Fungal Ecology 69 (2024) 101331

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Highly heterogeneous mycobiota shape fungal diversity in two globally distributed lichens

Agnese Cometto [a], Claudio G. Ametrano [a,*], Roberto De Carolis [a], Steven D. Leavitt [b], Martin Grube [c], Alberto Pallavicini [a], Lucia Muggia [a]

[a] Department of Life Sciences, University of Trieste, Via L. Giorgieri 10, 34127, Trieste, Italy
[b] Department of Biology, Brigham Young University, 84602, Provo, Utah, USA
[c] Institute of Biology, University of Graz, Holteigasse 6, A-8010, Graz, Austria

ARTICLE INFO

Handling Editor: Prof. Benjamin E. Wolfe

Keywords:
Ascomycota
Basidiomycota
DNA metabarcoding
Ecology
Fungal community
Symbiosis

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 mycobiota. Because lichen mycobiota diversity is still largely unknown, we aimed to characterize it in two cosmopolitan lichens, *Rhizoplaca melanophthalma* and *Tephromela atra*. The mycobiota were investigated across a broad distribution using both a culture-dependent approach and environmental DNA metabarcoding. The variation of the mycobiota associated with the two lichen species was extremely high, and a stable species-specific core mycobiota was not detected with the methods we applied. Most taxa were present in a low fraction of the samples, and no fungus was ubiquitously present in either lichen species. The mycobiota are thus composed of heterogeneous fungi, and some taxa are detectable only by culture-dependent approaches. We suspect that lichens act as niches in which these fungi may exploit thallus resources and only a few may establish more stable trophic relationships with the major symbiotic partners.

1. Introduction

Symbioses are self-sustaining interactions between organisms of different kingdoms, known as symbionts, which result in new structures, metabolic activities (de Bary, 1879; Frank, 1877; Douglas, 1994), and genomic interactions (Guerrero et al., 2013). The symbionts interact to develop a more or less stable unit of selection in which they are interlinked by metabolic relationships, which can range from antagonistic to mutualistic (Margulis and Fester, 1991; Douglas and Werren, 2016). The so established symbiosis is determined by the presence of the different partners and their specialization in different 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, 1877) which are, among the terrestrial symbioses, iconic examples of the living together of a 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 lichen symbioses, i.e., the lichen thallus.

The lichen thallus contains, though, a multitude of associated microorganisms as well, such as prokaryotes, other microalgae and microfungi (e.g., Muggia et al., 2008; Arnold et al., 2009; Grube et al., 2009; Aschenbrenner et al., 2017; Moya et al., 2017; Spribille, 2018; Hawksworth and Grube, 2020). The lichen-associated microorganisms are acknowledged as the microbiota (Grube et al., 2009), its fungal part has been described as the mycobiota (Fernández-Mendoza et al., 2017), while the term phycobiota (Barreno et al., 2022) was recently introduced to define the microalgal diversity of the lichen thallus. However, their functional and taxonomic diversity is largely unknown (Spribille, 2018; Hawksworth and Grube, 2020).

In particular, the lichen mycobiota (U'Ren et al., 2010; Fernández-Mendoza et al., 2017; Muggia and Grube, 2018; Banchi et al., 2020), is represented by that fraction of microfungi which were supposed to develop diverse trophic relationships (parasitic, commensals, or saprotrophic) with either the mycobiont or the photobionts (Lawrey and Diederich, 2018). These fungi have been discovered in the early 19th century and have been traditionally referred to as "lichenicolous fungi" for over a century. They have been (and still are) recognized by visible

* Corresponding author. Department of Life Sciences, University of Trieste, Via L. Giorgieri 10, I-34127, Trieste, Italy.
E-mail address: claudiogennaro.ametrano@units.it (C.G. Ametrano).

https://doi.org/10.1016/j.funeco.2024.101331
Received 21 June 2023; Received in revised form 10 January 2024; Accepted 16 January 2024
Available online 6 March 2024
1754-5048/© 2024 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

infection or fertile structures on the host thalli (Lawrey and Diederich, 2003; Diederich et al., 2018). There are, however, also fungi that cryptically occur in lichen thalli (e.g., Harutyunyan et al., 2008; Arnold et al., 2009; Muggia et al., 2016). Similar to endophytism, these taxa became known as "endolichenic fungi" (Arnold et al., 2009; U'Ren et al., 2010, 2012, 2014; Muggia et al., 2016; Fernández-Mendoza et al., 2017; Banchi et al., 2018a). Owing to ambiguities in distinguishing the two groups of fungi, Hafellner (2018) proposed a re-definition of lichenicolous fungi, i.e., of the lichen mycobiota, as "all lichen-inhabiting fungi, both non-lichenized and lichenized, either obligate or facultative, with a colonization inducing symptoms on the host or not." Hafellner (2018) recognized three subgroups of lichenicolous fungi: 1) lichenicolous fungi s. str. (living exclusively on lichens, discernible by their symptomatic traits of infection; about 1800 described species among Ascomycota and Basidiomycota; Diederich et al., 2018); 2) endolichenic fungi (cryptically occurring, 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; and 3) lichen epiphytes, fungi usually lichenized that grow on the lichen thalli, usually recognized among Lecanoromycetes (Poelt 1958; Moya et al., 2020).

According to the above definitions, the diversity of 'lichenicolous fungi s. str.' was studied more easily based on their morphological traits, whereas the diversity of the 'endolichenic fungi' was uncovered recently, and their evolutionary origin was studied only for a few members (Ertz et al., 2009, 2016; Muggia et al., 2016, 2019, 2021). In general, most of the lichenicolous fungi seem to be phylogenetic distantly related from the lichen mycobionts (Lutzoni and Miadlikowska, 2009) and many taxa have been found to represent new lineages in the fungal tree of life (e.g., Muggia et al., 2021, Cometto et al., 2022). Arnold et al. (2009) proposed that the cryptically occurring lichenicolous fungi 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 analogy with plant endophytes is the absence of infection symptoms on lichen thalli (Arnold et al., 2009), 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 any lichen phenotype or ecological conditions.

Microscopic analyses of morphology and culture isolation were the main approaches to study the diversity of the lichen mycobiota before the introduction of molecular methods to fungal diversity studies (Petrini et al., 1990; Crittenden et al., 1995; Girlanda et al., 1997; Lawrey and Diederich, 2003; Lawrey et al., 2007; Moya et al., 2017; Diederich et al., 2018). In general, several attempts are required to isolate and grow any those symptomatic lichenicolous fungi in culture, while their inconspicuous, microscopic mycelium hampers their identification inside the lichen thallus. Still, culture isolations are essential for the morphological characterization of those taxa that occur cryptically in the thalli, especially when they reveal to be new monophyletic lineages and new species are discovered and formally described (Muggia et al., 2021, Cometto et al., 2022). In this context, isolated strains represent only a minor part of the whole lichen mycobiota. 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). On the other hand, a fraction of culturable fungi is not detected by DNA metabarcoding (as reported in the series of studies done on alpine lichen communities by Muggia et al., 2016, Fernadez-Mendoza e t al. 2017, Banchi et al., 2018a,b).

Data from high-throughput sequencing (HTS) complement the results of culture-dependent approaches and help to uncover more comprehensively the diversity of the lichen associated fungi (U'Ren et al., 2014; Muggia et al., 2020). The understanding of lichen mycobiota has already benefited from HTS technology at different scales (Bates et al., 2012; Zhang et al., 2015; Fernández-Mendoza et al., 2017; Banchi et al., 2018b; Smith et al., 2020; Yang et al., 2022) and identified three major ecological fractions of the lichenicolous fungi (Fernández-Mendoza et al., 2017). These are: 1) a "generalist environmental pool" of fungi unspecific to the lichen host; 2) a more specific "parasitic pool" that grow and complete their life cycle specifically on their lichen host; and 3) a pool of "occasionally occurring 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 mycobiota are thus represented by diverse lineages of Ascomycota and Basidiomycota, and both filamentous and yeast species are recognized. The major representatives belong to the large 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 (Sterflinger and Krumbein, 1995; Wollenzien et al., 1995), plant pathogens and opportunistic fungi on animals (Harutyunyan et al., 2008; Muggia et al., 2016, 2019, 2021; 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). Basidiomycetes yeasts are represented by tremelloid (Fernández-Mendoza et al., 2017; Cometto et al., 2022) and cystobasidioid taxa (Spribille et al., 2016; Cometto et al., 2022; Tagirdzhanova et al., 2021) from lichens growing in alpine, subalpine and boreal habitats. Furthermore, Smith et al. (2020) suggested that the lichen growth forms build diverse microhabitats and can act as further selecting factor of the mycobiota composition.

The present study aimed to characterize the range of diversity of lichen mycobiota of two widespread lichen species, *Rhizoplaca melanophthalma* (DC.) Leucker & Poelt and *Tephromela atra* (Huds.) Hafellner to understand its possible main drivers on a wide geographic range. These two lichen species were selected as study models and studied across a broad range of their distribution as they are often found to co-occur in alpine environments. Our specific goals were to 1) understand if the two target species host significantly different mycobiota and if other factors such as altitude or location have an influence on the diversity of these fungal communities, 2) detect if there is a stable (across the geographic and altitude range) and specific lichenicolous fungal composition associated with either lichen species that could be recognized as the 'core mycobiota', and 3) identify similarities and differences between the mycobiota that can be isolated in culture and the mycobiota detected by metabarcoding analyses.

2. Materials and methods

2.1. Sampling

The lichens *Rhizoplaca melanophthalma* and *Tephromela atra* were selected as study organisms (Fig. 1). These two species were chosen because they are well-characterized at a global scale for their morphological plasticity and genetic diversity. They have been the focus of numerous studies that described the intraspecific variation of the mycobionts and the photobionts, as well as the culturable fraction of

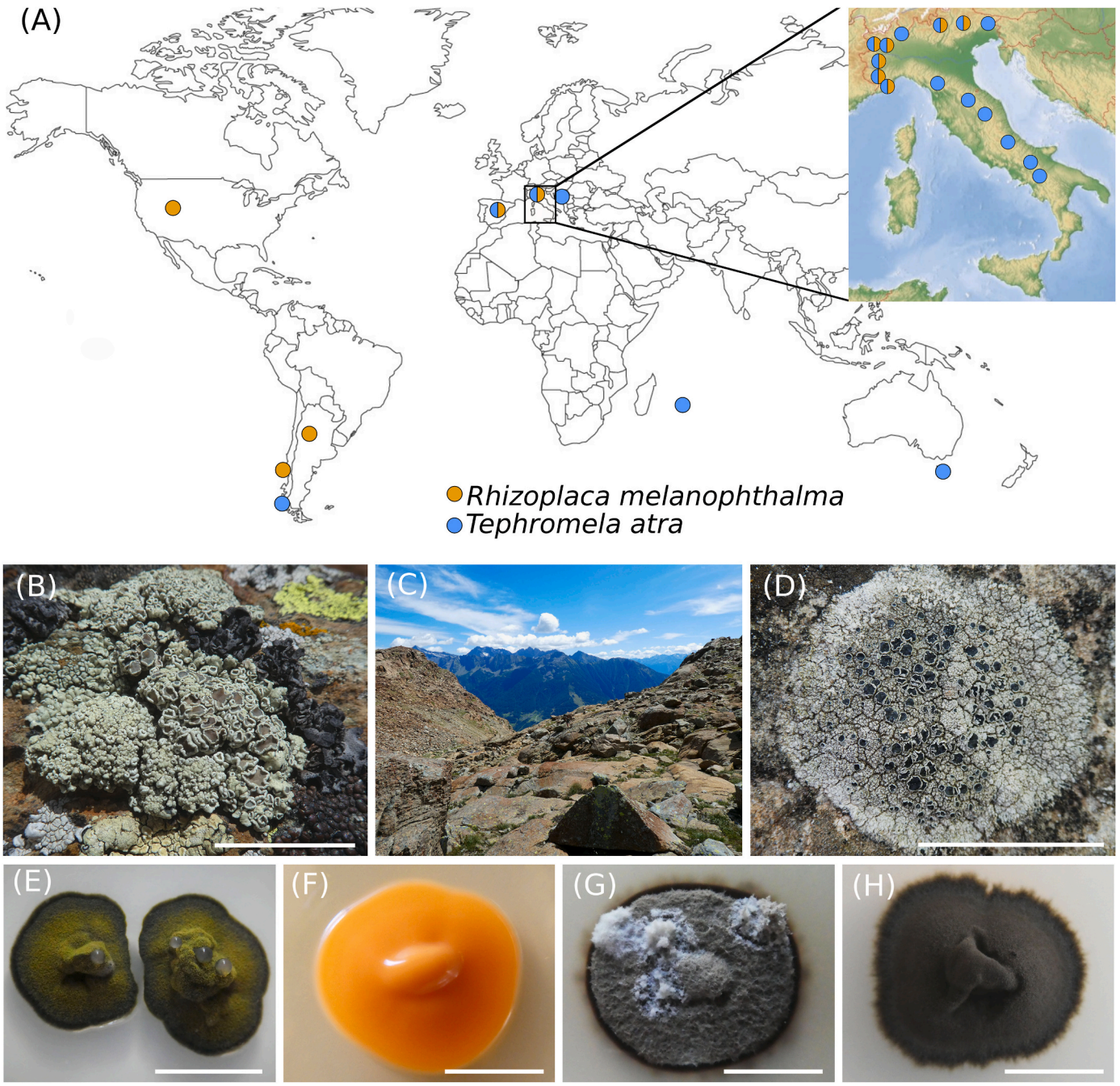

Fig. 1. (A) Map of the localities from which *Rhizoplaca melanophthalma* and *Tephromela atra* were collected. **(B)** *R. melanophthalma* collected in the Alps (Italy), **(C)** collecting localities at Laghetti Sassersa in Italian Alps (Valtellina, Lombardy; Italy), **(D)** *T. atra* collected in the Apennines. **(E)** colony shape of Mycosphaerellales sp. L3082 and **(F)** Microsporomycetaceae sp. L2343 isolated from *R. melanophthalma*, **(G)** Coniochaetales sp. L3093 and **(H)** Chaetothyriales sp. L3059 isolated from *T. atra*. Scale bars: **(B–D)** 2 cm, (e–h) 1 cm.

filamentous and yeast ascomycetes and basiodiomycetes (Muggia et al., 2008, 2010, 2014; Leavitt et al., 2011, 2016; De Carolis et al., 2022; Cometto et al., 2022, 2023). *Rhizoplaca melanophthalma* has an umbilicate, fruticose to subfoliose, thallus (attached at a single point), whereas *T. atra* builds a crustose thallus composed of adjacent areoles (Muggia et al., 2008, 2010, 2014; Leavitt et al., 2011, 2016). 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-sandstone, siliceous and calcareous rocks (Appendix S1, see Supplemental Data with this article). 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), Indian Ocean (Mauritius) and Oceania (Tasmania) (Fig. 1). Both lichen species were found together in only nine localities (two in Spain and seven in Italy; hereafter referred to as 'small 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).

2.2. 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 to avoid including fungi randomly present on the thalli. For *R. melanophthalma* one marginal lobe and one apothecium were taken, while for *T. atra* one marginal areole and one apothecium. The fragments were surface-sterilized by washing three times for 15 min with sterile water, followed by 30 min of washing with 500 μl of a 1:10 dilution of Tween 80, and a final washing step of 15 min for three times with sterile water. The cleaned samples were dried under a laminar flow hood and stored at −20 °C until they were processed for DNA extraction. The DNA extraction was performed following the CTAB protocol of Cubero et al. (1999), with minor adjustments. DNA extractions (~50 ng/ml) of ten individuals belonging to the same population were pooled together to represent eventually a single sample in the molecular analyses.

2.3. 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 lichenicolous fungi, algae and lichen mycobionts that were isolated in axenic culture from the thalli of *R. melanophthalma* and *T. atra* included in the molecular analyses. These strains were isolated at the beginning of the research project and their DNA extractions was already available and used for previous phylogenetic studies based on the same sampling campaign included here (Cometto et al., 2022, 2023; De Carolis et al., 2022). For each mock community (one specific to *Tephromela atra*, and one to *Rhizoplaca melanophthalma*), fungal and algal strains comprised: *i)* one of the two *R. melanophthalma* and *T. atra* mycobionts *ii)* the corresponding *Trebouxia* photobiont isolated from their thalli (see De Carolis et al., 2022), *iii)* a number (see here below) of lichenicolous fungal strains (filamentous fungi and yeasts; Fig. 1e–h) isolated from thalli of either lichen species that were included in this metabarcoding analysis. 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, 2023; De Carolis et al., 2022). The lichenicolous 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 for *R. melanophthalma* (n. 1) was composed of 36 strains isolated from thalli of *R. melanophthalma*, while the mock community for *T. atra* (n. 2) included 27 strains isolated from thalli of *T. atra*. Either mock community was composed of *i)* the mycobiont DNA at the concentration of 5 ng/μl, *ii)* the photobiont DNA at the concentration of 2,5 ng/μl, *iii)* the DNA of the other fungi at three different concentrations (0,5 ng/μl, 0,05 ng/μl and 0, 005 ng/μl) to simulate the presence of lichenicolous fungi likely to be differently abundant within the thalli.

2.4. 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 s, 54 °C for 30 s and 72 °C for 30 s. 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, Norcross). 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 PCRs were run under the following conditions: 94 °C for 2 min and 6 cycles at 94 °C for 50 s, 60 °C for 20 s and 72 °C for 30 s. 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 bp (BMR Genomics, Padua, Italy).

2.5. Bioinformatic analyses and statistics

Raw Illumina paired-end reads (2 × 300 bp) were demultiplexed, quality checked with FastQC (Andrews, 2018), trimmed by Trimmomatic (Bolger et al., 2014), denoised and dereplicated to amplicon sequence variants (ASVs) 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) and using UNITE as the reference database (Abarenkov et al., 2010). Based on this taxonomy, the table containing the features (ASVs) 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 mycobiota ASV table; diversity accumulation curves were also plotted. Krona plots (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 mycobiota; in this way we discarded samples with extremely low sequencing depth that are likely to miss a relevant part of the fungal diversity and can influence the mean sequencing depth used for normalization. The normalized table was used to calculate alpha diversity indices. ASV richness, Shannon and Simpson indices were selected, as the first does not consider taxa relative abundance, while the other two do. Shannon (H') emphasizes on community evenness, the higher the index the higher diversity and evenness; Simpson ($0 < D < 1$) emphasizes on dominance, one being the lowest possible diversity (one taxa only, complete dominance) and zero being infinite diversity. Alpha diversity distributions (complete dataset) of *R. melanophthalma* and *T. atra* samples were plotted as violin plots and compared using the non-parametric Wilcoxon test. A further comparison was performed only retaining the nine localities where both lichen species were collected (hereafter referred to as 'small dataset'). This reduced dataset allowed fair comparisons of the fungal communities associated with the two lichens and helped identify when the lichen host can be an influential factor in determining the lichen-associated fungal diversity.

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. The metadata were tested for their homogeneity of dispersion using the function betadisper from vegan package. The results of the homogeneity test were then used to run an ANOVA with a Tukey post-hoc. Then, the possible correlation of lichen host, altitude (whose range was divided

into four relevant altitude classes) or geographic origin of the samples was assessed by PERMANOVA (PERMutational ANalysis Of VAriance) in R using adonis with 10^4 permutations. The analysis was carried out using the same dissimilarity matrix of the NMDS ordination. Pairwise comparisons were also made, for the group of samples with at least $n \geq 4$, the obtained p values were corrected for multiple comparisons using the Benjamini-Hochberg correction.

We aimed to find whether there was a stable, lichen species-specific fraction of fungi associated with one or both lichens, which can be referred to as the 'core mycobiota'. Thus, we searched for the core mycobiota in a frequentist fashion, i.e., as the portion of the diversity which can be consistently recovered in a relevant portion of the sample set. Therefore, we tested its possible presence, progressively relaxing the similarity threshold to cluster ASVs into operational taxonomic units (OTUs), the required relative abundance threshold for each ASV or OTU within each sample, and progressively increasing the sample fraction (percentage of samples containing that ASV or OTU) required to be included in the core taxa. We selected ASVs (no similarity threshold), 99% and 97% similarity clustered ASVs (OTUs), 0.050, 0.010, 0.005, 0.001 relative abundance thresholds, and 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 sample fraction thresholds. These analyses were performed both on the complete and the small datasets, for both lichen hosts. 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) and converted to cladograms to ease the visual comparison in FigTree; taxonomy was assigned to ASVs on the final cladograms (assign_qiime_taxonomy_to_fasta_alignment.py).

The correspondence of the mycobiota 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).

2.6. Culture isolation

Fungal isolation was performed from four thalli for each population of *R. melanophthalma* and *T. atra* following the protocol of Yoshimura 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 as previously described (Yoshimura et al., 2002; Muggia et al., 2016). The clean fragments were ground in sterile water under the hood and tiny thallus fragments were picked with a sterile bamboo needle and transferred into agar tubes. Six different media were used to promote the growth of as many different filamentous and yeast fungi as possible: Lilly and Barnett (LB, Lilly and Barnett, 1951), *Trebouxia* medium (TM, Ahmadjian, 1993), 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 $m^{-2} \times s^{-1}$, 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. These isolates were genetically identified (by sequencing the ITS and the LSU loci; see Cometto et al., 2022; Cometto et al. under review) and preserved as cryostocks (at University of Trieste).

3. Results

3.1. DNA sequencing and data analysis

The initial average read count per sample was 237,000 (st. dev. 77,000). 15.5×10^6 reads passed the quality filter, and the average reads count per sample was 228,000 (st. dev. 74,000) after filtering out low quality and short reads. After paired reads assembly, denoising and singletons exclusion 1430 ASVs were identified. ASVs belonging to the mycobionts, the photobionts (mainly identified as different species of *Trebouxia*) and any other non-fungal taxa were filtered out and 980 ASVs were retained: 392 ASVs in *R. melanophthalma* and 646 ASVs in *T. atra* samples.

Accumulation curves were generated for each sample, both before and after filtering out the main symbionts. Most of the curves leveled off and reached a plateau with a rather small sampling effort (Appendix S2-S4). The mean values of ASVs abundance were 3326 (95% CI: 2015–4928) and 4884 (95% CI: 2966–7658) for *R. melanophthalma* and *T. atra*, respectively. The median abundance percentage of mycobiota assigned ASVs was 0.89% and 2.65% for *R. melanophthalma* and *T. atra*, respectively.

3.2. Mock communities and negative control

In the *R. melanophthalma* mock community, 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 (Appendix S5; Appendix S6, Supporting Information). In the *T. atra* mock community, 27 out of 28 taxa were detected: only taxon L2875, which was in the mock community at the lowest concentration (0.005 ng/μl) and identified by BLAST (98.25% similarity) as Teratosphaeriaceae sp., was not identified among the ASVs (Appendix S7, S8). In some cases (e.g., L3816; Appendix S5) multiple ASVs corresponded to a single Sanger sequence obtained from a cultured strain.

The negative control was clean when checked on RT-PCR; its PCR was then run about 10–15 cycles longer than any other sample. ASVs corresponding to *Capnodiales* sp., *Coniosporium* sp., *Filobasidium* sp., *Malassezia restricta, Pseudeurotium* sp., *Rhizoplaca* sp., 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 four samples, *Trebouxia* sp. in one sample), while the other ASVs were only present in the negative control.

3.3. Taxonomic composition of lichen mycobiota

Taxonomy was assigned to ASVs using BLAST or the machine-learning sklearn approach (Appendix S9): 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. 2% of ASVs taxonomic assignments were in conflict and, in these cases, taxonomy was assigned manually.

The majority of the ASVs of the *R. melanophthalma* mycobiota were Ascomycota (93%);, the remainder were classified as Basidiomycota (6%), unidentified fungi (0,5%), Olpidiomycota (0,2%) and Chytridiomycota (0,02%). At the class level (Fig. 2a), the most represented

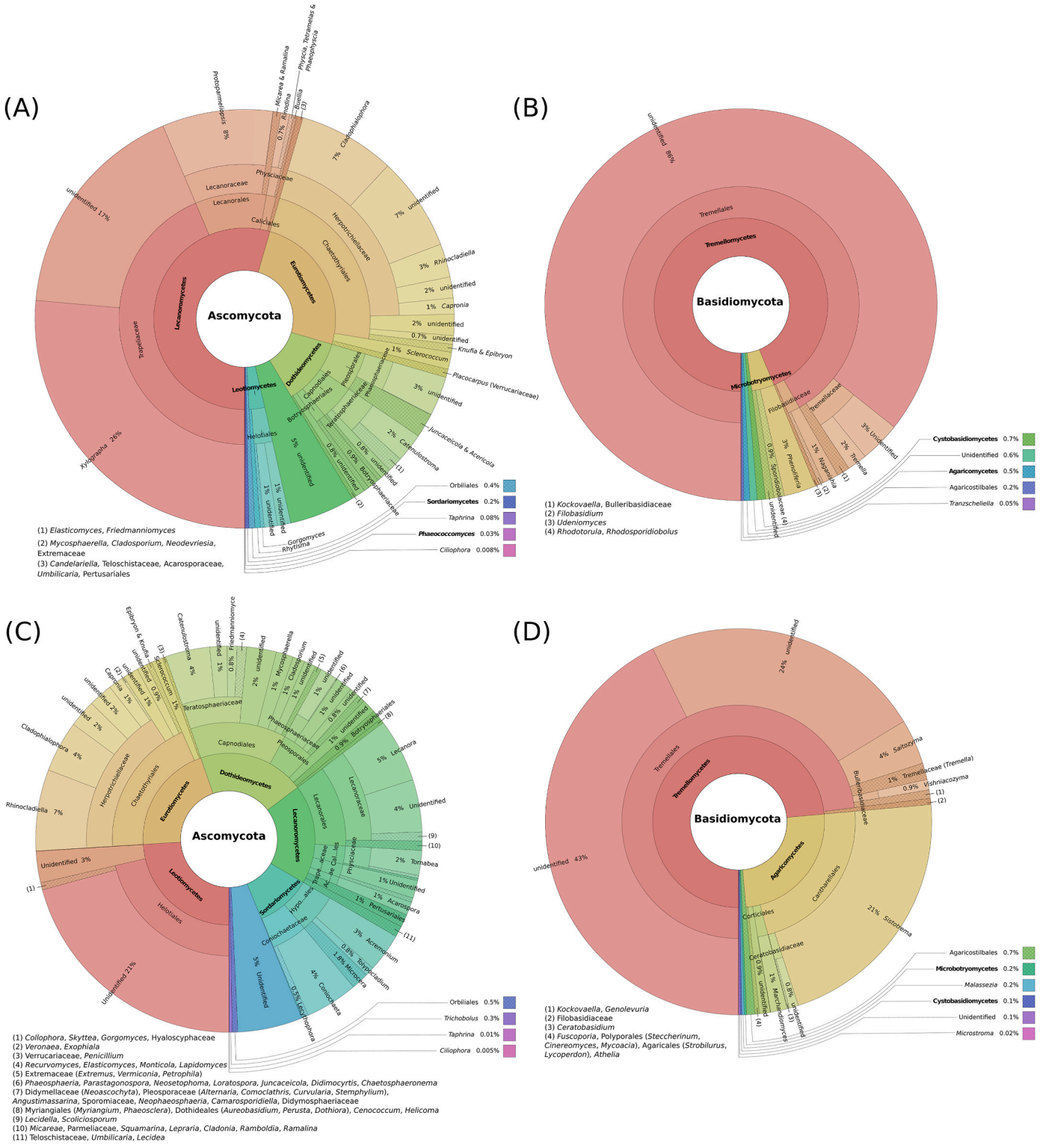

Fig. 2. Krona plots describing the proportion of ASVs assigned to different taxa according to UNITE database (see methods) in **(A, C)** Ascomycota and **(B, D)** Basidiomycota at the genus level in the mycobiomes of **(A, B)** *R. melanophthalma* and **(C, D)** *T. atra* using the non-normalized ASV table.

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* mycobiota 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%),

Lecanoromycetes (19%), Sordariomycetes (10%) and unidentified classes (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%).

3.4. Alpha, beta diversity and the core mycobiota

After discarding the samples with less than 500 reads assigned to the mycobiota, 29 populations of *R. melanophthalma* and 30 populations of *T. atra* were retained. Six populations (1, 11, 13, 20, 41 and 51) were discarded (Appendix S1). ASV richness and Shannon index (complete dataset) showed the alpha diversity higher in *T. atra* than in *R. melanophthalma,* but at the same time *T. atra* community was slightly less even. 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 (Fig. 3a and 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 small dataset had median values of 31 (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 small dataset showed no significant differences between the *R. melanophthalma* and *T. atra* distribution, even though the median values of alpha diversity in *T. atra* were still higher, showing slightly higher diversity, but also a *T. atra* community slightly more dominated by the most abundant taxa (Fig. 3d–f).

Relevant metadata including lichen host, altitude class (except class 1) and country of origin having four or more samples passed the homogeneity test and were used in PERMANOVA. NMDS plots (Fig. 4, Appendix S10) showed that the samples and the mock communities were grouped according to the lichen host ($p = 10^{-5}$), geographic origin ($p = 10^{-5}$) and altitude ($p = 10^{-5}$) as highlighted by PERMANOVA using the complete dataset. Pairwise comparisons showed correlations with lichen mycobiont (*Rhizoplaca* or *Tephromela*) and extreme altitudes (class 4) versus all the other altitude classes ($0.00006 < p < 0.002$) except class 1, which was excluded because its distribution of distance to centroid was significantly different from the distributions of the other three classes. Additional significant correlations included South American versus European samples ($0.0002 < p < 0.008$) and North American samples ($p = 0.01$), while the correlation with location within Europe ($0.02 < p < 0.03$) and within Italy ($0.07 < p < 0.17$) was less significant. The pairwise comparisons between North American group and European groups were also significant ($0.007 < p < 0.03$).

The ordination also showed that *T. atra* samples from remote localities (sample n. 15 from Tasmania and sample n. 22 from Mauritius) were segregated from the European samples, but being single samples they were not included in pairwise comparisons. The small dataset (localities with both lichen species) did not show significance except for geographic origin, but pairwise comparisons did not show significance after being corrected for multiple comparisons. The limited number of samples with both lichen hosts and the limited altitude range (class 2 and 3) prevented further tests. Notably, the negative control sample was completely separated from any other samples, thus strengthening the reliability of the results.

Line plots (Fig. 5a–c, d, f; Appendix S11-S14) showed how the core ASVs and OTUs size decreased quickly when stricter (higher) relative abundance and stricter (lower) sample fractions threshold were

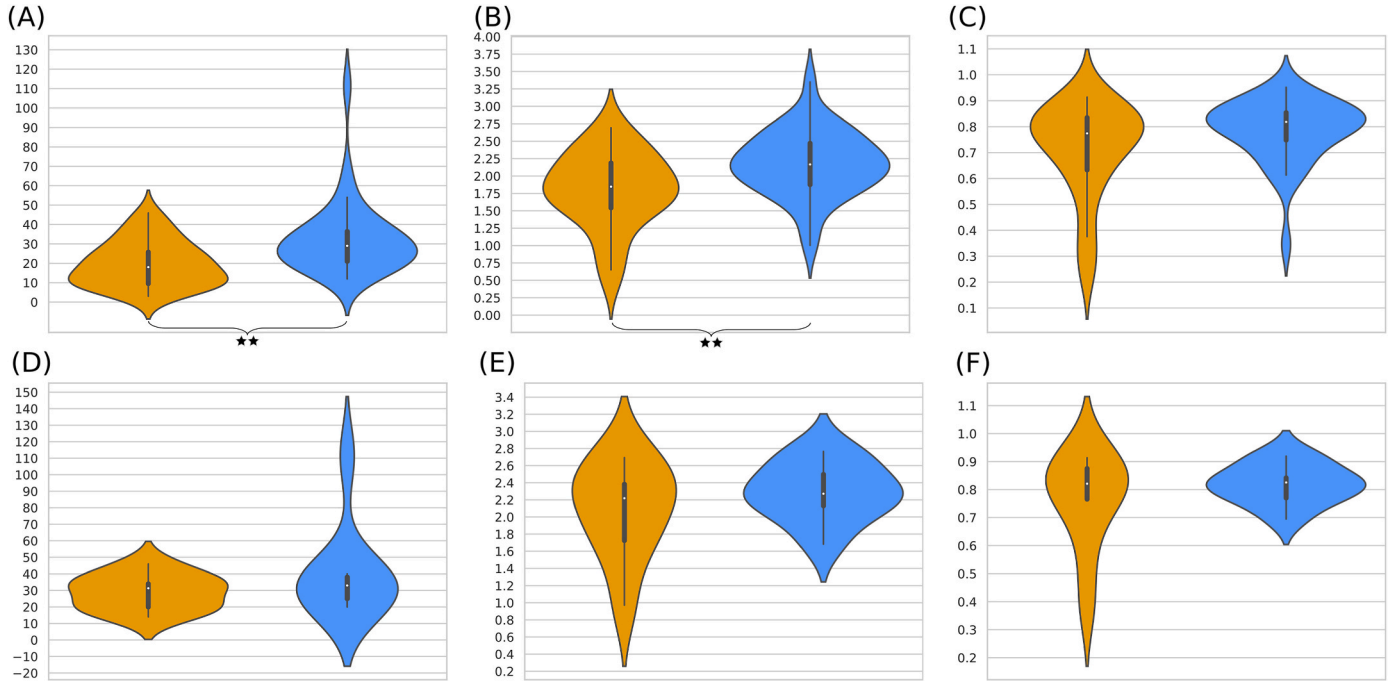

Fig. 3. Violin plots of alpha diversity metrics calculate with **(A, D)** ASV richness, **(B, E)** Shannon and **(C, F)** Simpson indices. **(A–C)** Complete dataset, **(D–E)** small dataset for *R. melanophthalma* (orange) and *T. atra* (blue). The violin shape shows the probability density of the distribution; 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 $p < 0.01$.

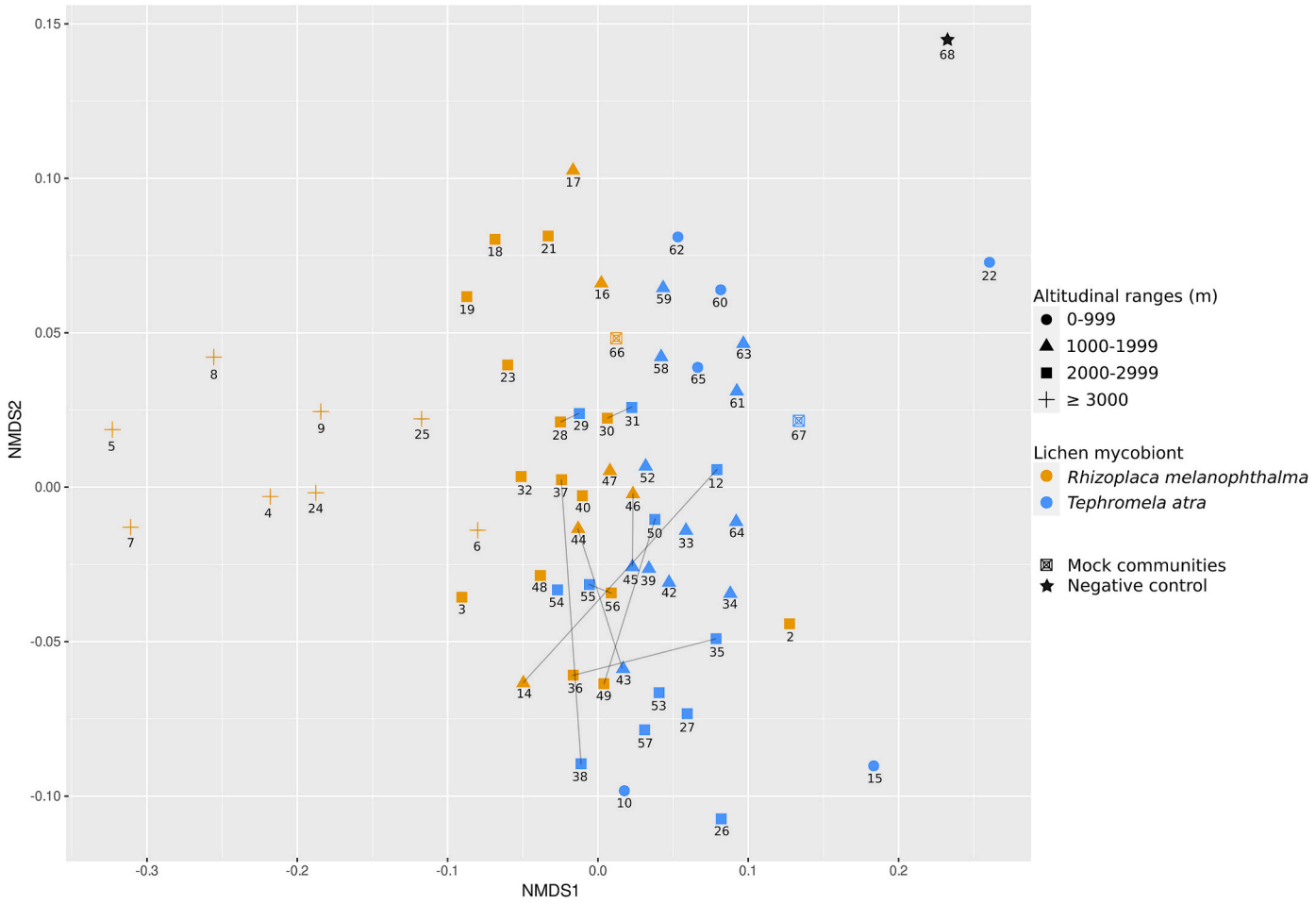

Fig. 4. Non-metric Multi Dimensional Scaling (NMDS) ordination based on the Bray-Curtis dissimilarity matrix calculated from the normalized (by median sequencing depth) OTU table. Symbols and colors correspond to altitudinal ranges (m a.s.l.) and lichen species, respectively. Numbers correspond to samples, as described in table S1 (Population ID). Samples n. 66 and 67 correspond to the mock communities n.1 and n.2 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.

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* (Fig. 5a–c), while 146 and 179 core OTUs, for *R. melanophthalma* and *T. atra* respectively, were found in the small dataset (Fig. 5d–f). Among them 15 were shared between the two cores (53 in the small 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 Orbiliomycetes (Orbiliales sp.). The number of core OTUs decreased quickly when applying higher sample fraction thresholds (Fig. 5b–e; Appendix S15, S16). Using a 0.5 sample fraction threshold (0.7 in the small dataset) it reached a single core OTU, *Cladophialophora* sp. (and two OTUs in the small dataset, Dothideomycetes sp. and *Xylographa*) for *R. melanophthalma*, and two core OTUs, Dothideomycetes sp. and Tremellales sp. (but none in the small dataset) for *T. atra*. There was no shared core OTU between the two mycobiota.

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 small dataset; Appendix S11, S12). Of these, 16 core OTUs were shared between the two lichen mycobiota (58 core OTUs were shared in the small dataset). The shared core composition overlapped the 99% OTU core, except for an unidentified Lecanoromycetes sp. (in the small 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 small 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 small dataset) for *T. atra* were retained using a 0.5 sample fraction threshold (0.7 in the small dataset); there were no shared core OTU (Appendix S11, S12, S17 and S18).

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 small dataset; Appendix S13, S14). Among them 11 OTUs were shared between the two cores (and 49 core OTUs in the small 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 small dataset; instead, the new OTU Botryosphaeriales sp. was present). The number of the core OTUs decreased to zero using a 0.5 sample fraction threshold (and 0.7 in small dataset; Appendix S13, S14, S19 and S20).

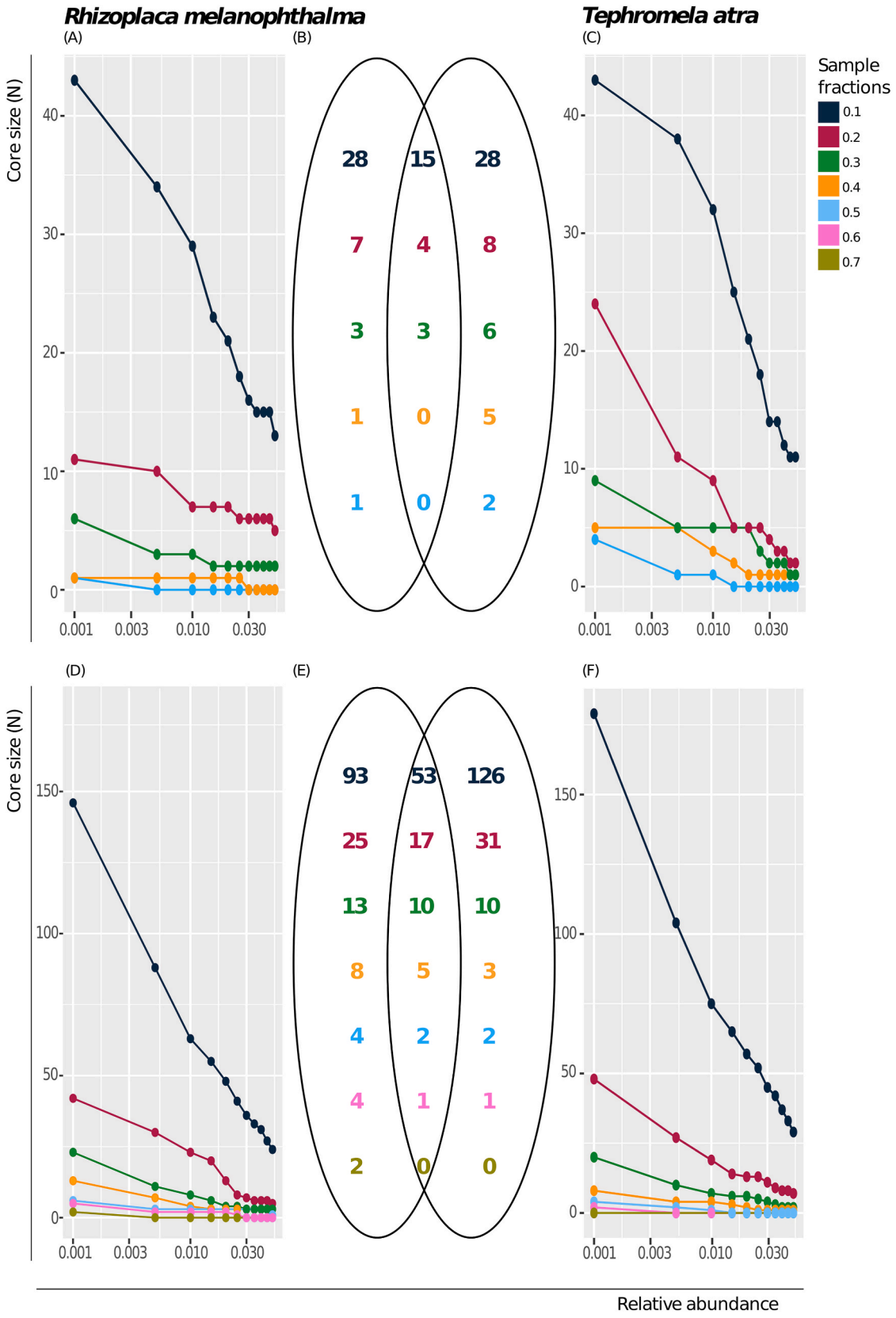

Fig. 5. Mycobiome core taxa of *R. melanophthalma* and *T. atra* using 99% similarity clustered ASVs (OTUs) of the complete **(A–C)** and small **(D–F)** dataset. **(A, C, D, F)** Line plots show the number of core OTUs retained by varying the read relative abundance and the sample fraction (i.e., percentage of samples containing that OTUs) thresholds. **(B, E)** Venn diagram 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.

3.5. 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 (Fig. 6a, Appendix S21).

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 (Fig. 6b–Appendix S22). The fraction of diversity detected only by the culture-dependent approach amounted to about 13% of the total detected diversity for both *R. melanophthalma* and *T. atra*, p [possibly slightly less considering that strains typically grouped into fewer ASVs (Fig. 6).

Of these strains the basidiomycetes (20%) have been phylogenetically identified in Cometto et al. (2022) and the ascomycetes (80%) were further analyzed in Cometto et al. (2023).

4. Discussion

We described the diversity and variation of lichen mycobiota in the two widespread lichen species *Rhizoplaca melanophthalma* and *Tephromela atra* by the combination of DNA metabarcoding analyses and a culture-dependent approach. We identified fungi that potentially represent the core mycobiota of either lichen; however, these mycobiota are variable and comprise taxa occurring in a relatively low sample fraction (none of the taxa had a frequency higher than 70%). 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 mycobiota of *R. melanophthalma* and *T. atra* are largely composed of heterogeneous lichenicolous fungi which do not develop any observable structure on the lichen thalli. A fraction of these mycobiota comprises generalists associated with both lichens. Furthermore, there is a relevant fraction undetected by PCR and found only by the culture-dependent approach. We assume this could be due to primer specificity and/or amplification biases (e.g. amplicon competition) of the less frequent (or abundant) lichenicolous fungi.

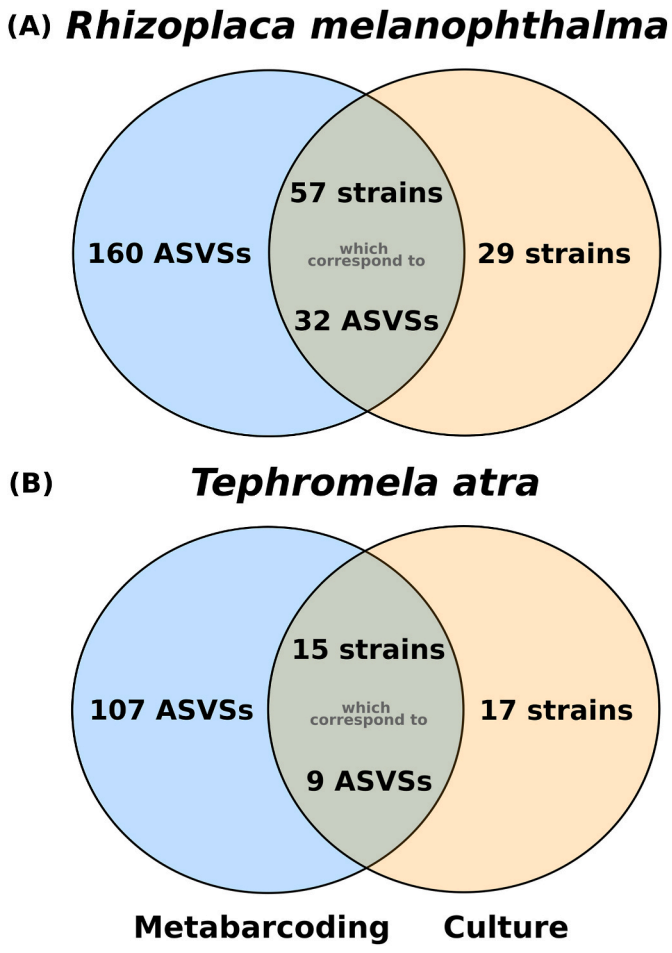

Fig. 6. Venn diagrams showing the comparison between culture-independent (metabarcoding, light blue) and culture-dependent (axenic isolation of strains, orange) approaches for **(A)** *R. melanophthalma* and **(B)** *T. atra* mycobiomes. The overlapping area of the diagram shows how many strains (and ASV) are shared in the two approaches.

4.1. Lichen mycobiota are variable and heterogeneous

The mycobiota of the two lichen species *R. melanophthalma* and *T. atra* are diverse and variable. They are mostly composed of Ascomycota, while Basidiomycota represent a less abundant fraction. This agrees with previous studies investigating the mycobiota of other lichens (Zhang et al., 2015; Fernández-Mendoza et al., 2017; Banchi et al., 2018a; Smith et al., 2020; Yang et al., 2022). The mycobiota diversity of *T. atra* seems 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; in addition, the *T. atra* communities tend to have more dominant taxa than *R. melanophthalma* communities. However, the generally low single sample diversity and the wide range in diversity measures, prevents to highlight conclusive, significant differences. 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 mycobiota diversity can be influenced by the lichen mycobiont species. Nevertheless, the broad geographic sampling and the number of individuals (lichen thalli) analyzed for each population, is enough to distinguish a fair segregation of two groups of lichen mycobiota, i.e., belonging to *R. melanophthalma* and *T. atra*. The distinction of the lichen mycobiota emerges in the NMDS ordination and PERMANOVA analysis when lichen host, geographic locations and altitude are considered. Indeed, the mycobiota of *T. atra* collected from Tasmania and Mauritius are very different from the others. Similarly, *R. melanophthalma* samples collected at high altitudes (over 3000 m a.s.l.) in South America are characterized by mycobiota 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. However, since the localities hosting both lichen species are limited to samples from Europe and they only partially represent the altitude range of the two lichen species, it is impossible to conclusively determine which factor among lichen host, geographic distance or (extreme) elevation has the prominent role in shaping the mycobiota diversity.

On the other side, the mycobiota of *R. melanophthalma* and *T. atra* seem to be equalized by the presence of those 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 taxa, retained at low sample fraction thresholds in the two mycobiota, 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 of symptomatic lichenicolous fungi (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 further supports the idea that thalli are open systems in which potentially multiple lichen-forming fungi can grow intertwined. Indeed, in rich lichen communities, where thalli develop next to each other, different lichen mycobionts in any thallus could derive from spores or hyphae extending from the neighbor thalli. Taxa shared by the two lichen mycobiota are identified only when relaxed thresholds are applied and are species known to be extremotolerant rock-inhabiting fungi (Ruibal et al., 2009) such as *Friedmanniomyces endolithicus* (Selbmann et al., 2005; Coleine et al., 2020), *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, which are phylogenetically closely related to these genera of rock-inhabiting fungi and pathogens, have been already isolated from lichens (Harutyunyan et al., 2008; Leavitt et al., 2016; Banchi et al., 2018b; Muggia et al., 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 (Cometto et al., 2023).

Basidiomycota 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 mycobiota in general. Indeed, it was reported as low as 0.1% of the total OTUs by Banchi et al. (2018a,b) 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. (2017) and Banchi et al. (2018a,b) 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 and metagenomics in some fruticose epiphytic and crustose epilithic lichens (Spribille et al., 2016; Tagirdzhanova et al., 2021; Tuovinen et al., 2019, 2021). Here, we detect many basidiomycetes yeast taxa also in the *R. melanophthalma* and *T. atra* mycobiota, and our results are also supported by the culture-dependent approach (Cometto et al., 2022). The most frequent basidiomycetes are taxa from the order Tremellales (Tremellomycetes) that were also recovered by Fernández-Mendoza et al. (2017) and Banchi et al. (2018a,b). ASVs belonging to Cystobasidiomycetes were recovered sporadically in our analyses. Although Cystobasidiomycetes of the order Cyphobasidiales were hypothesized to be ubiquitous in parmelioid macrolichens (Spribille et al., 2016), in accordance with Lendemer et al. (2019) and Smith et al. (2020), Cystobasidiomycetes seem to be not as frequent in the lichen mycobiota we analyzed. Our results place these taxa in the fraction of 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 mycobiota, 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 mycobiota, 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 (from *Rhizoplaca*) which is a fungus isolated from lichens from dry habitats many years ago (Harutyunyan et al., 2008), the lichenicolous fungus *Tremella macrobasidiata* AM453 (from *Tephromela*; Millanes et al., 2011; Zamora et al., 2011), and a Dothideomycetes (from *Tephromela*) fungus with high BLAST similarity to black fungi isolated from rocks (Gueidan et al., 2011). We thus refrain from proposing a true shared core mycobiota.

The kind of mycobiota 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.

4.2. Metabarcoding studies in lichens can be partial because of thallus properties, DNA amplification biases and the nature of the data itself

DNA metabarcoding by high-throughput sequencing (HTS) uncovers in a 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 (Bates et al., 2012; Zhang et al., 2015; Fernández-Mendoza et al., 2017; Banchi et al., 2018a,b). This results in a low sampling depth of the lichen associated fungi and possibly uneven depth in different lichen species (Fernández-Mendoza et al., 2017; Banchi et al., 2018a,b). Our data also showed a relatively low abundance of taxa in the whole mycobiota and an uneven sequencing depth between *R. melanophthalma* and *T. atra* mycobiota, having *T. atra* mycobiota 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. Accumulation curves from both lichens reached the plateau in most samples, highlighting a fairly low fungal diversity (per single lichen population) in the mycobiota of both lichens. However, some low abundance taxa may still be missed by the primer system and the experimental protocols we adopted.

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, mycobiota poor in Basidiomycota were described when the ITS2 barcode was used (Banchi et al., 2018a,b), 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 supposedly universal primers used in this study may not have been appropriate to efficiently amplify some of these rarer fungi in lichens and left them undetected in the metabarcoding results. Metagenomics is a powerful resource to investigate lichen mycobiota, 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 (Mande et al., 2012; Alneberg et al., 2014). 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 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 amounts 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 possibly 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 prepared mock communities 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 analyzed in the study (Lamb et al., 2019; Banchi et al., 2020).

Our characterization of the core mycobiota of the two lichen species was based on the fraction of samples in which each taxon occurred and their relative abundance within the sample. The mycobiota 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 from 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 mycobiota. 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 (Tedersoo et al., 2022). 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. However, future analyses are needed to tackle whether any species or higher level taxa of the mycobiota have specific functional role within the *R. melanophthalma* and *T. atra* lichen symbioses. This approach would align with the recent holobiosis framework which notes that holobionts often recruit microbes from the environment; within this model the microorganisms function would be more relevant than its taxonomy, to make it suitable to take part to the symbiosis (Doolittle and Booth, 2017).

4.3. A fraction of the mycobiota is detected only by culture isolation

Out of the thousands of metabarcoding studies performed since the early 2000s, a comparison between the diversity detected by DNA metabarcoding and culture-dependent approaches on the same samples is seldom reported. Some studies have performed such a comparison among different mycobiota and reported similar results and discrepancies between the two methods (U'Ren et al., 2014; Dissanayake et al., 2018; Pang et al., 2019; Durán et al., 2021; Oita et al., 2021; Yang et al., 2022). Our results, in accordance with results found in literature, show that DNA metabarcoding uncovers the largest part of the diversity of the lichen mycobiota, in which a part of fungi isolated in culture is found. However, and as reported twice for lichens (U'Ren et al., 2014; Yang et al., 2022), and also highlighted for endophytic fungi (Oita et al., 2021; Dissanayake et al., 2018), there are fungal species that do grow in culture but are not detected by the metabarcoding analysis. We suggest that this is likely due to several factors, from the amount and quality of the DNA extracted from the lichen thallus 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 mycobiota.

5. Conclusions

DNA metabarcoding studies on lichen mycobiota help reveal the diversity and variation of fungi associated with lichens, which may play a role in shaping the phenotypes of lichen thalli and making these symbioses ecologically successful worldwide. Future development of species-specific blocking primers may facilitate a more accurate characterization of the mycobiota diversity. 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 with the major symbiotic partners. Thus culture isolates will serve for further -omics and ad hoc *in vitro* experiments to study the other biological sides of the mycobiota. While 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 (Nash, 2008), the role of others seems varied. For example, lichen growth could 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), some yeasts seem very likely to influence the secondary metabolite production and the physiology of some lichens (Spribille et al., 2016), and it might be imagined that some lichenicolous fungi contribute to rejuvenating of thalli by local infections of older parts (Grube and Muggia, 2021). Even less is known about the activity of the many other lichenicolous fungi that we detect in this study by amplicon sequencing and culturing. Some fungi may be trapped in the lichen and others may wait for better conditions. Additional work is needed before we can assign a functional role to these poorly characterized lichenicolous fungi.

Author contributions

L. Muggia designed the study; A. Cometto, L. Muggia and S. D. Leavitt collected samples; A. Cometto performed molecular laboratory work; C. G. Ametrano and A. Cometto performed bioinformatics and statistics; A. Cometto, C. G. Ametrano, L. Muggia A. Pallavicini M. Grube, S. D. Leavitt and R. De Carolis wrote and revised the manuscript; All authors gave final approval for publication.

Data availability

The data that support the findings of this study are openly available as NCBI SRA under the Bioproject accession PRJNA1068006

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Special thanks to Diego Leiva, Julieta Orlando, Gintaras Kantvilas, Damien Ertz and Sergio Pérez Ortega for the help in lichen samples collection.

This work was supported by the Italian Ministry of Research (MUR, Ministero dell'Università e della Ricerca) project PRIN 2017, Grant Number 20177559A5.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2024.101331.

References

Abarenkov, K., Nilsson, R.H., Larsson, K.-H., Alexander, I.J., Eberhardt, U., Erland, S., Høiland, K., et al., 2010. The UNITE database for molecular identification of fungi – recent updates and future perspectives. New Phytol. 186, 281–285.

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

Alneberg, J., Bjarnason, B.S., Bruijn, I.D., Schirmer, M., Quick, J., Ijaz, U.Z., Lahti, L., et al., 2014. Binning metagenomic contigs by coverage and composition. Nat. Methods 11, 1144–1146.

Andrews, S., 2018. A Quality Control Tool for High Throughput Sequence Data.

Arnold, A.E., Miadlikowska, J., Higgins, K.L., Sarvate, S.D., Gugger, P., Way, A., Hofstetter, V., et al., 2009. A phylogenetic estimation of trophic transition networks for ascomycetous Fungi: are lichens cradles of symbiotrophic fungal diversification? Syst. Biol. 58, 283–297.

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. Mol. Ecol. 26, 2826–2838.

Bakker, M.G., 2018. A fungal mock community control for amplicon sequencing experiments. Molecular Ecology Resources 18, 541–556.

Banchi, E., Ametrano, C.G., Tordoni, E., Stanković, D., Ongaro, S., Tretiach, M., Pallavicini, A., et al., 2020. Environmental DNA assessment of airborne plant and fungal seasonal diversity. Sci. Total Environ. 738, 140249.

Banchi, E., Stankovic, D., Fernández-Mendoza, F., Gionechetti, F., Pallavicini, A., Muggia, L., 2018a. ITS2 metabarcoding analysis complements lichen mycobiome diversity data. Mycol. Prog. 17, 1049–1066.

Banchi, E., Stankovic, D., Fernández-Mendoza, F., Gionechetti, F., Pallavicini, A., Muggia, L., 2018b. ITS2 metabarcoding analysis complements lichen mycobiome diversity data. Mycol. Prog. 17, 1049–1066.

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.

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. Lichenol. 44, 137–146.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120.

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

Callahan, B.J., McMurdie, P.J., Holmes, S.P., 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. ISME J. 11, 2639–2643.

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. Nat. Methods 13, 581–583.

Černajová, I., Škaloud, P., 2020. Lessons from culturing lichen soredia. Symbiosis 82, 109–122.

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. nov. Fungal Biol. 123, 625–637.

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.

Chomicki, G., Renner, S.S., 2017. Partner abundance controls mutualism stability and the pace of morphological change over geologic time. Proc. Natl. Acad. Sci. U.S.A. 114, 3951–3956.

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 Biol. 124, 458–467.

Cometto, A., Leavitt, S.D., Grube, M., De Hoog, S., Muggia, L., 2023. Tackling fungal diversity in lichen symbioses: molecular and morphological data recognize new lineages in Chaetothyriales (Eurotiomycetes, Ascomycota). Mycolog. Prog. 22 (8), 53.

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 Biol. 126 (9), 587–608.

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 Phytol. 130, 267–297.

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 Systemat. Evol. 216, 243–249.

Davey, M.L., Currah, R.S., 2006. Interactions between mosses (Bryophyta) and fungi. Can. J. Bot. 84, 1509–1519.

De Bary, A., 1879. Die erscheinung der symbiose: Vortrag gehalten auf der versammlung deutscher naturforscher und aerzte zu cassel. Trübner.

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. Front. Microbiol. 13, 809804.

Diederich, P., Lawrey, J.D., Ertz, D., 2018. The 2018 classification and checklist of lichenicolous fungi, with 2000 non-lichenized, obligately lichenicolous taxa. Bryologist 121, 340–425.

Dissanayake, A.J., Purahong, W., Wubet, T., Hyde, K.D., Zhang, W., Xu, H., et al., 2018. Direct comparison of culture-dependent and culture-independent molecular approaches reveal the diversity of fungal endophytic communities in stems of grapevine (Vitis vinifera). Fungal Divers. 90, 85–107.

Dixon, P., 2003. VEGAN, a package of R functions for community ecology. J. Veg. Sci. 14, 927–930.

Doolittle, W.F., Booth, A., 2017. It's the song, not the singer: an exploration of holobiosis and evolutionary theory. Biol. Philos. 32, 5–24.

Douglas, A.E., 1994. Symbiotic Interactions. Oxford University Press, Oxon, GB, 1994.

Douglas, A.E., Werren, J.H., 2016. Holes in the hologenome: why host-microbe symbioses are not holobionts. mBio 7, e02099-15.

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.

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 Biol. 120, 1416–1447.

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*. Mycol. Res. 113, 141–152.

Estensmo, E.F.L., 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.

Fernández-Mendoza, F., Fleischhacker, A., Kopun, T., Grube, M., Muggia, L., 2017. ITS1 metabarcoding highlights low specificity of lichen mycobiomes at a local scale. Mol. Ecol. 26, 4811–4830.

Fleischhacker, A., Grube, M., Kopun, T., Hafellner, J., Muggia, L., 2015. Community analyses uncover high diversity of lichenicolous fungi in Alpine habitats. Microb. Ecol. 70, 348–360.

Frank, A.B., 1877. Über die biologischen Verhältnisse des Thallus einiger Krustenflechten. Beitr Biol. Pflanz. 2, 123–200.

Girlanda, M., Isocrono, D., Bianco, C., Luppi-Mosca, A.M., 1997. Two foliose lichens as microfungal ecological niches. Mycologia 89, 531–536.

Glenn, T.C., Pierson, T.W., Bayona-Vásquez, N.J., Kieran, T.J., Hoffberg, S.L., Thomas, J. C., Lefever, D.E., et al., 2019a. Adapterama II: universal amplicon sequencing on Illumina platforms (TaggiMatrix). PeerJ 2019, e7786.

Glenn, T.C., Pierson, T.W., Bayona-Vásquez, N.J., Kieran, T.J., Hoffberg, S.L., Thomas, J. C., Lefever, D.E., et al., 2019b. Adapterama II: universal amplicon sequencing on Illumina platforms (TaggiMatrix). PeerJ 2019, e7786.

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. ISME J. 3, 1105–1115.

Grube, M., Cernava, T., Soh, J., Fuchs, S., Aschenbrenner, I., Lassek, C., Wegner, U., et al., 2015. Exploring functional contexts of symbiotic sustain within lichen-associated bacteria by comparative omics. ISME J. 9, 412–424.

Grube, M., Muggia, L., 2021. 2.5 Complex lichen communities inhabiting rock surfaces. In: Life at Rock Surfaces, vols. 175–188. De Gruyter.

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 Biol. 115, 987–996.

Guerrero, R., Margulis, L., Berlanga, M., 2013. Symbiogenesis: the holobiont as a unit of evolution. Introduction in Microbiology 16, 133–143.

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

Harutyunyan, S., Muggia, L., Grube, M., 2008. Black fungi in lichens from seasonally arid habitats. Stud. Mycol. 61, 83–90.

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

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 49, 77–77.

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

Hocking, A.D., Pitt, J.I., 1980. Dichloran-glycerol medium for enumeration of xerophilic fungi from low-moisture foods. Appl. Environ. Microbiol. 39, 488–492.

Honegger, R., 2009. Lichen-forming fungi and their photobionts. Plant Rrelationships 307–333. Springer.

Kellogg, J.J., Raja, H.A., 2017. Endolichenic fungi: a new source of rich bioactive secondary metabolites on the horizon. Phytochemistry Rev. 16, 271–293.

Lahti, L., Shetty, S., Blake, T., Salojarvi, J., others, 2017. Tools for microbiome analysis in R. Versiones 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. Mol. Ecol. 28, 420–430.

Lawrey, J.D., Binder, M., Diederich, P., Molina, M.C., Sikaroodi, M., Ertz, D., 2007. Phylogenetic diversity of lichen-associated homobasidiomycetes. Mol. Phylogenet. Evol. 44, 778–789.
Lawrey, J.D., Diederich, P., 2018. Lichenicolous Fungi – Worldwide Checklist, Including Isolated Cultures and Sequences Available. George Mason University, pp. 340–425.
Lawrey, J.D., Diederich, P., 2003. Lichenicolous fungi: interactions, evolution, and biodiversity. Bryol. 106, 80–120.
Lear, G., Dickie, I., Banks, J., Boyer, S., Buckley, H.L., Buckley, T.R., Cruickshank, R., et al., 2018. Methods for the extraction, storage, amplification and sequencing of DNA from environmental samples. N. Z. J. Ecol. 42, 10.
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). Mol. Phylogenet. Evol. 59, 587–602.
Leavitt, S.D., Grewe, F., Widhelm, T., Muggia, L., Wray, B., Lumbsch, H.T., 2016. Resolving evolutionary relationships in lichen-forming fungi using diverse phylogenomic datasets and analytical approaches. Sci. Rep. 6, 1–11.
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. Am. J. Bot. 106, 1090–1095.
Li, W., Godzik, A., 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics 22, 1658–1659.
Li, Y., Steenwyk, J.L., Chang, Y., Wang, Y., James, T.Y., Stajich, J.E., Spatafora, J.W., et al., 2021. A genome-scale phylogeny of the kingdom Fungi. Curr. Biol. 31, 1653–1665.
Lilly, V.G., Barnett, H.L., 1951. Physiology of the Fungi. *Physiology of the Fungi*.
Lücking, R., Aime, M.C., Robbertse, B., Miller, A.N., Ariyawansa, H.A., Aoki, T., Cardinali, G., 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.
Lutzoni, F., Miadlikowska, J., 2009. Lichens. Curr. Biol. 19.
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.
Mande, S.S., Mohammed, M.H., Ghosh, T.S., 2012. Classification of metagenomic sequences: methods and challenges. Briefings Bioinf. 13, 669–681.
Margulis, L., Fester, R., 1991. In: Bellagio Conference and Book. Symbiosis as Source of Evolutionary Innovation: Speciation and Morphogenesis. Conference–June 25-30, 1989. Bellagio Conference Center, Italy. Symbiosis, pp. 93–101.
McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217.
Millanes, A.M., Diederich, P., Ekman, S., Wedin, M., 2011. Phylogeny and character evolution in the jelly fungi (Tremellomycetes, Basidiomycota, Fungi). Mol. Phylogenet. Evol. 61, 12–28.
Moya, P., Molins, A., Chiva, S., Bastida, J., Barreno, E., 2020. Symbiotic microalgal diversity within lichenicolous lichens and crustose hosts on Iberian Peninsula gypsum biocrusts. Sci. Rep. 10 (1), 14060.
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.
Muggia, L., Fleischhacker, A., Kopun, T., Grube, M., 2016. Extremotolerant fungi from alpine rock lichens and their phylogenetic relationships. Fungal Divers. 76, 119–142.
Muggia, L., Grube, M., 2018. Fungal diversity in lichens: from extremotolerance to interactions with algae. Life 8, 15.
Muggia, L., Grube, M., Tretiach, M., 2008. Genetic diversity and photobiont associations in selected taxa of the *Tephromela atra* group (Lecanorales, lichenised Ascomycota). Mycol. Prog. 7, 147–160.
Muggia, L., Nelsen, M.P., Kirika, P.M., Barreno, E., Beck, A., Lindgren, H., Lumbsch, H.T., Leavitt, S.D., 2020. Formally described species woefully underrepresent phylogenetic diversity in the common lichen photobiont genus *Trebouxia* (Trebouxiophyceae, Chlorophyta): an impetus for developing an integrated taxonomy. Mol. Phylogenet. Evol. 149.
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.
Muggia, L., Pérez-Ortega, S., Fryday, A., Spribille, T., Grube, M., 2014. Global assessment of genetic variation and phenotypic plasticity in the lichen-forming species *Tephromela atra*. Fungal Divers. 64, 233–251.
Muggia, L., Quan, Y., Gueidan, C., Al-Hatmi, A.M.S., Grube, M., de Hoog, S., 2021. Sequence data from isolated lichen-associated melanized fungi enhance delimitation of two new lineages within Chaetothyriomycetidae. Mycol. Prog. 20, 911–927.
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.
Nai, C., Wong, H.Y., Pannenbecker, A., Broughton, W.J., Benoit, I., de Vries, R.P., Gueidan, C., Gorbushina, A.A., 2013. Nutritional physiology of a rock-inhabiting, model microcolonial fungus from an ancestral lineage of the Chaetothyriales (Ascomycetes). Fungal Genet. Biol. 56, 54–66.
Nash, T.H., 2008. In: Lichen Biology, second ed. second ed.
Nilsson, R.H., Anslan, S., Bahram, M., Wurzbacher, C., Baldrian, P., Tedersoo, L., 2019. Mycobiome diversity: high-throughput sequencing and identification of fungi. Nat. Rev. Microbiol. 17, 95–109.
Oita, S., Carey, J., Kline, I., Ibáñez, A., Yang, N., Hom, E.F., et al., 2021. Methodological approaches frame insights into endophyte richness and community composition. Microb. Ecol. 82 (1), 21–34.
Ondov, B.D., Bergman, N.H., Phillippy, A.M., 2011. Interactive metagenomic visualization in a Web browser. BMC Bioinf. 12, 1–10.
Pagano, J., Levin, J.D., Trejo, W., 1958. Diagnostic medium for differentiation of species of Candida. Antibiot. Annu. 5, 137–143.
Pang, K.L., Guo, S.Y., Chen, I.A., Burgaud, G., Luo, Z.H., Dahms, H.U., Hwang, J.S., et al., 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.
Petrini, O., Hake, U., Dreyfuss, M.M., 1990. An analysis of fungal communities isolated from fruticose lichens. Mycologia 82, 444–451.
Poelt, J., 1958. Über parasitische Flechten. II. Planta 51 (3. H), 288–307.
Poelt, J., Mayrhofer, H., 1988. Systematics and evolution uber Cyanotrophie bei Flechten. Plant Systemat. Evol. 82, 265–281.
Quan, Y., Muggia, L., Moreno, L.F., Wang, M., Al-Hatmi, A.M.S., Menezes, N. da S., Shi, D., et al., 2020. A re-evaluation of the Chaetothyriales using criteria of comparative biology. Fungal Divers. 103, 47–85.
Rafferty, N.E., Caradonna, P.J., Bronstein, J.L., 2015. Phenological shifts and the fate of mutualisms. Oikos 124, 14–21.
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.
Rosenberg, E., Zilber-Rosenberg, I., 2011. Symbiosis and development: the hologenome concept. Birth Defects Res. Part C Embryo Today - Rev. 93, 56–66.
Ruibal, C., Gueidan, C., Selbmann, L., Gorbushina, A.A., Crous, P.W., Groenewald, J.Z., Muggia, L., et al., 2009. Phylogeny of rock-inhabiting fungi related to Dothideomycetes. Stud. Mycol. 64, 123–133.
Selbmann, L., Hoog, G.S.D., Mazzaglia, A., Friedmann, E.I., Onofri, S., 2005. Fungi at the edge of life: cryptoendolithic black fungi from Antarctic desert. Stud. Mycol. 51, 1–32.
Smith, H.B., Grande, F.D., Muggia, L., Keuler, R., Divakar, P.K., Grewe, F., Schmitt, I., et al., 2020. Metagenomic data reveal diverse fungal and algal communities associated with the lichen symbiosis. Symbiosis 82, 133–147.
Spribille, T., 2018. Relative symbiont input and the lichen symbiotic outcome. Curr. Opin. Plant Biol. 44, 57–63.
Spribille, T., Tuovinen, V., Resl, P., Vanderpool, D., Wolinski, H., Aime, M.C., Schneider, K., et al., 2016. Basidiomycete yeasts in the cortex of ascomycete macrolichens. Science 353, 488–492.
Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30 (9), 1312–1313.
Sterflinger, K., Krumbein, W.E., 1995. Multiple stress factors affecting growth of rock-inhabiting black fungi. Bot. Acta 108, 1438–8677.
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 (4), evab047.
Tedersoo, L., Bahram, M., Zinger, L., Nilsson, R.H., Kennedy, P.G., Yang, T., Anslan, S., Mikryukov, V., 2022. Best practices in metabarcoding of fungi: from experimental design to results. Mol. Ecol. 31, 2769–2795.
Teixeira, M.M., Moreno, L.F., Stielow, B.J., Muszewska, A., Hainaut, M., Gonzaga, L., Abouelleil, A., et al., 2017. Exploring the genomic diversity of black yeasts and relatives (Chaetothyriales, Ascomycota). Stud. Mycol. 86, 1–28.
Tuovinen, V., Ekman, S., Thor, G., Vanderpool, D., Spribille, T., Johannesson, H., 2019. Two basidiomycete fungi in the cortex of wolf lichens. Curr. Biol. 29, 476–483.
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. Environ. Microbiol. 23, 2484–2498.
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. Microb. Ecol. 60, 340–353.
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. Am. J. Bot. 99, 898–914.
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.
White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methodsand Applications. Academic Press, Inc., New York, N.Y, pp. 315–322.
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.
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. Sci. Total Environ. 167, 287–294.
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.
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.
Yoshimura, I., Yamamoto, Y., Nakano, T., Finnie, J., 2002. Isolation and Culture of Lichen Photobionts and Mycobionts. Protocols in Lichenology, vols. 3–33. Springer.
Yu, N.H., Park, S.Y., Kim, J.A., Park, C.H., Jeong, M.H., Oh, S.O., Hong, S.G., et al., 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.
Zamora, J.C., Pérez-Ortega, S., Rico, V.J., 2011. *Tremella macrobasidiata* (Basidiomycota, Tremellales), a new lichenicolous fungus from the Iberian Peninsula. Lichenol. 43, 407–415.
Zhang, T., Wei, 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. Sci. Rep. 5, 14850.