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SHORT-TERM DYNAMICS OF THE MYCOBIOME IN SPECIES OF THE GENERA *UMBILICARIA* AND *DERMATOCARPON* IN A SAXICOLOUS LICHEN COMMUNITY

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Lichens, which are fungi that form mutualistic relationships with algae or cyanobacteria, also serve as habitats for a variety of microbial communities. Recently, the diversity of fungi associated with lichens has garnered considerable research attention. However, despite such advances, the mycobiome of lichens and its variations remain inadequately explored in many areas. Over a six-week period in spring, three saxicolous lichen species from the genera *Dermatocarpon* and *Umbilicaria* were randomly sampled at two locations in a low mountain range in Bavaria, Germany. The mycobiomes associated with these lichens were analysed and taxonomically classified using a metabarcoding approach with Illumina sequencing. The results revealed that the microbial communities displayed a clear specificity to their host lichens. Overall, it appears that the similarity of the mycobiome is generally higher within a genus than between different genera. Additionally, no significant changes in the fungal community of any single lichen species were observed over the six-week period, suggesting that changes in lichen mycobiomes occur slowly within a vegetation season.

Key words: *Dermatocarpon*, endolichenic fungi, host-lichen specificity, lichenicolous fungi, lichens, mycobiome, seasonal fluctuation, *Umbilicaria*

INTRODUCTION

Lichens and their associated fungi have been the subject of research for over 30 years (Cometto *et al*. 2023, Peršoh and Rambold 2012, Rambold and Triebel 1992, Wang *et al*. 2016, Williams *et al*. 2017). These studies increasingly highlight that lichen thalli provide habitats for a wide range of microorganisms, including prokaryotes, fungi and other eukaryotes (Banchi *et al*. 2018, Grube and Berg 2009, Schwob *et al*. 2024). This community of diverse microorganisms, collectively referred to as the 'microbiome' (Berg *et al*. 2016), can be classified into various phylogenetic groups. Specifically, the fungi associated with lichens, forming the 'mycobiome', are often categorized as lichenicolous

fungi (Diederich *et al.* 2018, Kondratyuk *et al.* 2021, Zhurbenko *et al.* 2021). Among these lichenicolous fungi, a distinction is made between those that produce visible meio- or mitosporic reproductive structures on or within the lichen thallus and those that exhibit no visible morphological changes (Diederich *et al.* 2018, Lawrey and Diederich 2003, Lisci *et al.* 2003, Pichler *et al.* 2023, U'Ren *et al.* 2012). The latter, often referred to as asymptomatic or endolichenic fungi (Diederich *et al.* 2018, U'Ren *et al.* 2012), are typically found within the lichen thallus and can only be recognized with optical aids.

Before the advent of new cultivation methods in the 1990s (Biosca *et al.* 2016, Fernández-Mendoza *et al.* 2017, Geiser *et al.* 1994), fungal communities associated with lichens were scarcely studied in detail. However, recent methodological advancements have revealed that the diversity of lichen-associated fungi is much greater than previously thought (Muggia *et al.* 2014, Spribille *et al.* 2020). Modern high-throughput technologies have enabled the classification of mycobiomes into guilds (Nguyen *et al.* 2016, Nilsson *et al.* 2019) and have facilitated the rapid and largely accurate characterization of a wide range of mycobiomes (Paul *et al.* 2018). One significant advantage of metabarcoding the ITS region (internal transcribed spacer) is its ability to identify operational taxonomic units (OTUs) across different fungal phylogenetic groups and determine the mycobiome composition at the species level (Bazzicalupo *et al.* 2013, Schoch *et al.* 2012, U'Ren *et al.* 2010).

A key question that remains is understanding the factors influencing the alpha-diversity and composition of fungal communities associated with different lichen species. The vast diversity of these fungal communities suggests that they may play crucial roles in ecological processes, such as metabolite biosynthesis and the breakdown of older lichen components (Fazio *et al.* 2018, Grube 2018, Hodkinson and Lutzoni 2009). However, only a limited number of lichen species have been extensively studied in relation to their associated mycobiomes (Cometto *et al.* 2024, Yang *et al.* 2024). This is partly because current lichenological research often concentrates on the lichens themselves and their secondary metabolites. Lichens are known to produce over a thousand secondary metabolites (Huneck and Yoshimura 1996, Shanmugam *et al*. 2016, 2022, Stocker-Wörgötter 2008), some of which are being investigated for their antimicrobial, anticancer and antimycobacterial properties (Elsebai *et al*. 2011, Srinivasan *et al*. 2019).

For the three saxicolous lichen species of our study, the presence of gyrophoric and lecanoric acids as secondary metabolites in *Umbilicaria* species has already been confirmed several times (Grande *et al*. 2018, Jahn *et al*. 2017, Mohammadi *et al*. 2022, Posner *et al*. 1992), while no secondary metabolites are known from *Dermatocarpon* spp. so far (e.g. confirmed by LIAS light, Botanische Staatssammlung München 2001–2022, SNSB IT Center; Rambold *et al*.

2014). Elečko *et al*. (2022) recently described the antioxidant properties of lecanoric acid, highlighting how it safeguards both the mycobiome and the lichen itself from oxidative stress. Secondary metabolites, such as lecanoric acid, can influence community dynamics by either inhibiting or promoting the colonization of organisms (Calvo *et al.* 2002, Rangel *et al.* 2021).

Numerous studies on lichen fungi have demonstrated a correlation between the presence of certain fungal species and the identity of their host lichen species (Fleischhacker *et al.* 2015, Jüriado *et al.* 2019, Lawrey and Diederich 2003, Sierra *et al.* 2020). This relationship is also evident in the observation that microbial communities of closely related lichen species tend to be more similar to each other than to those of unrelated species (Ochman *et al.* 2010, Shapira 2016). However, since not all host lichen species have been examined, it remains uncertain whether these findings are universally applicable. In addition to examining the alpha-diversity of lichen-associated fungi, future research should also explore how different growth forms of lichens may influence specificity of host species. Many studied host lichen species are widely attached to their substrate, while foliose and umbilicate lichens, which adhere through a smaller attachment point or a tiny adhesive organ called 'holdfast', have been under explored (Brodo *et al.* 2001). Studying umbilicate lichen species, which are only loosely connected to their substrates (Ríos *et al.* 2002), can provide clearer insights into the mycobiome associated with the lichen thallus.

For the present study, two neighbouring rock sites in northern Bavaria with lichens of the genera *Dermatocarpon* and *Umbilicaria* with squamulose and umbilicate growth forms, respectively, were selected and examined for the possible presence of host specificity of their respective mycobiome. The second aspect of this study focused on the question as to whether the fungal community of lichens is subject to temporal changes or not. Previous research has investigated and demonstrated sporadic seasonal influences, mainly by comparing different seasons and mapping them to variations in temperature, precipitation and UV radiation intensity (Beck *et al*. 2014, Guevara-Araya *et al.* 2022, Oita *et al.* 2021, Oliveira *et al.* 2021). However, there are very few studies that deal with possible changes in communities over a much shorter period of time.

The aim of the current study was to investigate whether compositional shifts occur even over a short period of time and whether a trend can be derived from this. Samples of the three saxicolous lichen species *Dermatocarpon miniatum*, *Umbilicaria hirsuta* and *Umbilicaria pustulata* (syn: *Lasallia pustulata*) were taken at a site in Upper Franconia, Germany, over a period of around two months in spring 2018. We hypothesized that the differences in alpha- and beta-diversity between the lichen genera *Dermatocarpon* (Eurotiomycetes) and *Umbilicaria* (Lecanoromycetes) are more pronounced than the variations

within the genus *Umbilicaria* and are evident in the mycobiome associated with saxicolous lichen species with umbilical growth forms. Furthermore, we expected specific aspects of mycobiome compositions, especially the relative abundances of the phyla Ascomycota and Basidiomycota within the lichen-associated mycobiomes, to change during the six-week study period.

MATERIALS AND METHODS

Site description

As part of this study, samples of various lichens were collected in the southern Fichtelgebirge in Upper Palatinate, Bavaria, Germany. The rocky sampling points are located downhill from the castle ruin Waldeck (near Kemnath town). The area around the castle ruins was formed by tectonic faults (Schröder 1966) with calcareous soils and basaltic rock (Bayerisches Landesamt für Umwelt 2021, Heimat- and Kulturverein Waldeck, 2019). For this short-term study (i.e. within one season), 96 sample fragments of *Dermatocarpon miniatum*, *Umbilicaria hirsuta* and *U. pustulata* were taken on seven days during seven campaigns demarcated over six weeks from April to June 2018 (campaigns 1 to 7) at GPS-located points in true replicates.

Sampling

When removing the 96 fragments of lichen thalli, care was taken to ensure that the remaining parts of the respective thalli remained attached to the substrate. These were already pre-classified to species level in the field. These fragments were separated from the lichen thallus with sterile forceps and packed in sterile rectangular boxes labelled with a Universally Unique Identifier (UUID, version 4) and boxes were provided with an Operational Design Code (ODC) to uniquely identify and characterize each sample (Harjes *et al.* 2019, 2020). To ensure a traceable and reproducible workflow during field sampling, each lichen sample was registered with a GPS-enabled Windows Phone using the Diversity Mobile app together with the associated box and a photograph of the labelled box was taken at the respective location (Jablonski *et al.* 2009, Weibulat *et al.* 2013). All this information, i.e. identifiers and time-space coordinates, was stored together with the names of the host lichens in the Diversity Workbench Diversity Collection (DWB) database of a Diversity Workbench installation at SNSB IT Center, München, Germany (Hagedorn *et al*. 2014, Harjes *et al.* 2020, Triebel *et al.* 2018). The samples were stored at −20 °C in the freezer of the Department of Mycology at the University of Bayreuth, Germany, until further processing.

DNA extraction

Total genomic DNA was extracted from cleaned lichen thalli of *Dermatocarpon miniatum*, *Umbilicaria hirsuta* and *U. pustulata* using Charge Switch® gDNA Plant Kit (Invitrogen, Thermo Fischer Scientific Inc., Waltham, Mass., USA) according to the manufacturer's instructions. A Nanodrop® ND1000 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Mass., USA) was then used to check the samples for DNA concentration and quality (Peršoh *et al*. 2008). The isolated DNA samples are stored at −20 °C for long-term use at the Department of Mycology at the University of Bayreuth, Germany.

DNA library preparation and Illumina sequencing

DNA multiplexing to prepare the DNA library for Illumina sequencing was performed with different primers and consisted of a two-step PCR (polymerase chain reaction). To assess the diversity of the lichen mycobiome, the ITS region was amplified. The first PCR amplification step was performed with a reaction volume of 12.5 µl, consisting of 0.5 µl of total genomic DNA, 0.25 µl each of the specific primers, namely ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), which had specific 'TAG' sequences and part of the Illumina sequencing primer at the 5′ ends (Manter and Vivanco 2007, Martin and Rygiewicz 2005). In addition, 6.25 µl of GoTaq® G2 Hot Start Colorless Master Mix (Promega, Madison, USA) and 5.25 µl of RNase-free water were added to reach the final volume of 12.5 µl. PCR amplification was performed in a 'Primus 96' Thermal Cycler (MWG-Biotech, Ebersberg, Germany) and included an initial denaturation at 95 °C (3 min), followed by 30 cycles of denaturation at 94 °C (27 sec), annealing at 57 °C (1 min), elongation at 72 °C (1 min 30 sec) and a final elongation step at 72 °C (7 min). The obtained PCR product was subjected to ExoSAP digestion (Exonuclease I and shrimp alkaline phosphatase, New England Biolabs, Ipswich, USA) to purify it from excess nucleotides and residual primers. This ExoSAP was prepared with a total volume of 2.0 µl per reaction, consisting of 0.2 µl Exonuclease I (20,000 U/ml), 0.2 µl SAP (1,000 U/µl) and 1.6 µl RNase-free water. For the reaction, 5.0 µl of PCR-1 product was mixed with 2.0 µl of ExoSAP master mix and incubated at 37 °C (15 min), followed by inactivation at 80 °C (15 min). The second PCR amplification was performed in a total reaction volume of 25.0 µl with 5.0 µl of the purified first PCR product under the same conditions as the first PCR reaction, except for only 5 denaturation cycles at 94 °C. For this purpose, 1.0 µl of primer mixture consisting of Illumina sequencing primers, INDEX sequences and P5- and P7-adapter sequences, as well as 12.5 µl of GoTaq® and 6.25 µl of RNase-free water were added to

the reaction mixture. The TAG (PCR step 1) and INDEX sequences (PCR step 2) amplified in the two PCR steps were equimolarly pooled and the success of amplification was checked by gel electrophoresis on agarose (0.8% w/v, 0.5× TBE buffer, 85 V, 40 min) followed by staining of DNA with ethidium bromide and imaging under UV light (GelDoc TM Station, MWG-BIOTECH, Ebersberg, Germany) (Guerreiro *et al.* 2018). The amplified fragments of the second PCR reaction were quantified using ImageJ software (version 1.50b) (Schneider *et al.* 2012) and subsequently equimolarly pooled. Clean PCR® Nucleic Acid Cleanup (CleanNA, GC Biotech B.V.) was used to purify the PCR pool by removing excess nucleotides and primers. After purification, the PCR pools were re-visualized in 0.8% agarose gels and signals from each pool were quantified as described above to create Illumina library pools. High-throughput sequencing of the final pool was performed at the Department of Genetics, Ludwig Maximilian University, Munich, Germany (Dr A. Brachmann) using the Illumina MiSeq® sequencer (Illumina, Inc., San Diego, CA, USA) with 2 × 250 bp paired-end sequencing (MiSeq Reagent Kit v3 Chemistry, Illumina, Inc., San Diego, CA, USA).

Processing of sequence data

The raw readings were quality checked using FastQC (Andrews 2010) and then demultiplexed applying the 'extract_barcodes.py' and 'split_libraries_fastqc.py' scripts implemented within the QIIME1 pipeline (Caporaso *et al.* 2010) to remove any sequences with ambiguous base calls. Before the demultiplexed reads were imported into the QIIME2 pipeline, they were sorted using the 'filter_fasta.py' script (Bolyen *et al.* 2018) based on the used forward primer. Using the CASAVA 1.8 format, the sorted reads were then imported into QIIME2. The remaining primer sequences were separated from the reads using the 'cutadapt' plugin, leaving the SSU nrDNA sequence (Martin 2011). Subsequently the reads from each library were individually denoised and dereplicated using the QIIME2 plugin 'DADA2'. About 10% of all reads were supplied as training data and a maximum expected error of 2 per read was applied to account for the different error rates of each sequencing run (Callahan *et al.* 2016, Nearing *et al.* 2018). The obtained sequence files and the corresponding feature tables of the amplicon sequence variants (ASVs) of each sequencing run were then merged using the command 'feature-table merge', resulting in a single ASV feature table. Subsequently, the OTUs were determined based on the ASVs output from DADA2 using VSEARCH and a 97% sequence similarity threshold.

Taxonomic classification and ecological guild assignment

Taxonomic identification was performed by a manual search in the UNITE database (Nguyen *et al.* 2016). Based on all results of a BLAST search, the identity with the highest taxonomic identity was selected, using a threshold of 97% sequence similarity. At the same time, only identities that had at least an E-value of 2e-65 were selected. Based on this taxonomic classification, the corresponding guilds were assigned to the previously assigned genera using the Fungal Traits database (Põlme *et al.* 2020). For subsequent analysis, the identified guilds were further divided into four groups: 'biotrophic mutualists' (including all lichen-inhabiting fungi), 'biotrophic mycoparasites' (including all lichen-parasitic and pathogenic fungi), 'saprotrophic fungi' (including all saprotrophic wood, soil and litter fungi) and 'unidentified' fungi (which could not be assigned to any guild).

Statistical analysis

The 96 samples collected were reduced to 64 samples due to poor quality in the last above-mentioned processing steps. Starting from this original dataset with 502 OTUs and 64 samples, the samples that had less than 10,000 reads were removed. This threshold was calculated by determining the total sum of all reads within a sample and the median of all these totals; the threshold value is approximately half the median. In addition, OTUs that had no reads and those that had a relative frequency of less than 0.01% were removed from the data set. After this process, 124 OTUs and 43 DNA amplicons remained in the data set for further analysis. For statistical analysis, these data were imported into the program RStudio (v. RStudio Desktop 2022.07.1+554, RStudio for Mac) to create a rarefaction curve representing diversity as a function of sequencing depth using the RStudio package 'vegan' (v. 2.5-7) and the *rarecurve* function. Analysis of alpha-diversity was performed using the packages 'phyloseq' (v. 1.36.0) (McMurdie and Holmes 2013) and 'ggplot2' (v. 3.3.5) (Villanueva and Chen 2019) in RStudio (R Core Team 2022). Variations in species richness (Observed, Chao1), Pielou's species evenness, and Shannon diversity were mapped by the *estimate_richness* function for each host species and the different campaigns.

In addition, parts of the further analyses were conducted using a transformed 'phyloseq' document that used the *transform_sample_counts* function to map the relative abundance data. A one-way ANOVA was used to test host types as well as campaigns for the presence of significant differences. Beta-diversity was analysed based on Bray-Curtis distance and shows variation in

OTU composition between samples using abundance data based on OTUs; as part of this analysis, non-metric multidimensional scaling (NMDS) plots were created to show the differences and similarities in OTU composition among host species and over time. To visualize the dissimilarities, the OTU composition of the lichen-associated mycobiome was visualized using principal coordinate analyses (PCoA) based on the Bray-Curtis distance and tested for linear responses of the mycobiome along explanatory variables. The 'phyloseq' package in RStudio was again used for this purpose. All the processed data is accessible as supplementary information in the Zenodo digital repository accessible via (Karthik *et al.* 2024).

RESULTS

Taxonomic composition and lifestyles of the lichen-associated mycobiome of different hosts at different campaigns

After quality filtering, 43 DNA amplicons and 124 OTUs were obtained, of which 110 OTUs could be assigned to the fungal kingdom. The remaining 14 OTUs could not be assigned with certainty to the fungal kingdom and remain in the data set as 'not identified'. Of the 43 amplicons, 9 came from the host species *Dermatocarpon miniatum*, 26 from *Umbilicaria hirsuta* and 8 from *U. pustulata*. Originally 96 lichen fragments were collected; the campaigns (C-1 to C-7) are also represented by different numbers of DNA amplicons: C-1 contains 14 from all hosts, C-2 contains 5 from all hosts, C-3 contains 7 from all hosts, C-4 contains 4 from the two *Umbilicaria* species, C-5 contains 4 from the two *Umbilicaria* species, C-6 contains 4 from the hosts *D. miniatum* and *U. hirsuta*, and C-7 contains 5 from the two *Umbilicaria* species (Supplementary Fig. 1, Supplementary Table 1).

Taxonomic classification of the mycobiomes revealed that Ascomycota was the dominant phylum in all three host species and across all campaigns, followed by Basidiomycota and a few unidentified sequences. The smallest proportion of reads could be assigned to the phylum Chytridiomycota (Fig. 1). Among the three host species, abundance of Basidiomycota was highest in *U. pustulata* and lowest in the amplicons of *U. hirsuta*. Regarding the absolute abundance of Ascomycota, the number of reads is highest in *U. hirsuta*. In terms of the absolute abundance of Basidiomycota, the number of reads is highest in *U. pustulata.* Looking at the abundance of phyla over time within a host, no trend towards changes in composition can be identified (Fig. 1). Looking at the classes in the amplicons of *D. miniatum*, the class of Eurotiomycetes dominates, followed by Agaricomycetes and about the same proportion of Dothideomycetes and Chytridiomycetes (Fig. 2). Considering the classes in

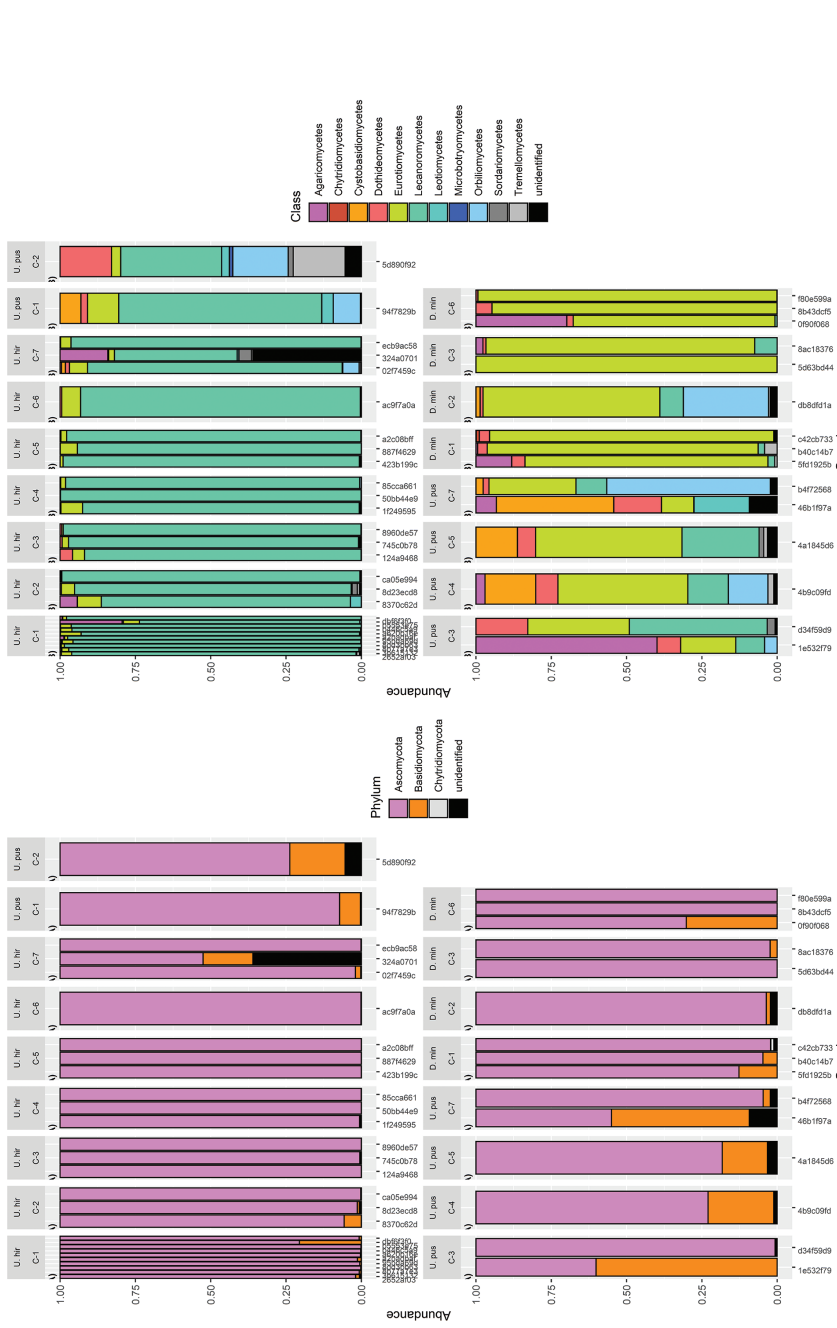

Fig. 1. Taxa barplot showing relative abundances of phyla and class of all samples separated by hosts (*Umbilicaria hirsuta*, *U. pustulata* and *Dermatocarpon miniatum*) and campaigns. The functions transform_sample_counts and tax_glom from the 'phyloseq' package were used to display this plot. Phyla and Class labelled as 'unidentified' could not be taxonomically assigned

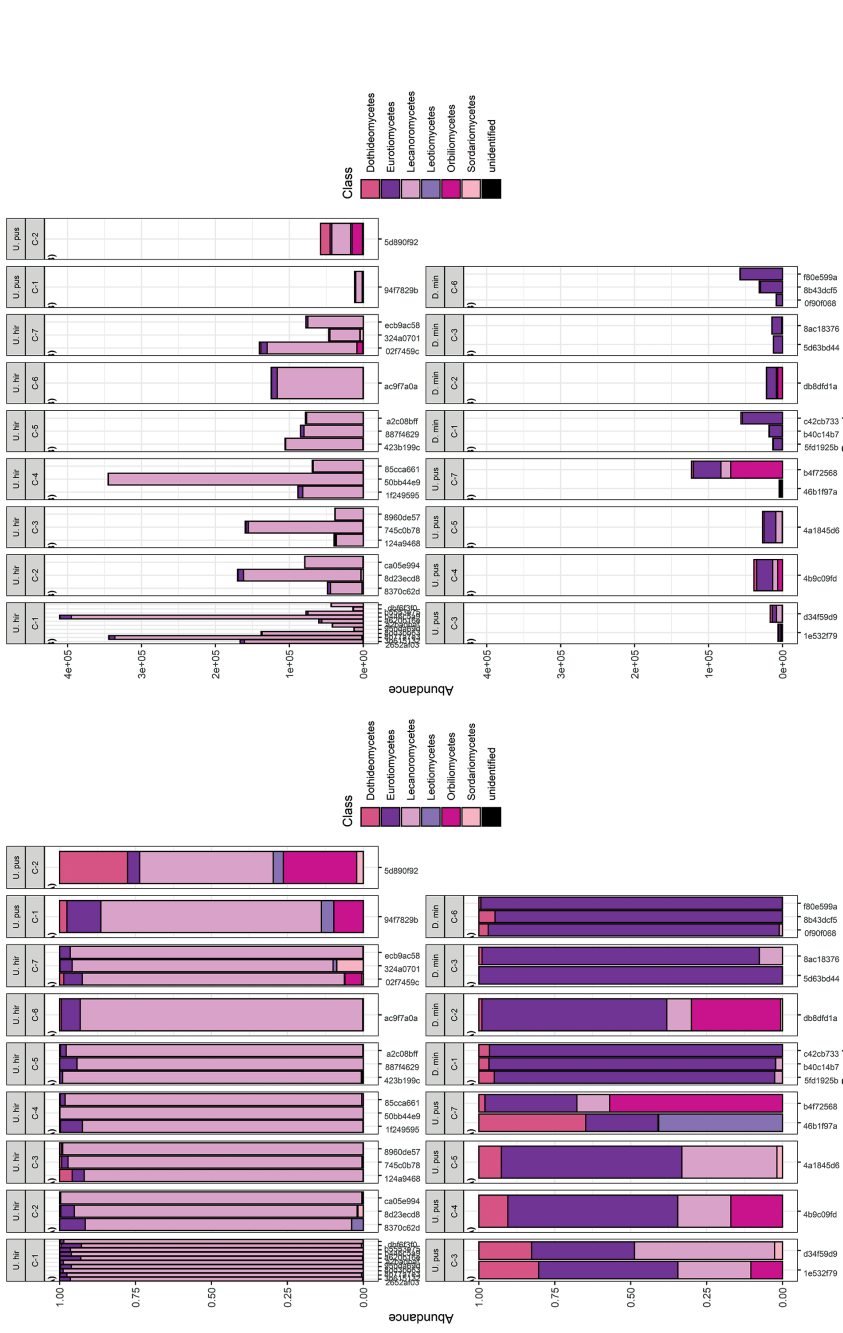

Fig. 2. Taxa barplot showing relative abundances of classes of all samples separated by hosts and campaigns. The functions transform_sample_counts and tax_glom from the 'phyloseq' package were used to display this plot. Classes labelled as 'unidentified' could not be taxonomically assigned

the amplicons of *U. hirsuta*, the Lecanoromycetes dominate the community in almost every sample. The second and third most common classes, namely Eurotiomycetes and Agaricomycetes respectively, account for a comparatively small proportion. When looking at the classes of the amplicons of *U. pustulata*, Eurotiomycetes are most frequently represented, followed by Lecanoromycetes and Orbiliomycetes.

To further elucidate the lichen-associated mycobiome, the different lifestyles of the fungi identified to generic level were investigated (Supplementary Table 2). Of the 124 OTUs, 15 could be assigned as biotrophic mutualists, 27 as biotrophic mycoparasites and 34 as saprotrophs. The remaining 48 OTUs could not be determined taxonomically in sufficient depth to assign a lifestyle and remain in the dataset as 'unidentified'. The biotrophic mutualistic lifestyle dominates the overall lichen-associated mycobiome of all three host species combined across campaigns, with occasional 'unidentified' or saprotrophic lifestyles also present (Fig. 3). Considering the fungal lifestyles for individual host lichen species, saprotrophic fungi dominate the mycobiome in *U. pustulata* as opposed to the other two host species; here, this lifestyle even dominates over the biotrophic mutualistic species. In contrast, the two host species, *D. miniatum* and *U. hirsuta* are dominated by biotrophic mutualistic fungi (Fig. 3).

Alpha-diversity of the lichen-associated mycobiome of the different hosts at different campaigns

To test alpha-diversity, the entire data set with all 124 OTUs and 43 DNA amplicons was analysed for fungal species richness, Shannon diversity and Pielou's evenness. One-way analysis of variance (ANOVA) was used to test for any significant differences. In the present case, there was no significant difference in species richness (ANOVA $p > 0.05$), Shannon diversity ($p > 0.05$) and evenness ($p > 0.05$) when comparing the different host lichen species (Supplementary Table 3). The expected OTUs were evaluated with the 'Chao1' index. Here, the median is highest for *U. pustulata* (Fig. 4). The medians of *U. hirsuta* and *D. miniatum*, which hardly differ from each other, are at a considerable mycobiome composition distance. Thus, the number of expected OTUs is highest for *U. pustulata*. The index 'Observed' evaluates the species richness of the observed OTUs. The distribution of observed OTUs is very similar to the one of the expected OTUs in the Chao1 diagram (Fig. 4). The 'Shannon' index assesses the differences in diversity between species. A look at the Shannon plot shows the differences between the three hosts, with *U. pustulata* showing the highest diversity and again having a clear gap to *D. miniatum* and *U. hirsuta*. According to the Shannon index, the host *U. hirsuta* exhibits the lowest species diversity. 'Pielou's evenness' index evaluates the

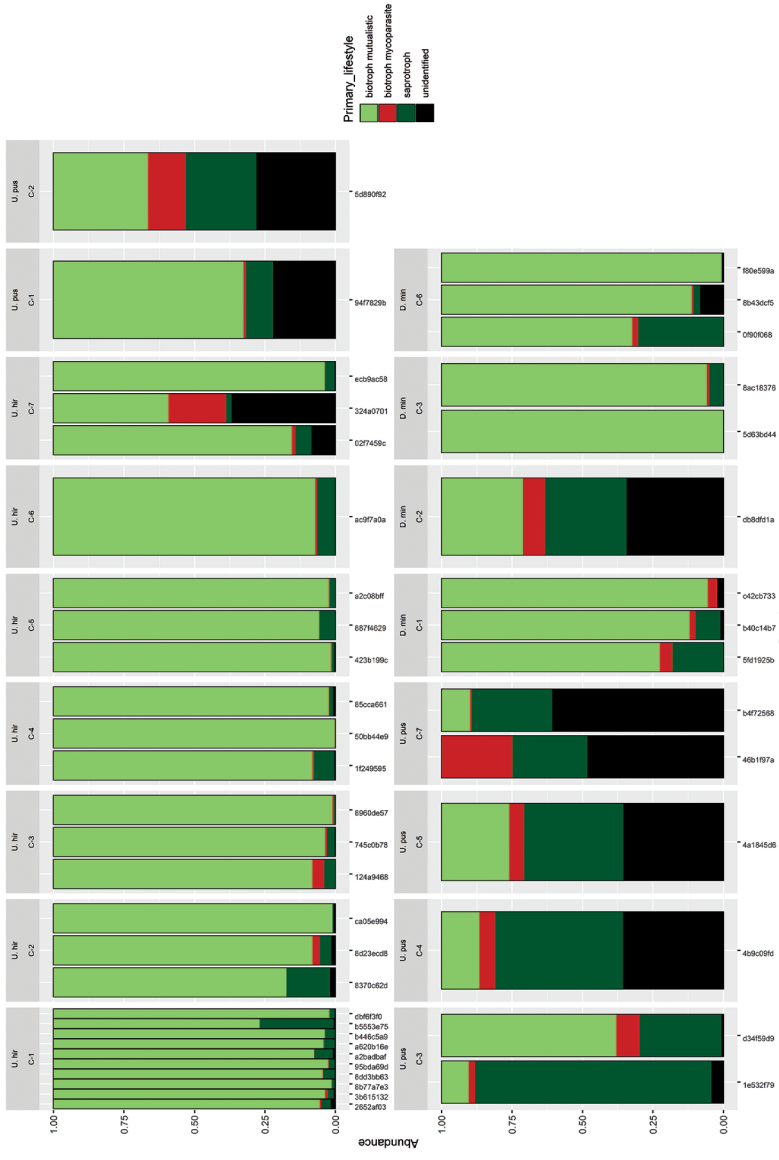

Fig. 3. Barplot showing relative abundances of primary lifestyles in the different samples, separated by hosts and campaigns. The transform_sample_counts and tax_glom functions from the 'phyloseq' package were used to plot this graph. In addition, lifestyles were grouped into four guilds for mapping: biotroph mutualistic (lichenized), biotroph mycoparasite (lichen parasite, pathogen, pathotroph), saprotroph (wood saprotroph, soil saprotroph, litter saprotroph) and unidentified. Those marked as 'unidentified' could not be taxonomically assigned

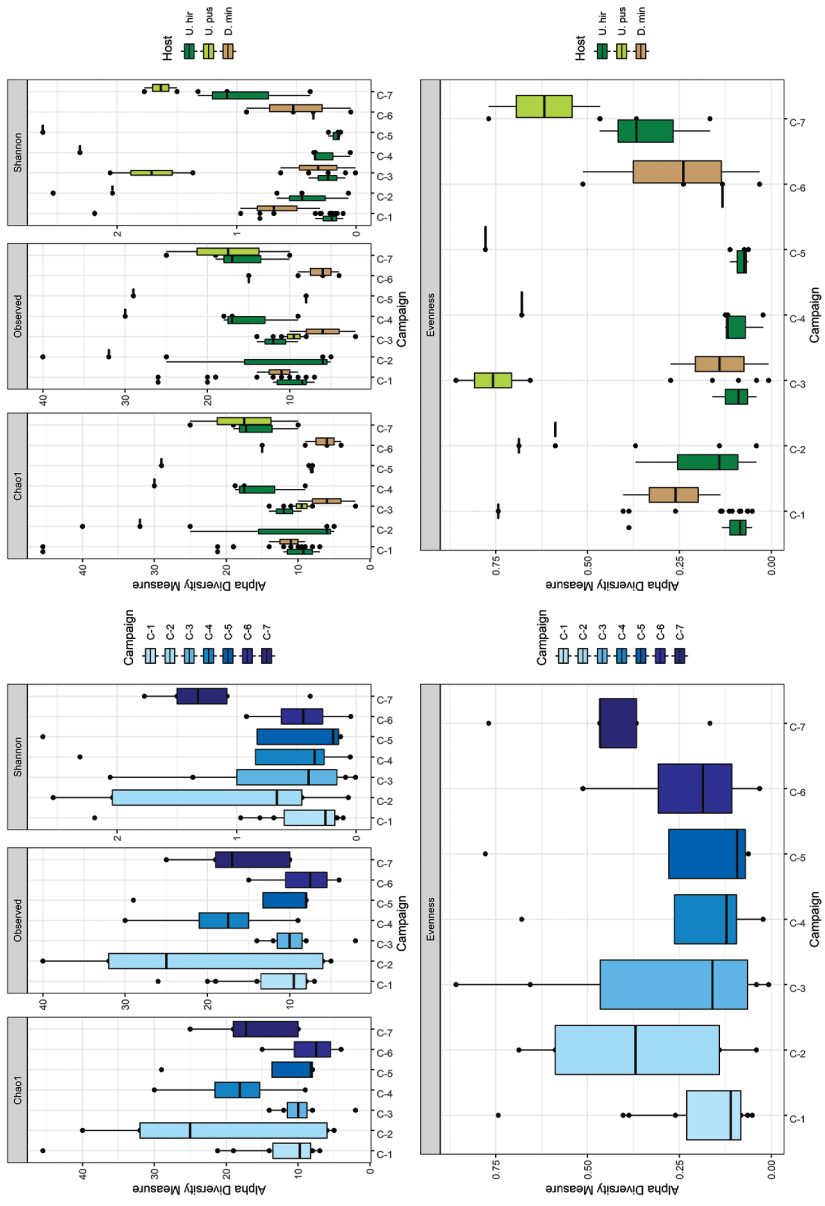

Fig. 4. Alpha diversity measure of the rarefied dataset plotted by the different campaigns and the hosts within them. For this purpose, the data were rarefied using the rarefy_even_depth function of the 'phyloseq' package. The plot shows the distribution for estimated species richness (Chao1), abundance (Observed) and diversity (Shannon). Data were analysed according to Pielou's species evenness. Median values and interquartile ranges have been indicated in the plots

commonness or rarity of species in a community. Evenness was highest in *U. pustulata*, with a clear gap again to *D. miniatum* and *U. hirsuta.*

To analyse the data set equally when considering the different campaigns, the entire data set of 124 OTUs and 43 DNA amplicons was analysed for fungal species richness, Shannon diversity and Pielou's evenness. ANOVA was again used to test significant differences. Again, there was no significant difference for all deployed indices when comparing the different campaigns to each other (Supplementary Table 5). OTUs of all three hosts are not present in every campaign, which is why not every host in every campaign is represented by a boxplot, but sometimes only by a dot or a line (one sample being present) or even no geometric figure (no sample being present). *U. pustulata* shows the highest number of expected OTUs compared to *D. miniatum* and *U. hirsuta* in every campaign where OTUs are present, except for C-3 (Fig. 4). In C-3, the median of *U. hirsuta* is higher than that of *U. pustulata*. Looking at the distribution of medians of individual hosts across campaigns, we notice a relatively constant range for both *D. miniatum* and *U. hirsuta*. Only the distribution for *U. pustulata* extends over a larger range (Fig. 4). For all three hosts and across campaigns, the distribution of observed OTUs is very similar to the one of the expected OTUs in the Chao1 diagram (Fig. 4). The Shannon index and therefore the differences in diversity between the host species can best be evaluated in the first three campaigns (C-1, C-2 and C-3), as all three hosts are represented here. Differences in species diversity can be seen here both between the hosts and between the campaigns, with *U. pustulata* showing considerably higher species diversity than the other two host species in C-3 and C-7 (only two host species). Evenness was relatively similar among campaigns, but *U. pustulata* again showed considerably higher evenness compared to the other host species in campaigns C-3 and C-7 (again, only two host species) (Fig. 5).

Beta-diversity of the lichen-associated mycobiome of the different host lichen species and genera at different campaigns

Beta-diversity (using Bray-Curtis dissimilarity on square root transformed reads) for lichen-associated fungi was assessed for all fungi found. Host species had no significant effect on fungal community structure (PERMANOVA $p > 0.05$). Sample ordination was visualized using non-metric multidimensional scaling (NMDS) (Fig. 6A, B, C), combining samples from one host across all campaigns to obtain a better overview. The community composition of *U. pustulata* overlaps with those of *U. hirsuta* and *D. miniatum* (Fig. 6A). However, the latter two do not overlap (Fig. 6C), indicating that the two *Umbilicaria* species have a more similar mycobiome composition (Fig. 6A). In addition, one sample of *D. miniatum* is clearly further away from the others and its ordination lies directly in the middle of the samples of *U. pustulata*.

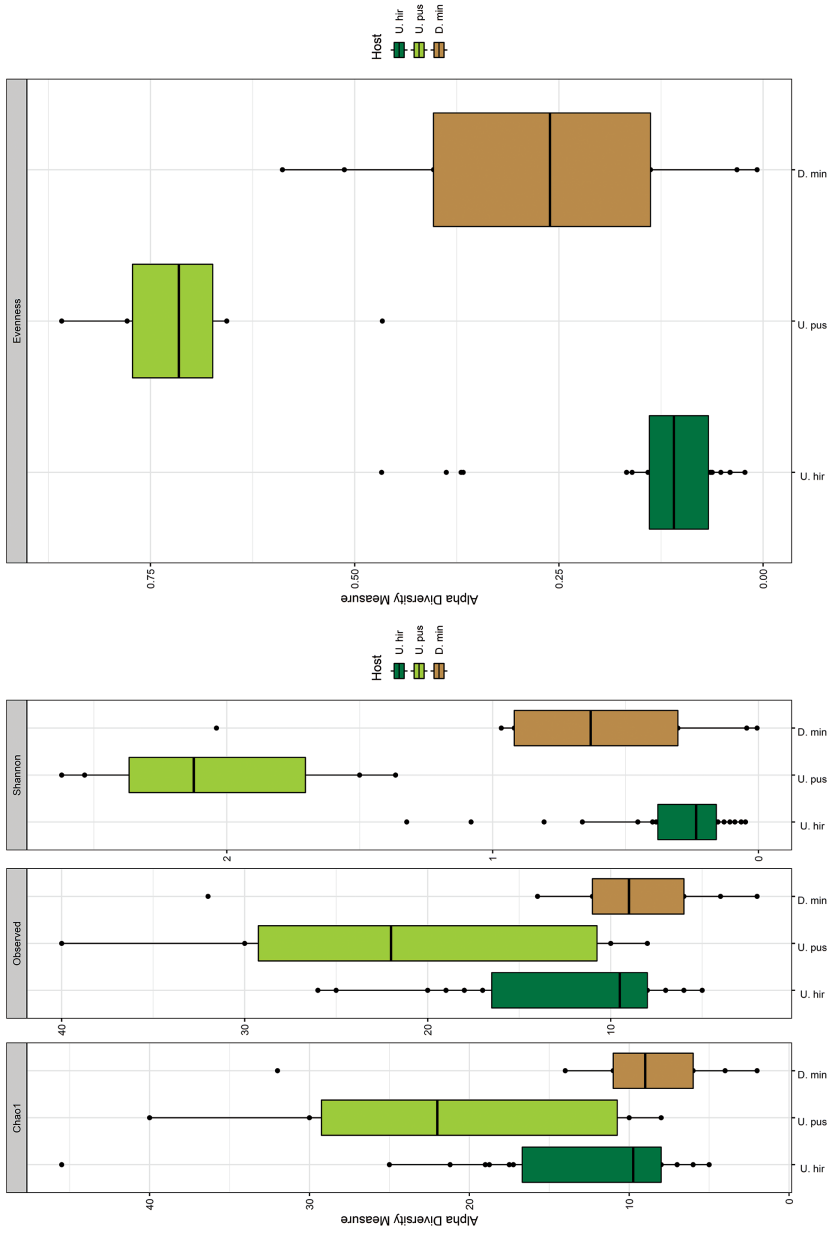

Fig. 5. Alpha diversity measure of the rarefied dataset plotted by the three different hosts. Samples were rarefied using the rarefy_even_depth function in the 'phyloseq' package and evaluated for estimated species richness (Chao1), abundance (Observed), diversity (Shannon) and evenness. Median values and interquartile ranges have been indicated in the plots

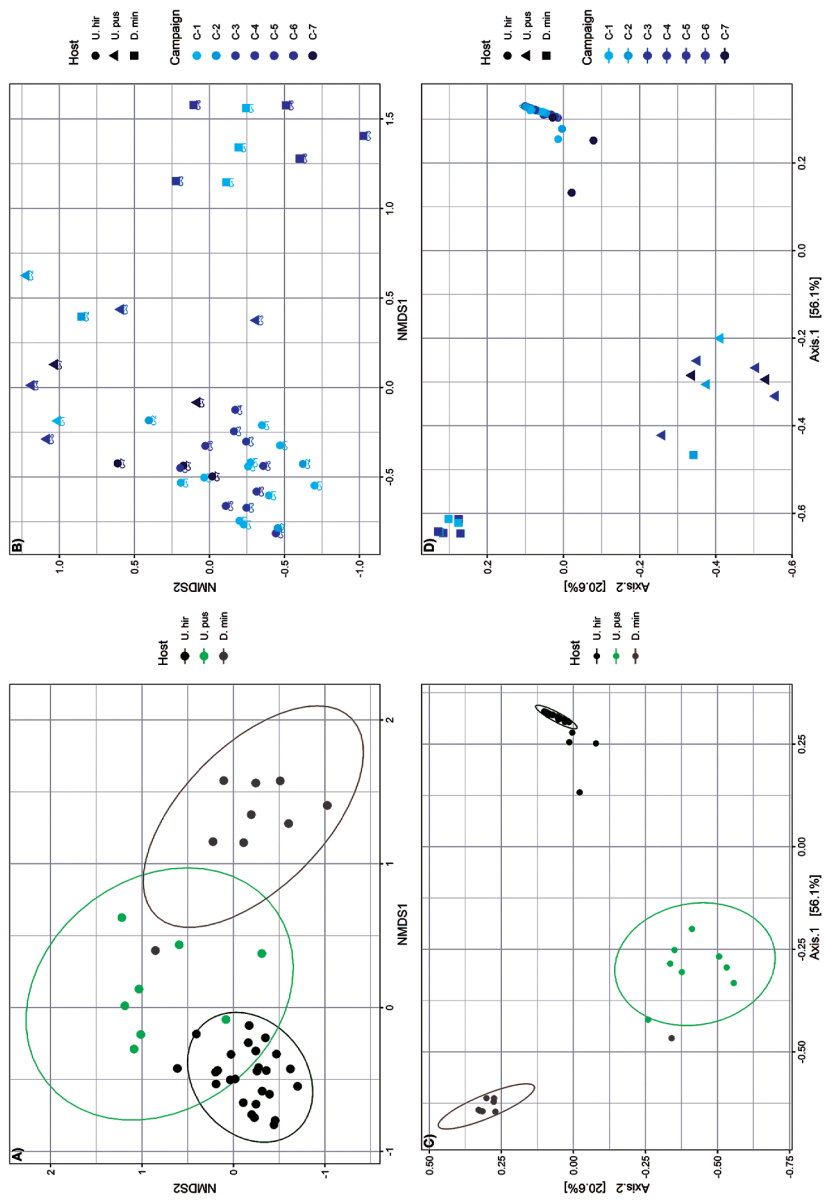

Fig. 6. Visualization of the variation of the mycobiome composition among the three different hosts and different campaigns by NMDS. Based on the Bray-Curtis dissimilarity matrix, the plot shows the relative abundances of OTUs. The function ordinate from the 'phyloseq' package was used to display this plot. Ellipsoids represent a 95% confidence interval surrounding the data points of each factor group. NMDS ordination stress value included

Overall, the results therefore indicate a higher intrageneric mycobiome similarity (Fig. 6C). The diversity shifts were also considered from the perspective of the various campaigns. For this purpose, the campaigns were visualized in addition to the existing division according to host lichen genera and species in a NMDS plot (Fig. 6B and D). Campaign also had no significant effect on fungal community structure (PERMANOVA $p > 0.05$).

DISCUSSION

The presented results demonstrate that sample design, DNA amplification primer selection and other processing parameters were suitably chosen and carried out for both environmental and laboratory work. However, the results suggest that when the study is repeated, a larger scale should be selected to have enough samples accessible for comparison for every host species in every campaign. Here, it would be necessary to account for the potential rejection of individual samples due to low DNA content during DNA extraction, or insufficient reads during downstream data processing, to ensure that there are sufficient samples accessible for data analysis later. In this study, we considered both endolichenic and lichenicolous phylotypes for the assessment of the entire lichen-associated fungal communities and therefore omitted respective methodological precautions, such as comprehensive surface sterilization of the lichen thalli.

Diversity of the mycobiome as a possible indicator for host-lichen specificity

Almost all samples in this study showed that Ascomycota dominate over Basidiomycota and other phyla in the lichen-associated mycobiomes of the analysed host species. Numerous other studies have already established the strong presence of Ascomycota and Basidiomycota in lichens (Smith *et al.* 2020, Stocker-Wörgötter 2008, Tuovinen *et al.* 2019, Wang *et al.* 2016). Presumably, the basidiomycetes found in our study are also yeasts, which, as previously described (Spribille *et al.* 2016), reside asymptomatically in the structurally important lichen cortex. Furthermore, we focused on the different fungal classes present in the different host lichens (Fig. 2). In the host lichen *D. miniatum*, most of the fungal species belonged to the Eurotiomycetes (Ascomycota), suggesting a great ecological importance of this class. However, it should be considered that the host species is a member of the family Verrucariaceae, which belongs to the class Eurotiomycetes. Therefore, we searched for the mycobiont species in the mycobiomes of the host lichens, which are known to lead a lichenized (in this study 'biotrophic mutualistic') lifestyle (Fig. 3, Supplementary Table 4). The results indicate that the host species itself is re-

sponsible for the amount of Eurotiomycetes in *D. miniatum*, which explains the dominance in the different samples. The same is true for *U. hirsuta*, where the dominance of Lecanoromycetes in the current study is because the host species belongs to the Umbilicariaceae family, which in turn belongs to the Lecanoromycetes class. Although the host species could be identified in the samples of *U. pustulata*, no substantial dominance could be detected. In addition, the mycobiome of the *U. pustulata* samples appears to be more diversified compared to the other two host species, possibly due to the spores and other fungal debris on the thallus of the organism. Due to its increased surface area, the shaggy appearance of the lichen thallus makes it easier for other fungal species to colonize this lichen species (Muggia and Grube 2018, Łubek *et al.* 2019). In addition, lichens with umbilical growth forms adhering to the substrate were only specifically selected for the study design to prevent contamination of the lichen-associated mycobiome by substrate-colonizing fungi (Muggia and Grube 2018). Despite the chosen strategy, contamination leading to a shift in the associated mycobiome of the *U. pustulata* samples cannot be completely ruled out.

Another focus of this study was on the lifestyle of the fungi identified at generic level (Fig. 3). Not surprisingly, the biotrophic mutualistic lifestyle dominated in terms of relative abundance in the associated fungi of the host lichen species *U. hirsuta* and *D. miniatum*. As already hinted, this is due to the dominance of the mycobiont, i.e. the host species, in the respective mycobiome. However, it was astonishing that in the mycobiome of *U. pustulata* the saprotrophic fungi even outnumbered the biotrophic mutualistic fungi. Such a constellation indicates that a larger number of specialized lichenicolous fungi may be present here compared to the other two host lichen species (Fernández-Mendoza *et al.* 2017, Hawksworth 2003, Lawrey and Diederich 2003). Overall, mycoparasitic fungi played a subordinate role in the mycobiomes of all three host lichen species. This is not surprising, as we took special care to collect specimens without visible infections when sampling the lichen thalli. A low relative abundance of parasitic fungi was also found for other lichen species in the asymptomatic state (Fernández-Mendoza *et al.* 2017).

This work unequivocally shows that, while exhibiting a great deal of diversity (Figs 1–4), the mycobiomes of various lichen hosts also exhibit a great deal of similarity among samples within the same lichen host species. Generally, lichen hosts belonging to the same species have a more similar microbiome, and consequently mycobiome, than hosts belonging to different species (Ochman *et al.* 2010, Shapira 2016, Sierra *et al.* 2020). In any case, the study design does not allow any individual-related statements, but only species-related inferences, as only fragments of the lichen thalli were collected during sampling. Based on the results of the beta-diversity analysis, it is evident that there is no clear host specificity for the mycobiomes of the three lichen species

(Fig. 6). According to the work of Fernández-Mendoza *et al*. (2017), the lack of host specificity of lichen-associated mycobiomes can be explained by a shared species pool, consisting of generalist, specialized and transient species, from which the communities are subsampled. From the ordination, however, it becomes clear that the mycobiomes of *Umbilicaria* species are more comparable to one another, even if there are also compositional similarities between *U. pustulata* and *D. miniatum*, implying higher intrageneric similarity in lichen-associated mycobiome structure. The similarities described between the two *Umbilicaria* species are in line with the findings of Smith *et al.* (2020) and could be due to common anatomical characteristics, such as growth form, which provide similar micro-niches for lichenicolous fungi. Based on these results and the ordination in the NMDS plot (Fig. 6) it can be assumed that the host *D. miniatum* sample from C-2 is more akin to the samples of *U. pustulata*. A possible explanation for this would be that the host identity was determined incorrectly during environmental monitoring in the field. For this sample, a further laboratory determination would be required to confirm this assertion beyond a reasonable doubt.

Given the observed, albeit minor, differences in lichen-associated mycobiome structure, it is also important to consider the potential impact of parent secondary metabolites. Calvo *et al.* (2002) and Rangel *et al.* (2021) state that the effects of secondary metabolites on community dynamics include facilitating or inhibiting the colonisation of foreign organisms. This in turn may generally help to explain the differences between the various mycobiomes studied, supporting the theory of host species specificity in lichens. Therefore, it is reasonable to assume that lichen species reflect extremely unique microhabitats that are distinguished by a variety of environmental and intrageneric features (Wang *et al.* 2016).

Short-time dynamics in the lichen-associated mycobiome

This study aimed to explore potential alterations in the mycobiome linked with lichens in a brief seven campaigns demarcated in six weeks duration. Up until now, research has solely focused on determining if the lichen-associated mycobiome experiences seasonal variations or changes over the course of several calendar years (Beck *et al.* 2014, Guevara-Araya *et al.* 2022, Oita *et al.* 2021). Depending on the day of sampling, the *D. miniatum, U. hirsuta, and U. pustulata* samples used in this study were separated into campaigns during the spring of 2018. Based on the beta-diversity analysis results acquired from this temporal aspect, all samples and campaigns (C-1 to C-7) indicate that the mycobiomes associated with lichens are not sufficiently altered in the spring within a brief timeframe (Fig. 1). Hence, there is no significant changes over time in the mycobiomes of *D. miniatum, U. hirsuta,* and *U. pustulata.*

Nevertheless, the results of the taxonomic profiling (Fig. 1) and the alpha-diversity analysis (Fig. 4) show that intraseasonal differences are present, albeit small. We argue that these are not directional (deterministic) changes, but short-term oscillatory fluctuations based more on stochastic processes. Presumably, the different host species hardly select specialized (endolichenic) species over time to form distinct 'mature' communities, but rather temporarily harbour generalist and transient species and immature life stages (spores, propagules and mycelia) that are acquired randomly. This way, the host lichens rather represent propagule banks and suboptimal habitats where some species may maintain an immature state within the thalli until they shift to a more suitable habitat (Fernández-Mendoza *et al.* 2017). This might explain the short-time changes in diversity observed in this study, which aligns with reports from the literature stating that time determines intraspecific differences in mycobiome aspects of lichens (Fleischhacker *et al.* 2015, Lawrey and Diederich 2003, Łubek *et al.* 2019).

In our study, comparability between some campaigns is limited due to the number of samples. This is evident, for instance, in C-1 where 10 samples of *U. hirsuta* were compared to one sample of *U. pustulata* and three samples of *D. miniatum*. Since the natural mycobiome of a host lichen species cannot be depicted in a sufficiently representative way based on a single sample, such comparisons are not very meaningful. For campaigns lacking samples for specific host species (e.g. *D. miniatum* is absent from C-4 and C-5), a similar inference should be made. As mentioned above, future studies should therefore increase both sampling and sequencing depth to ensure a sufficient number of samples and thus comparability. However, the study's cautiously anticipated conclusion may be that even this slight short-term dynamic in relative abundances indicates that fungi linked with lichens are communities where there is an intense struggle for resources and space rather than latent organisms.

However, it might be demonstrated that further claims, such as those regarding the significance of lichen substances for mycobiome composition and dynamics as well as the temporal aspect, could be made with a similar study design (sample survey combined with the selected methodological approach) on a wider spectrum of umbilicate lichens on tendentially nutrient-poor silicate sites. Lichen communities containing distinct species from the genera *Ramalina* and *Usnea* would be especially plausible for such a new version of the research methodology.

CONCLUSIONS

Our research demonstrates that the mycobiome associated with lichens in saxicolous species exhibits host-lichen uniqueness and also raises questions about the short-term dynamics of the fungal community within them.

The identification of dominant classes in the mycobiome implies that certain fungi are more suited to, or even in charge of, surviving within particular host species and producing secondary metabolites there. We may conclude from this investigation that the short-term dynamics of the mycobiome offered an intriguing viewpoint on the taxonomy of lichens. However, more detailed research is needed to test this concept in order to draw more definitive conclusions. Longer-term and more rigorous sampling may be a viable solution for this, ensuring that there are enough samples and enabling a comprehensive comparison across multiple six-week time periods. A longer study duration would also allow for various six-week periods in different seasons, which would provide conclusions regarding when the alterations are most prominent in addition to maybe revealing shifts in the mycobiome. Such research would yield valuable information for future inquiries regarding potential community studies as well as further in-depth examinations of certain genera. The investigation of potential temporal alterations in the lichen-associated mycobiome is still an uncharted territory that needs further focus in the future.

FUTURE PERSPECTIVES

Further studies should focus on identifying fungal taxa at a finer taxonomic scale to understand their specific roles and interactions. Investigating the functional roles of dominant and interacting fungal species can provide insights into their ecological roles and contributions to the lichen ecosystem. Exploring the influence of specific environmental factors (e.g. humidity and temperature) on fungal community dynamics can enhance our understanding of their ecological preferences and adaptability. This comprehensive analysis provides a detailed understanding of the fungal communities associated with different host lichens and over temporal scales, offering a foundation for future ecological and conservation studies.

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