) with branches (Figs. 8i, j, l). Two strains (L2896 and L3122), isolated from *R. melanophthalma* collected in North America (Utah), are closely related to *Microcera physciae* (Crous et al. 2021) and other *Microcera* species as well as two *Fusarium* sp. samples, *Nectria cinnabarina* and *Cosmospora quaranticola*. These strains were characterized by an orange mycelium (Fig. 8m) made of hyaline hyphae (4 μ m diameter) with branches (Figs. 8n, o).

Taxonomy

Cladophialophora endolichena Cometto, de Hoog, Muggia, sp. nov.

Mycobank: MB 848887.

Etymology: residing inside lichens.

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 *R. melanophthalma* (L2638).

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 (not shown). Mostly of the hyphae are composed by rectangular and cylindrical cells ($4 \times 12–17 \mu$ m) from which branches generate (Fig. 3ai). Apical muriform conidiogenous cells ($10 \times 12 \mu$ m, Fig. 3ah) and lateral conidiogenous cells ($8 \times 10 \mu$ m; 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: *Lecanora bicincta*, *Lecanora polytropa*, *Protoparmeliopsis muralis*, *R. melanophthalma*, *T. atra*.

Material examined: SOUTH AMERICA, Argentina, prov. Mendoza, on basaltic boulders, alt. 1450–2000 m a.s.l., endolichenic fungi isolated from *R. melanophthalma* lichen thalli, 2019, L. Muggia, strain numbers L2612, L2614, L2619 and L2871. 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, L3240, L3251, L3264, L3766 and L3770. OCEANIA, Tasmania, three Thumbs, on dolorite rocks, alt. 545 m a.s.l., endolichenic fungi isolated from *T.*

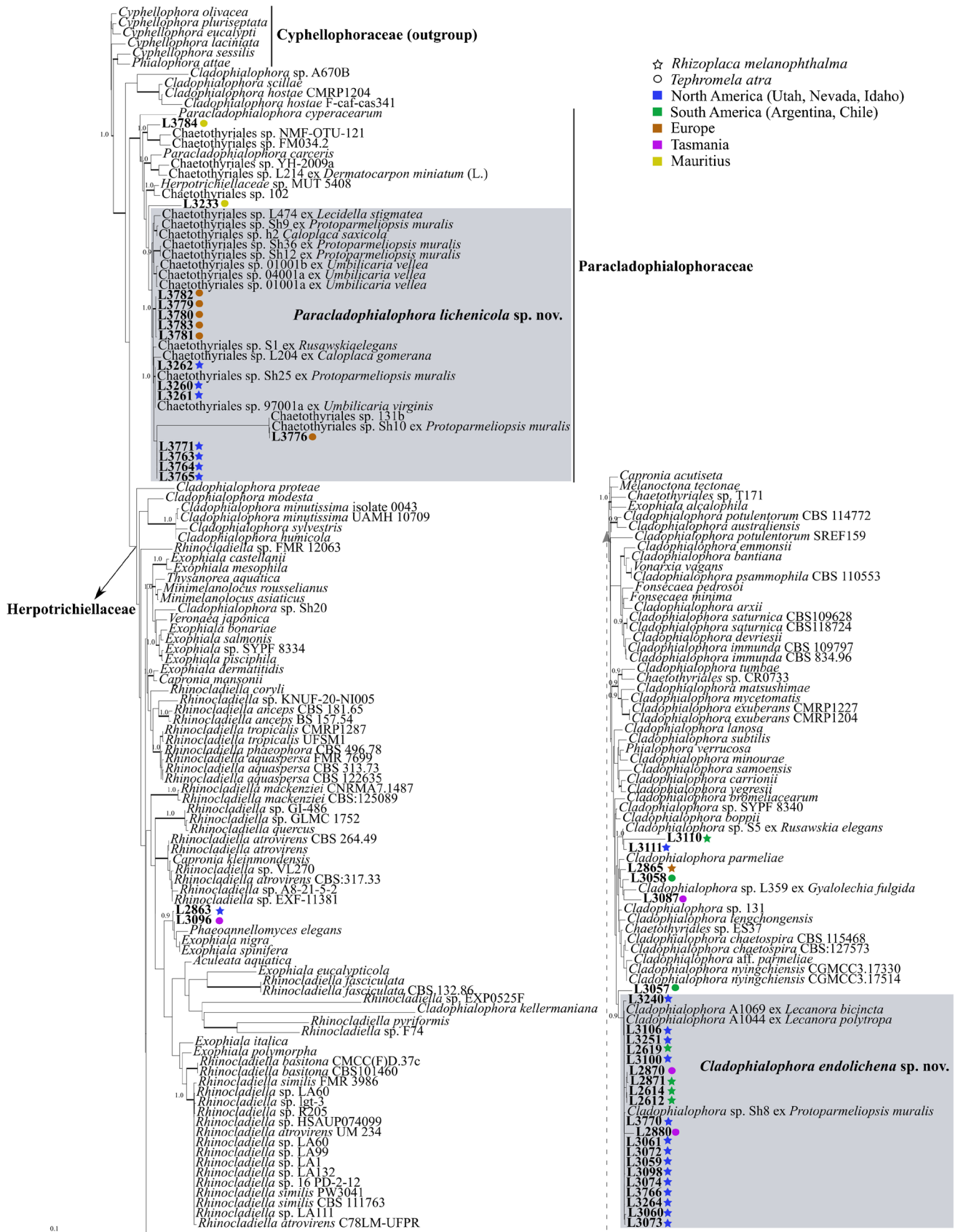


Fig. 4 Phylogenetic inference of *Herpotrichiellaceae* (*Chaetothyriales*): Maximum Likelihood analysis based on the concatenated nuclear ITS-LSU dataset; branches in bold denote RAxML bootstrap support $\geq 75\%$; Bayesian posterior probabilities ≥ 0.8 are reported above branches. Newly obtained sequences are in bold. Symbols and colours indicate the different lichen host and the localities from where the fungal strains were isolated, respectively. The newly identified lineages are highlighted in grey

atra lichen thalli, 2019, G. Kantvilas, strain numbers L2870 and L2880. ASIA, 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, Austria, between the states Styria and Carinthia, Koralpe mountain, siliceous-schist/ gneissic rocks, alt. 1800–2100 m a.s.l., endolichenic fungi isolated from *L. bicincta* and *L. polytropa* lichen thalli, 2012, L. Muggia, strains number A1044 and A1069.

***Paracladophialophora lichenicola* Cometto, de Hoog, Muggia, sp. nov.**

Mycobank: MB 848888.

Etymology: associated to lichens.

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 *T. atra* (L2570).

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 grey 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 ($4 \times 10\text{--}15 \mu\text{m}$; Fig. 3aa) intercalate to spherical cells (5 to $15 \mu\text{m}$ diameter; Fig. 3ab). Branching has originated from rectangular (Fig. 3aa). Chain of conidia ($2\text{--}5 \mu\text{m}$ 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*, *R. melanophthalma*, *Rusawskia elegans*, *T. atra*, *Umbilicaria virginis* and *Umbilicaria 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, R othelstein, on limestone rocks, alt. 1260 m a.s.l., endolichenic fungi isolated from *L. stigmatea*, 2006, L. Muggia and J. Hafellner, strain number L474.

Discussion

The culture approach applied in the present study captured a great diversity of the microfungi associated with two cosmopolitan, epilithic lichens – *R. melanophthalma* and *T. atra* – collected in the broad range of their distribution and including sites characterized by harsh environmental conditions. Here, we detected both already known fungal lineages and two new lineages, i.e. the species *Cladophialophora endolichena* sp. nov. and *Paracladophialophora lichenicola* sp. nov., which seem to be recurrently associated to lichen thalli. Indeed, many strains belonging to the two 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 or, more in general, to the micro conditions that the symbiotic lichen thalli offer.

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 the selective constraints in which the lichen grew, that were not simulated in the culture conditions. Thus, the culture

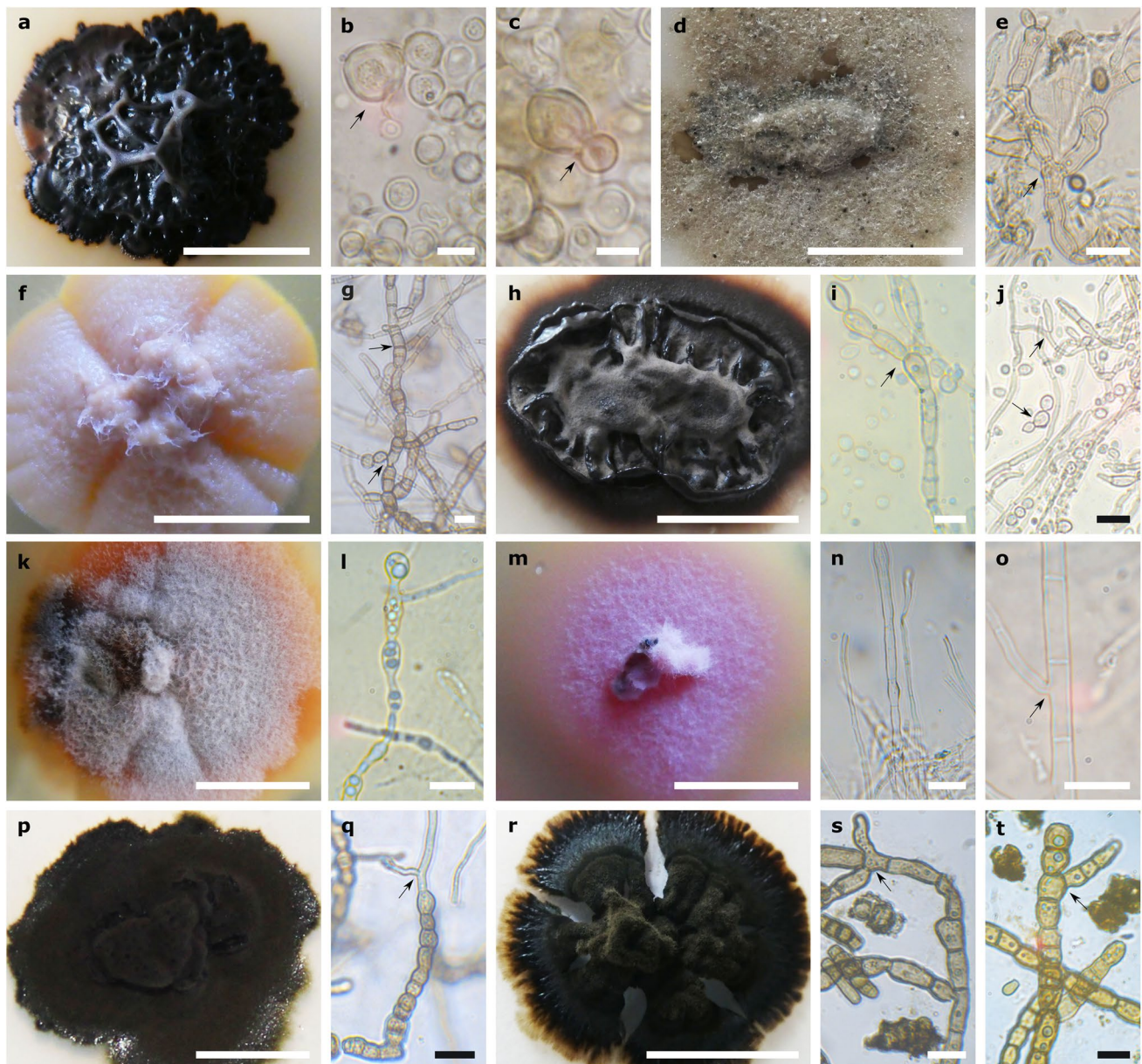


Fig. 6 Morphology of six-month to one-year old representative cultured fungal strains belonging to *Dothideomycetes* and included in the phylogenetic analysis of Fig. 3. Strains **a–c** L3077; **d, e** L3021; **f, g** L3056; **h–j** L3094; **k, l** L3048; **m** L2890; **n–o** L3890; **p, q** L2879; **r** L3104; **s–t** L3120. **a, d, f, h, k, m, p, r** Colony appearance on solid

medium after six-month to one year of growth. **b, c** Yeast-like black mycelium with budding cells. **e, g, i, j, n, o, s, t** Filamentous, septate hyphae with branches. **j, l, q** Conidia-like cells. Scale bars: **a, d, f, h, k, m, p, r** 1 cm; **b, c** 5 μ m; **e, g, i, j, i, n, o, q, s, t** 10 μ m

conditions applied seem to have not favoured in vitro the development of mycelia, particularly of those fungi that would be more extremophilic. 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 the original lichen thalli presented a lower and different mycobiome diversity in the DNA metabarcoding analyses

(Cometto et al. 2022), 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 (Urzi 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

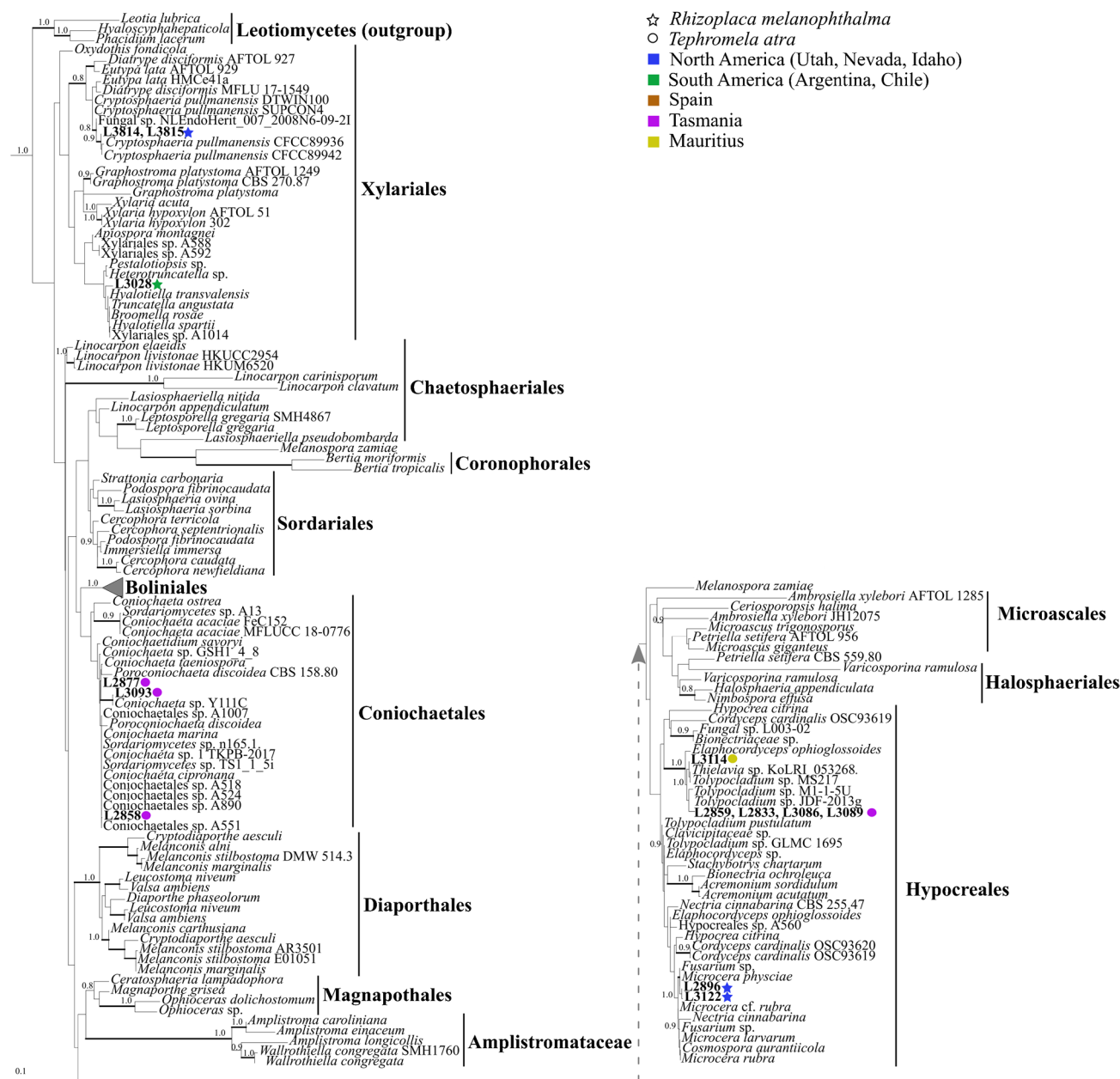


Fig. 7 Phylogenetic inference of *Sordariomycetes*: Maximum Likelihood analysis based on the concatenated nuclear ITS-LSU dataset; branches in bold denote RAxML bootstrap support $\geq 75\%$; Bayesian posterior probabilities ≥ 0.8 are reported above branches. Newly

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

et al. 2016, 2017) collected on soil and trees (Peršoh and Rambold 2012; Harutyunyan et al. 2008; Lawrey et al. 2012; Muggia et al. 2016, 2017; Crous et al. 2021). The other identified strains form either small, still unnamed (because still too poorly represented) lineages 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 *Knufia* (*Eurotiomycetes*, *Chaetothyriales*, *Trichomeriaceae*), *Elasticomyces* (*Dothideomycetes*,

Teratosphaeriaceae), or the endophytic *Neophaeococcomyces* (*Eurotiomycetes*, *Chaetothyriales*, *Trichomeriaceae*), *Paraphaeosphaeria* (*Dothideomycetes*, *Pleosporales*), and *Cryptosphaeria* (*Sordariomycetes*, *Xylariales*), *Tolypocladium* and the lichenicolous *Microcera* (*Sordariomycetes*, *Hypocreales*) 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

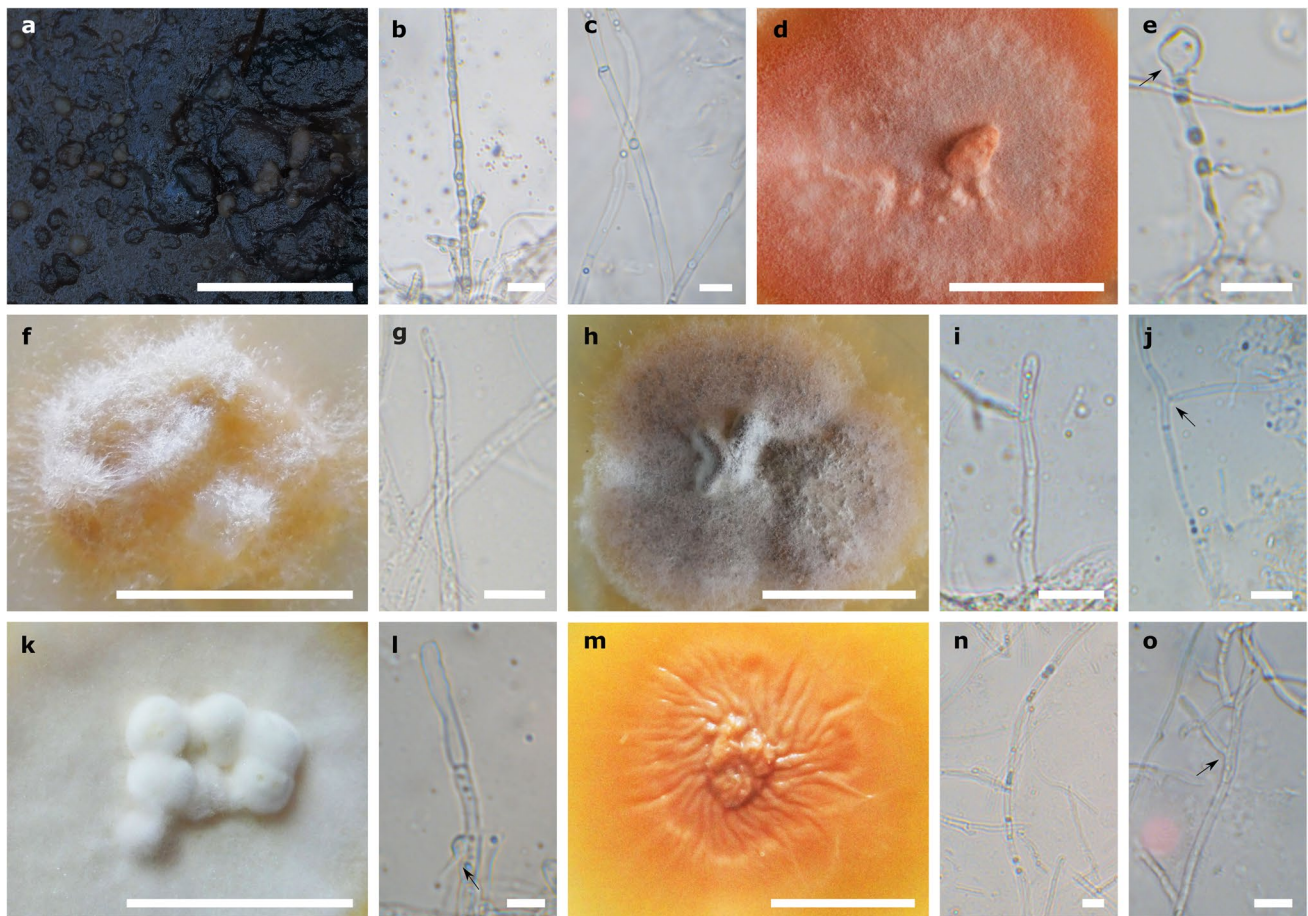


Fig. 8 Morphology of six-month to one-year old representative cultured fungal strains belonging to *Sordariomycetes* and included in the phylogenetic analysis of Fig. 4. Strains **a** L3814; **b, c** L3815; **d, e** L3028; **f, g** L2877; **h–j** L3114; **k, l** L3086; **m–o**

L3028. **a, d, f, h, k, m** Colony appearance on solid medium after six-month to one year of growth. **b, c, e, g, i, l, n** Slight and hyaline hyphae. **j, o** Branching hyphae. Scale bars: **a, d, f, h, k, m** 1 cm; **b, c, e** 10 μ m

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*’ sensu Muggia et al. (2015, 2019) was indeed isolated from a thallus of *T. atra* (which is the original lichen host species of *Muellerella atricola* and *Lichenodiplis lecanorae*; Aienza 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 inferences are topologically

congruent with those of Crous et al. (2016, 2018), Quan et al. (2020) and Wijayawardene et al. (2020). The new specie level lineage of *Paracladophialophora lichenicola* sp. nov. is included in *Paracladophialophoraceae*, being closely related to *Paracladophialophora cyperacearum* and *P. carceris* (Fig. 2, S1). *Paracladophialophora lichenicola* sp. nov. is well represented by multiple strains isolated here form both *R. melanophthalma* and *T. atra* (n. 15), but also previously from other lichens collected in the Mediterranean basin and Armenia (i.e., *Caloplaca gomerana*, *L. stigmataea*, *Protoparmeliopsis muralis*, *Umbilicaria virginis* and *U. vellea*; Harutyunyan et al. 2008). Members of this lineage occur at different altitudes and on different rock substrates (basalt, calcareous and siliceous). Harutyunyan et al. (2008) assigned the first isolated 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, our results strengthen the idea that *Paracladophialophora lichenicola* has developed a certain preference for the lichen-associated lifestyle, thus presenting additional data to support its formal recognition.

The second new lineage – here described as the new species *Cladophialophora endolichena* – 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*, *L. polytropa*, *P. muralis* and *Rusavskia elegans*; Harutyunyan et al. 2008; Muggia et al. 2016, 2017). *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; Quan et al. 2020) and our results are not unexpected – the lichen-associated lifestyle is ancestral in this order (Quan et al. 2020, Quan et al. 2023 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 of *Cladophialophora endolichena* 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 *Elastiomyces elasticus* or *Saxomyces penninicus* (Selbmann et al. 2008, 2013; Ruibal et al. 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 and Rambold 2012). Four strains are nested with *Phoma cladonicola*, *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.

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Author contributions All authors contributed to the study conception and design. Material preparation was performed by Lucia Muggia, Agnese Cometto, Steven Leavitt; data collection and analysis were performed by Agnese Cometto and Lucia Muggia. The first draft of the manuscript was written by Agnese Cometto and Lucia Muggia and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request, while sequences are public available in NCBI Genbank.

Declarations

Ethics approval and consent to participate (include appropriate approvals or waivers) Not applicable.

Consent for publication (include appropriate statements) Not applicable.

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