Full Paper

New Phytologist

Contrasting co-occurrence patterns of photobiont and cystobasidiomycete yeast associated with common epiphytic lichen species

Kristiina Mark[1,2], Lauri Laanisto[1], C. Guillermo Bueno[3], Ülo Niinemets[1,4], Christine Keller[2] and Christoph Scheidegger[2]

[1]Chair of Crop Science and Plant Biology, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Fr. R. Kreutzwaldi 5, Tartu 51006, Estonia; [2]Department of Biodiversity and Conservation Biology, Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Zürcherstrasse 111, Birmensdorf 8903, Switzerland; [3]Department of Botany, Institute of Ecology and Earth Sciences, University of Tartu, Lai 40, Tartu 51005, Estonia; [4]Estonian Academy of Sciences, Kohtu 6, Tallinn 10130, Estonia

Author for correspondence:
Kristiina Mark
Tel: +37 2731 3259
Email: kristiina.mark@emu.ee

Received: *2 October 2019*
Accepted: *4 February 2020*

New Phytologist (2020) **227:** 1362–1375
doi: 10.1111/nph.16475

Key words: Cystobasidiomycetes, endophytic fungi, interaction network, lichen, lichenicolous fungi, specialization, Trebouxia.

Summary

- The popular dual definition of lichen symbiosis is under question with recent findings of additional microbial partners living within the lichen body. Here we compare the distribution and co-occurrence patterns of lichen photobiont and recently described secondary fungus (Cyphobasidiales yeast) to evaluate their dependency on lichen host fungus (mycobiont).
- We sequenced the nuclear internal transcribed spacer (ITS) strands for mycobiont, photobiont, and yeast from six widespread northern hemisphere epiphytic lichen species collected from 25 sites in Switzerland and Estonia. Interaction network analyses and multivariate analyses were conducted on operational taxonomic units based on ITS sequence data.
- Our study demonstrates the frequent presence of cystobasidiomycete yeasts in studied lichens and shows that they are much less mycobiont-specific than the photobionts. Individuals of different lichen species growing on the same tree trunk consistently hosted the same or closely related mycobiont-specific *Trebouxia* lineage over geographic distances while the cystobasidiomycete yeasts were unevenly distributed over the study area – contrasting communities were found between Estonia and Switzerland.
- These results contradict previous findings of high mycobiont species specificity of Cyphobasidiales yeast at large geographic scales. Our results suggest that the yeast might not be as intimately associated with the symbiosis as is the photobiont.

Introduction

Symbiotic relationships play an important role in the growth, adaptation and evolution of many ecologically successful groups of organisms, despite their complexity and vulnerability (Moran *et al.*, 2005; Rosenberg *et al.*, 2007; Printzen *et al.*, 2013; Bennett & Moran, 2015; Divakar *et al.*, 2015). The stability of a symbiotic organism, often consisting of a multitude of organisms interlinked on the mutualistic–antagonistic continuum of relationships, termed 'holobiont' (Margulis & Fester, 1991; Douglas & Werren, 2016), is determined by the symbionts' interaction intimacy, specialization, and stability of environmental conditions and partner availability (Rafferty *et al.*, 2015; Chomicki & Renner, 2017).

Lichens are a well-known and reasonably well-studied example of obligate fungal symbiosis (Honegger, 2009; Nash III, 2010). A heterotrophic fungal partner, also called the mycobiont, and photosynthetic algae or cyanobacteria (photobiont) together form a common body called the lichen thallus. In this intimate and long-term relationship, the fungus hosts and harvests carbon products from the photobiont, and in return provides water, mineral nutrients, and protection from herbivores and adverse environmental conditions. While the mycobiont can be highly specific in selecting its photobiont (Piercey-Normore & DePriest, 2001; O'Brien *et al.*, 2013; Leavitt *et al.*, 2015; Magain *et al.*, 2016), mycobiont generalists (i.e. with low photobiont specificity) are also common (Wirtz *et al.*, 2003; Muggia *et al.*, 2013; Sadowska-Deś *et al.*, 2014). The exact factors driving photobiont selection are unclear, but are assumed to be determined by phylogenetic specialization, mycobiont reproductive strategy, availability of motile and airborne photobiont cells, as well as key ecological factors for the symbionts (i.e. climate, substrate; Scheidegger, 1985; Marshall & Chalmers, 1997; Yahr *et al.*, 2006; Printzen *et al.*, 2013; Singh *et al.*, 2017; Ertz *et al.*, 2018; Pardo-De la Hoz *et al.*, 2018).

While the dependence of the mycobiont on photosynthetic products is fairly well established, lichens, similarly to plants, host, in addition, a variety of bacteria with unclear significance to

the complex organism (Bates *et al.*, 2011; Partida-Martinez & Heil, 2011). Lichen-specific bacterial communities and various secondary fungal lineages inhabiting lichens have been discovered in next-generation sequencing studies (Grube *et al.*, 2009; Park *et al.*, 2015; Spribille *et al.*, 2016; Tuovinen *et al.*, 2019). These previously unseen symbionts may act as potential functional components in forming and structuring the lichen thallus and/or modulating the response to environmental variables (Liba *et al.*, 2006; Grube *et al.*, 2014; Cernava *et al.*, 2017).

Recently, a specific group of basidiomycetous yeasts, Cyphobasidiales (Pucciniomycotina, Basidiomycota), was detected as a constituent of the lichen microbiome (Spribille *et al.*, 2016). The abundance of the yeast within a lichen was found to correlate with differing concentrations of vulpinic acid (secondary fungal metabolite putatively related to herbivore and microbial defense) in two genetically inseparable epiphytic lichens *Bryoria fremontii* and *Bryoria tortuosa*. The genetic variation of the internal transcribed spacer (ITS) marker of the yeast, sequenced from two boreal lichen species – *Letharia vulpina* sensu lato and *Bryoria fremontii* collected from North America and Europe – was investigated in the same study. In this population genetic analysis, the yeast remained highly mycobiont species-specific, regardless of the origin, indicating a strong specificity on the yeast symbiont. Later, previously unknown cystobasidiomycete symbionts from the Microsporomycetaceae were found in a number of widespread northern hemisphere *Cladonia* species (Černajová & Škaloud, 2019). The consistent presence in addition to phenotypic correlation as shown by Spribille *et al.* 2016 raised speculation of the basidiomycetous yeast being the third mutualistic partner within the lichen complex. While the biological relationship with the lichen host has remained unknown, the possibility of the yeast triggering major phenotypic changes (direct or indirect production of a specific fungal secondary compound that could result in improved resistance to herbivory) or contributing to construction of the symbiotic organism's body were hypothesized (Spribille *et al.*, 2016).

By contrast, other authors (i.e. Millanes *et al.*, 2016; Oberwinkler, 2017) have considered *Cyphobasidium* spp. as lichenincolous fungi that can form galls on lichen thalli and not as an overlooked third mutualistic partner. Furthermore, a recent broad metagenomic study of 339 lichen species collected from the Appalachian Mountains in North America failed to detect basidiomycete yeasts in over 97% of sampled species, questioning their ubiquity in lichens (Lendemer *et al.*, 2019).

With added partners, the complexity of species interactions within such fused supraorganisms rapidly increases. Understanding the network of interactions within a holobiont is not easy, especially for lichens, where experimental data are scarce (Honegger, 2012). Studying lichen symbiont distribution patterns over ecological (biotic and abiotic) space has been a common method to elucidate aspects on partner selectivity and dependency (e.g. Hodkinson *et al.*, 2012; Chagnon *et al.*, 2016; Singh *et al.*, 2017; Rolshausen *et al.*, 2018). Alternatively, network-based approaches are used to explore multispecies interaction patterns as the web architectural structure could potentially elucidate factors responsible for sustaining communities (Dupont *et al.*, 2003; Blüthgen *et al.*, 2006; Thébault & Fontaine, 2010; Chagnon *et al.*, 2018). Guimarães *et al.* (2007) and Pires Mathias & Guimarães (2013) demonstrated the importance of interaction intimacy in shaping network structures. They found that highly intimate interactions, defined by the degree of biological association between partners (whether mutualistic or antagonistic), were less connected with higher modularity and more specialized compared with low-intimacy (nonsymbiotic) webs. Similarly, Chagnon *et al.* (2016) found a strong effect of interaction type on shaping the observed network structure.

Most symbiont association studies focus on a single interaction type at a time, thus making the comparison between different interaction types problematic (Bosch & McFall-Ngai, 2011). Here we used molecular techniques to elucidate and compare the interaction patterns within the lichen holobiont, between the mycobiont, the photobiont, and the cystobasidiomycete yeast. We sequenced the ITS marker for the three symbionts in six frequently co-occurring and common circumboreal to temperate and montane lichen species – *Hypogymnia physodes*, *Hypogymnia tubulosa*, *Lecanora pulicaris*, *Parmelia sulcata*, *Physcia adscendens* and *Pseudevernia furfuracea*.

The aims of this study were: to map symbiont associations within these six lichen species from a very fine ecological scale (a single tree trunk) to a broader geographic scale (two distant areas in Europe); to estimate and compare symbiont specialization with each other and with their lichen host (i.e. evaluate phylogenetic restrictions) through comparative network analyses; and to evaluate whether and to what extent the symbiont distribution patterns are subject to environmental (climatic and spatial) restrictions.

Materials and Methods

Sampling design

Six common epiphytic species – *Hypogymnia physodes*, *Hypogymnia tubulosa*, *Lecanora pulicaris*, *Parmelia sulcata*, *Physcia adscendens*, and *Pseudevernia furfuracea* – were collected systematically in Estonia and Switzerland. At least one specimen per each species was collected from seven trees in every site (Supporting Information Fig. S1). Such a sampling scheme was carried out in a total of 25 collection sites during June 2014: 10 sites in Estonia and 15 in Switzerland, selected to account for environmental variation in each country (Table S1). In the Swiss Alps, specimens were collected in two areas: the river of the Rhône valley in the canton of Valais (sites 1−9) and the river of the Inn valley in the Engadine in the canton of Grisons (sites 10−15; Fig. S1). In both valleys, the sampling was conducted in the valley bottom (*c.* 600–700 m above sea level (asl) in Valais and *c.* 1100–1400 m asl in Engadine) and on the north- and south-facing slopes of each mountain (*c.* 1700–2000 m asl in Valais and *c.* 1900–2100 m asl in Engadine). In Estonia, the specimens were collected in five locations: three in the eastern part of the country (Järvselja (sites 16, 17), Pedassaare (sites 18, 19), and Tallikeste (sites 24, 25)) and two in the western part: Soomaa (sites 20, 21) and Häädemeeste (sites 22, 23). In each location two sites were

sampled, one in a more open forest dominated by Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*), and the other in a denser, mixed spruce forest. Altitude, substrate tree species, visual estimation of canopy coverage, as well as most frequent tree species on site were recorded for each location (Table S1).

Hypogymnia physodes, *H. tubulosa*, *P. sulcata* and *P. furfuracea* specimens were identified in the field to species level. *Physcia adscendens* specimens were collected together with *P. tenella* as the two species are inseparable when juvenile. We do not attempt to differentiate the two species and consider them as a species complex, as the specimens cannot be separated by ITS sequence alone (Lohtander *et al.*, 2000). *Lecanora* specimens were identified in the field to genus level, followed by detailed morphological, chemical and anatomical characterization in the laboratory. Chemical analyses were performed with thin layer chromatography (TLC) using solvent systems A, B and C (Culberson & Ammann, 1979; White & James, 1985).

In total, 838 lichen specimens were collected from 25 sites – 157 *H. physodes*, 146 *H. tubulosa*, 160 *P. sulcata*, 86 *P. adscendens/tenella*, 155 *P. furfuracea*, and 134 *L. pulicaris* aggr. specimens. Subsequent *Lecanora* morphological/anatomical, and chemical investigations clarified that *Lecanora* sampling included five *Lecanora* morphospecies (Methods S1). Only the most frequently occurring *Lecanora* species – *L. pulicaris* – forming a monophyletic clade on the ITS gene tree (Fig. S2), was included in subsequent data analyses, together with the five macrolichen species. All specimens were stored at the Swiss Federal Research Institute WSL.

Molecular methods

About 2–5 mg of healthy and visually uncontaminated lichen thallus was lyophilized and then pulverized in a stainless bead mill Retsch MM300 (Düsseldorf, Germany) for 1 min at 30 Hz. The full genomic DNA from each lichen thallus was extracted using the DNEasy 96 Plant Kit (Qiagen) following the manufacturer's DNEasy 96 protocol for lyophilized material for initial mycobiont-photobiont genetic variation screening (48 specimens, eight individuals per species from different Swiss sites). The rest of the DNA extractions (790) were done using an automated and customized protocol with LGC sbeadex™ plant kit (LGC Genomics, Berlin, Germany) on a KingFisher™ Flex Purification System (Thermo Scientific, Dreieich, Germany). The full nuclear rDNA ITS was amplified for the lichenized fungus, green algal photobiont, and cystobasidiomycete yeast symbionts. The ITS region of the nuclear ribosomal RNA cistron has been used as the primary fungal barcode marker (Schoch *et al.*, 2012) but has also been used effectively in delimiting closely related green algal lineages in lichens (Helms *et al.*, 2001; Leavitt *et al.*, 2015; Moya *et al.*, 2017). Fungal-specific primers ITS1F (Gardes & Bruns, 1993) and ITS4 (White *et al.*, 1990) were used to amplify the mycobiont, algal specific nr-SSU-1780-5′ (Piercey-Normore & DePriest, 2001) and ITS4T (Kroken & Taylor, 2000) to amplify the trebuxioid photobiont, and *Cyphobasidium*-specific primers ITS_symrho_1F and LR0_symrho_R (Spribille *et al.*, 2016) were used to amplify the yeast symbiont. Each PCR reaction (12.5 µl) consisted of a JumpStart REDTax ReadyMix (Sigma Aldrich), 5µM of the forward and reverse primers and 0.8 µl of template DNA.

The thermal cycle conditions for the mycobiont and the photobiont followed the same protocol: initial denaturation at 94°C for 2 min, then 10 cycles of denaturation for 30 s at 94°C, primer annealing for 45 s at 62°C, and extension for 45 s at 72°C, then 25 cycles of denaturation for 30 s at 94°C, primer annealing for 45 s at 52°C, and extension for 45 s at 72°C, and finally extension for 10 min at 72°C. The PCR program for the yeast was: initial denaturation at 94°C for 2 min, 40 cycles of denaturation for 30 s at 94°C, primer annealing for 45 s at 49°C, and extension for 45 s at 72°C, and final extension for 10 min at 72°C.

The PCR products were visualized on 1% agarose gel stained with EZ-Vision In-Gel Solution 10,000X (Amresco, Solon, OH, USA) and purified with Exo-SAP (Fermentas, St Leon-Rot, Germany) treatment. The forward and reverse strands of the ITS markers were amplified using the same primers with BigDye Terminator v.3.1 cycle sequencing mix (Applied Biosystems, Foster City, CA, USA), following purification with BigDye xTerminator Purification Kit (Applied BioSystems). Sanger sequencing of the symbionts, carried out in the ETH Zurich Genetic Diversity Centre (GDC, Zürich, Switzerland) with a 3730 DNA Analyzer and a 3130xl DNA Analyzer (Applied Biosystems), was chosen to obtain high-quality ITS sequences of the dominant symbionts of the three focal organisms within the holobiont (Paul *et al.*, 2018).

ITS phylogenies and OTU delimitation

The sequence traces were observed and complementary strands aligned using Geneious v.7.1.9 (Kearse *et al.*, 2012). Contig identities were checked against the GenBank nucleotide database using the MegaBlast function (Madden, 2002). High-quality sequences from each species were aligned using the Mafft v.7.017 (Katoh *et al.*, 2002) with G-INS-I algorithm and 200PAM/k = 2 scoring matrix. All unique mutations were manually validated against their sequencing chromatograms before using the data matrices for downstream analyses. Haplotypes for all three symbionts were calculated in DnaSP v.6.10.03 (Rozas *et al.*, 2017). However, owing to the high number of haplotypes and haplotypic singletons within datasets, especially within cystobasidiomycete data (Table S2), operational taxonomic units (OTUs) were calculated for photobiont and yeast datasets.

We preferred evolutionarily meaningful OTUs over simple distance-based clusters (Nguyen *et al.*, 2016). To assign sequences into clusters, we used the ultrametric tree-based methods 'complete' and 'UPGMA' and the sequence-based and iteratively likelihood-maximizing 'ML' method of the 'IdClusters' function from the package Decipher (Wright, 2016). Additionally, sequence clusters sharing common evolutionary history were evaluated in Cluster Picker v.1.2 (Ragonnet-Cronin *et al.*, 2013) using Bayesian ITS gene trees constructed from Mafft alignments and inferred from Beast v.1.8.4 (Drummond *et al.*, 2012) using CIPRES Science Gateway v.3.3 (Miller *et al.*, 2010). Cluster Picker uses a clade support and within-clade

genetic distance thresholds to identify clusters. We applied an *a posteriori* probability of ≥ 95% and genetic distance threshold of 0.03 to identify strongly supported monophyletic clades within the photobiont and yeast datasets. The cutoff value of 0.03 was also used in the 'IdCluster' function. Bayesian trees were estimated from two independent Bayesian/Markov chain Monte Carlo analyses run for each symbiont dataset; each analysis was run for 200 million generations, sampling every 10 000th state. Convergence of analyses was assessed in TRACER v.1.5 and a maximum clade credibility trees with posterior probabilities (PP) were calculated from a set of trees excluding 10% burn-in in TREEANNOTATOR, and visualized in FIGTREE (Rambaut, 2009).

The final OTUs were assigned using a conservative consensus approach, where an OTU was assigned when at least three out of four methods supported this group. The mycobiont was treated as the traditional morphological species, as no conflict was found between OTU delimitation and the six focal species (each morphospecies corresponded to one OTU, except for the *P. adscendens/tenella* complex which formed a single OUT; Table S2). The full list of specimens together with haplotype and OTU assignments are given in Table S3.

Interaction network analyses

Bipartite network analyses were conducted to understand whether there is variation in symbiont specialization to sequenced partners and whether there are differences in photobiont and yeast network structures with the mycobiont. Only data from the six focal species evenly distributed across the sampling sites were used for network analyses.

Symbiont interaction is defined here as a co-occurrence of symbionts in a lichen thallus. Bipartite quantitative interaction matrices were constructed between lichen species and photobiont OTUs, between lichen species and cystobasidiomycete yeast OTUs, and between photobiont and cystobasidiomycete yeast OTUs using the number of thalli where symbionts were found co-occurring as the frequency measure. Thus, our datasets provided weighted estimates of interactions between the symbionts. We calculated the species-level specialization index (d') for each taxon with the function 'dfun' in the R package BIPARTITE (Dormann *et al.*, 2008). The differences between mean symbiont specializations for all interaction types were tested using Wilcoxon rank sum test but considering only taxa with $n \geq 5$ occurrences because of potential overestimation of specialization for rare taxa (Vázquez & Aizen, 2003).

Connectance (i.e. realized proportion of possible links), mean number of links per species, number of compartments (i.e. independent subsets of the web), degree of nestedness/segregation, modularity, and network-level of specialization (H2′) were used as the basic properties to characterize and compare photobiont and yeast interaction with the mycobiont.

Connectance, number of links and compartments are widely used as straightforward metrics in characterizing network α-properties (Delmas *et al.*, 2019). Nestedness and modularity are other commonly used and extensively evaluated network characteristics with different metrics described for their quantification (e.g. Almeida-Neto *et al.*, 2008; Fortuna *et al.*, 2010; Thébault & Fontaine, 2010). To measure segregation into modules, we chose a more recently developed node overlap and segregation measure (NOS; Strona & Veech, 2015). This symmetric measure can distinguish segregated and nested networks and ranges from −1 (complete segregation) to 1 (maximum node overlap), where values close to 0 indicate randomness. Specialization values (both H2′ and d') indicate the degree of deviation of a species or a network of interactions from a random interaction pattern, ranging from 0 for extreme generalization to 1 for perfect specialization. These specialization metrics are robust to varying species frequencies and sampling intensities when comparing networks (Blüthgen *et al.*, 2006). For comparing network raw properties, similar bipartite quantitative interaction matrices as described earlier were constructed for each site. Network structure metrics were calculated with R v.3.5.2 (R Core Team, 2013) using the functions 'networklevel' and 'nos' in the package BIPARTITE (Dormann *et al.*, 2008; Strona & Veech, 2015).

Variance partitioning analyses

First, we explored the OTU distribution in geographical space with point-biserial correlation between relative sampling frequency and country of origin. Then, ecological statistical analyses were used to explain whether and to what extent the photobiont and the cystobasidiomycete yeast co-occurrences are driven by abiotic and biotic factors. Only data from the six focal species evenly distributed across the sampling sites were used for variance partitioning analyses. The response matrices were defined by the photobiont or yeast OTU occurrence within each lichen sample. Two explanatory biotic matrices – the host/mycobiont species and the secondary symbiont species (either the photobiont or the yeast, depending which symbiont dataset was investigated) – and different environmental variables were set for each sample. These environmental variables included on-site estimated vegetation properties potentially affecting microclimatic conditions for epiphytes: forest type, substrate tree species, and canopy cover, and macroclimatic parameters. All available climatic variables, average monthly climatic data from 1970 until 2000, including temperature- and precipitation-related bioclimatic variables (BIO1–BIO19), water vapor pressure, wind speed and solar radiation for the 25 sampling sites were extracted from the WORLDCLIM v.2 30 s resolution datasets (Fick & Hijmans, 2017; http://worldclim.org/version2, accessed 14 July 2018). Principal component analysis (PCA) of the climatic variables of each collection site was carried out using the 'prcomp' function in the STATS core package in R (Zuur *et al.*, 2009) and the first and second principal components (PC1 and PC2) were used for subsequent variance partitioning analyses (Fig. S3).

Our sampling design described the geographical space with four different levels of spatial variation – country ($n = 2$; Estonia and Switzerland), geographic region ($n = 3$; Valais, Grisons, Estonia), site ($n = 25$) and tree ($n = 175$). We first investigated whether any of these levels were completely redundant and could create collinearity issues. We used the OTU occurrence response matrices in variance partitioning analyses to estimate unique and

shared explained variance of each spatial level. To account for the sampling design in variance partitioning analyses we performed a redundancy analysis (RDA) with OTU occurrences as response matrices and sites and tree identity variables as predictors (country and region did not explain any additional variation; Methods S1). The residuals of each correspondent RDA model were used as response matrices in the final variance partitioning analyses. As collinear variables can bias the coefficient and variance partition estimations, we dropped the redundant factors correlated with the response matrices with a Pearson correlation > 0.6 and a variance inflation factor (VIF) > 3 (Methods S1; Zuur *et al.*, 2009; Dormann *et al.*, 2013). The partition of variance used was based on sequential partial RDAs (Legendre & Legendre, 2012), running with the photobiont and yeast response matrices using the 'varpart' function in the R package VEGAN (Oksanen *et al.*, 2013). The impact of each (noncollinear) environmental factor was further explored by analyzing their marginal effects in a permutation test for RDA under reduced model (Methods S1). The significance of factors was tested using an ANOVA type III.

In addition, the significance of lichen medullary chemotype (i.e. composition of consistent secondary metabolites as determined by TLC analyses) on photobiont and yeast OTU distribution was tested using the 'adonis' function in the package VEGAN. The presence of cortical substances was not used for analyses as all of the studied species produce the same substance, atranorin, in the cortex. Horn–Morista distances were calculated from symbiont OTU occurrence matrices and the specific chemotype was defined for each sample (Table S3), but excluding individuals where no medullary compounds were detected (some *Lecanora* and all *Physcia*).

Results

Molecular data

Sequencing the three symbionts of 838 lichen specimens resulted in total of 2117 ITS contigs (786 for mycobiont, 830 for photobiont, and 500 for yeast). Acquiring good-quality sequences was most successful for the photobiont (99% of the samples) and almost as good for the mycobiont (94%). Sequencing success for cystobasidiomycete yeast was comparatively lower (60%), usually as a result of a poor-quality mixed chromatogram signal. Sequencing of the yeast was most successful for *H. physodes* ($n = 121$, 77%), *P. furfuracea* ($n = 114$, 74%), and *H. tubulosa* ($n = 105$, 72%), but less successful for *Physcia* ($n = 19$, 22%), *Lecanora* ($n = 59$, 44%), and *Parmelia* ($n = 83$, 52%). Newly generated ITS sequences are deposited in GenBank under accession codes MN654478 – MN654900 (Table S4).

Symbiont OTU diversity and phylogeny

Photobiont OTU delimitation resulted in a total of 14 clusters, including one singleton (i.e. PC09), all belonging to the genus *Trebouxia* (Clorophyta; Fig. S4). The six targeted lichen species hosted eight photobiont OTUs: PC02, PC05, PC06, PC07, PC11, PC12, PC13, PC14 (Fig. 1a). The most frequent photobiont, PC14 ($n = 421$), together with three smaller clusters (PC11–PC13) were related to *Trebouxia simplex* (BP = 1). Most other sequences (forming clusters PC01–PC07) were related to *Trebouxia impressa* (BP = 0.99). Additionally, *T. decolorans* and *T.* sp. were found from nontargeted *Lecanora* species; *T. decolorans* (PC08, PC09) was found associated with *L. carpinea* sensu lato, and *T.* sp. (PC10) with *L. argentata* and *L. chlarotera*.

Yeast OTU delimitation detected 27 units (Fig. S5). The six targeted lichen species hosted 23 yeast OTUs, of which eight had $n \geq 5$ (YC01a, YC01b, YC04, YC07, YC08a, YC08b, YC08c, YC13), and eight singleton clusters. Basidiomycete yeasts sequenced in the framework of our study all belonged to the fungal class Cystobasidiomycetes, with the majority within the order Cyphobasidiales (YC01–YC08; total $n = 450$; Fig. 1b). Within the Cyphobasidiales, three major groups were detected: group 1 included YC01a and YC01b and was closely related to *Cyphobasidium hypogymniicola* (BP = 1, total $n = 141$); group 2 included YC02–YC04 (BP = 0.97, total $n = 171$); group 3 included closely related subgroup of YC08a, YC08b, and YC08c (BP = 1, total $n = 107$), and more distantly related clades YC05, YC06, and YC07 (BP = 0.18, total $n = 29$) – all related to *Cyphobasidium usneiicola* (see Fig. S4 for details). Other yeast clusters were at lower frequency, fell outside of Cyphobasidiales and were related to different Cystobasidiomycetes groups (total $n = 58$): YC09–YC13 clustered within Erythrobasidiacea and were most closely related to *Hasegawazyma* spp. (BP = 0.99, total $n = 29$); YC14 was closely related to *Buckleyzyma aurantiaca* (BP = 1, $n = 6$); YC18–YC21 (total $n = 20$) were related to *Microsporomyces spp.* The yeast YC18 was closely related to *Microsporomyces pini* (BP = 1, $n = 3$), while YC21c ($n = 3$) corresponded to the recently described *Lichenozyma pisutiana*.

Symbiont specialization and distribution among lichen hosts

The *T. 'impressa'* group included closely related clusters PC02, PC05 and PC06 that were all associated with *P. adscendens/tenella*. *Parmelia sulcata* photobiont PC07 was closely related, but distinct from the *T. 'impressa'* from *Physcia*. *Hypogymnia physodes*, *H. tubulosa*, *P. furfuracea* and *L. pulicaris* hosted photobionts from the *T. 'impressa'* group (Fig. 1a). Among the photobionts of the six lichen species, all OTUs besides PC13 and PC14 were associated with a single lichen species. PC13 was shared among *L. pulicaris* ($n = 36$), *H. tubulosa* ($n = 22$), *H. physodes* ($n = 7$) and *P. furfuracea* ($n = 4$). PC14 was shared among *P. furfuracea* ($n = 150$), *H. physodes* ($n = 135$), and *H. tubulosa* ($n = 134$).

None of the more frequently occurring yeast taxa were uniquely sequenced from a single lichen species (Fig. 1b). Only YC01a, YC07, YC08b, and YC13 were recovered from two to three different lichen species, while all other were shared among five to six lichen species. At the same time, some yeast taxa showed a higher degree of host preference, with strong associations of YC01a and YC01b to *Hypogymnia* species (100% and 89% of sequences, respectively), YC7, YC8a and YC8c to *P. furfuracea* (96%, 81%, and 53% of sequences, respectively), and YC13 to *P. sulcata* (82%).

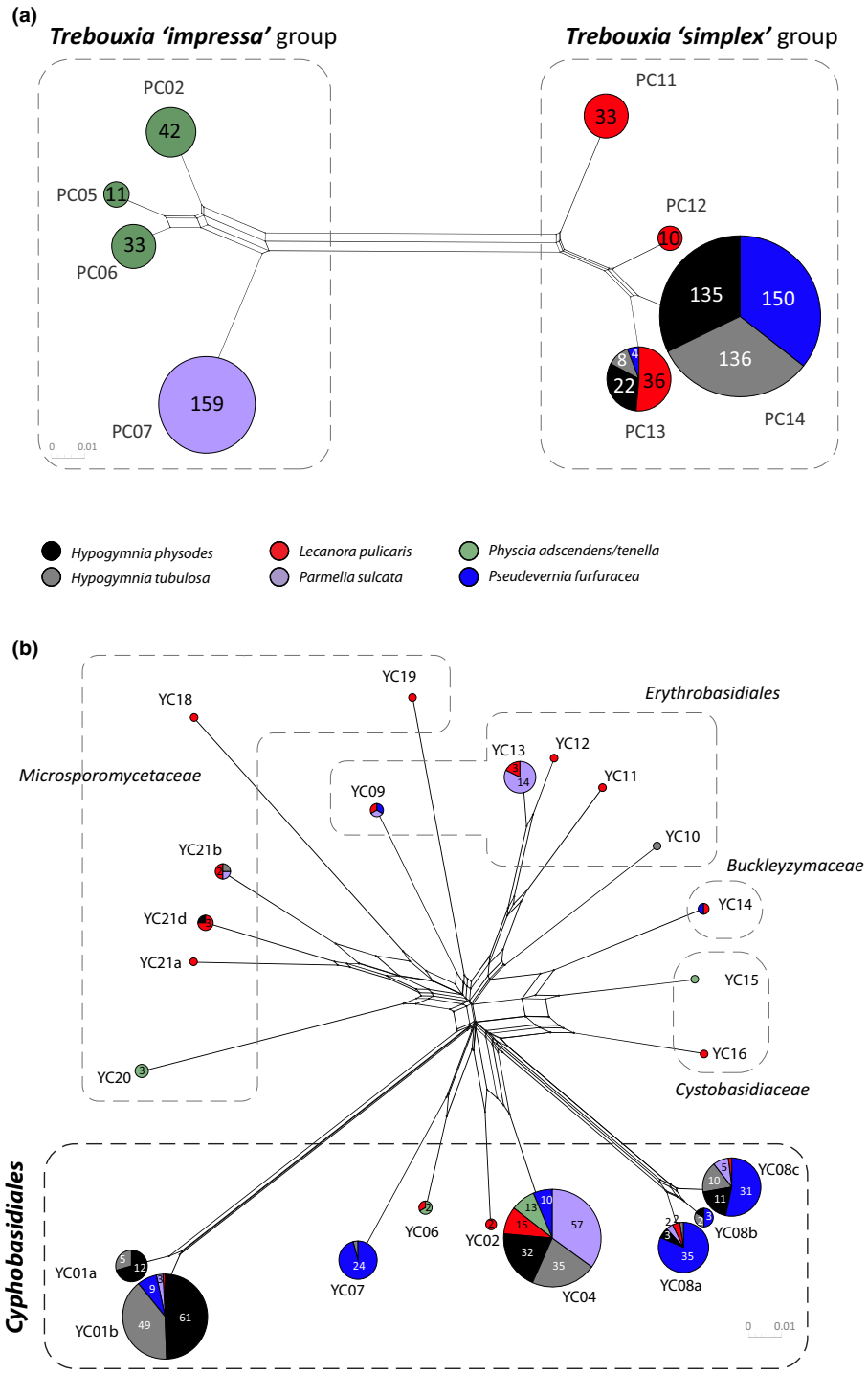

Fig. 1 Photobiont (a) and yeast (b) operational taxonomic unit (OTU) phylogenetic networks constructed in SplitsTree v.5 (Huson, 1998; Huson & Bryant, 2005). The Hamming distances method (Hamming, 1950) was used to obtain distance matrices, and weighted splits networks were constructed using the 'EqualAngleConvexHull' algorithm (Dress & Huson, 2004). Owing to computational constraints, only a single internal transcribed spacer sequence per OTU was used in the alignments. Node edges represent OTUs and are sized using a natural logarithmic scale in proportion to sample size (n; node diameter = $2 \log_e n$). Pie charts reflect proportions of different lichen hosts for symbiont OTUs. *N* values for each proportion are given within or next to the node. Bar indicates genetic distances between nodes.

On average, the photobionts were significantly more specialized on the mycobiont than were the cystobasidiomycete yeast taxa ($P = 0.003$; Table 1). Yeast specialization to photobiont was the lowest of the interaction combinations. Network-level specialization was also higher for the photobiont than for the yeast network ($P < 0.001$, Fig. 2f). With an average of 0.95 across the sites, photobiont interactions with lichen host were almost entirely specialized, while in yeast, interaction specialization was significantly lower,

with an among-site average of 0.64. Interaction web analyses further revealed that mycobiont–photobiont interactions had lower web connectance, a lower number of links per species, and were segregated into more compartments compared with the mycobiont–yeast webs (all $P < 0.001$; Fig. 2; Table S5).

Symbiont distribution between sampling sites and in climatic space

The photobionts PC14 (from *P. furfuracea H. physodes* and *H. tubulosa*) and PC07 (from *P. sulcata*) were evenly distributed across all sites. *Lecanora pulicaris* and *Physcia* photobionts showed some degree of spatial/climatic differentiation (Fig. 3a; also see later).

Cystobasidiomycetes yeast taxa were generally more unevenly distributed between the sampling sites (Fig. 3b). Switzerland and Estonia showed distinct yeast communities with different dominant OTUs across their respective sites – YC01b in Estonia and YC04 in Switzerland ($r = -0.934$ and $r = 0.892$, respectively, with both $P < 0.0001$). YC08c occurred at high frequency in Swiss mountain sites, was less common in valley bottom sites, and sequenced only once from Estonia ($r = 0.626$, $P = 0.001$). YC08b was only found in Switzerland, in Valais mountain sites 2 and 7 ($r = 0.236$, $P =$ ns). On the other hand, YC13 and YC07 were only common in Estonia (YC13, $r = -0.580$, $P = 0.002$; YC07, $r = -0.560$, $P = 0.004$). YC08a occurred at approximately equal frequencies in both countries ($r = 0.030$, $P = 0.887$).

The study sites grouped into five bioclimatic groups (Fig. 4), separating Estonia (sites 16–25), Valais valley bottom (sites 1, 3, 6), Valais mountain (sites 2, 4, 5, 7, 8, 9), Grisons valley bottom (sites 12 and 15), and Grisons mountain (sites 10, 11, 13, 14). PC1 and PC2 accounted for 60.1% and 23.8% of the variability of the climatic data, and were most highly correlated with annual precipitation (BIO12, $r^2 = 0.97$) and mean temperature of coldest quarter (BIO11, $r^2 = 0.97$), respectively.

Of the *Physcia* photobionts, PC02 was positioned at the drier end of the precipitation gradient with most samples in Valais valley bottom and Estonia, while PC05 occurred more frequently in Swiss mountain sites with higher precipitation (Fig. 4a). Of the *L. pulicaris* photobionts, PC11 had a preference for drier sites (predominantly found in Estonia), while PC13 and PC12 occurred at greater frequency in Swiss mountain forests.

Cyphobasidiales YC01 (including YC01a, predominantly from Swiss valley bottom sites 6, 12 and 15, and YC01b, predominantly from Estonia) was distributed at the drier end of the precipitation gradient (Fig. 4b). YC08b and YC8c had distribution centers towards wetter climates in Swiss mountain sites (i.e. infrequent or not present in Swiss valley bottom sites or Estonia).

Multivariate analyses

Variance partitioning analyses confirmed the photobiont's strong dependency on the mycobiont, while the host species did not play as important a role for the cystobasidiomycete yeast (Table 2). The role of environment (i.e. vegetation parameters and microclimate) was very low in both photobiont and yeast, yet still made a minor but significant contribution to photobiont data explanation. The yeast data showed a weak relationship with all the studied variables; the host species and photobiont taxa were significant but also with very low explanatory power. The effect of chemotype on structuring symbiont communities was significant for photobiont ($r^2 = 0.80$ at $P = 0.028$) when permutations were calculated within lichen species, while there was no such evidence for the yeast data ($r^2 = 0.13$ at $P = 0.196$).

Discussion

Symbiont association and distribution analyses revealed contrasting patterns between the trebouxioid photobiont and cystobasidiomycete yeast in common co-occurring lichen species. The photobiont from the genus *Trebouxia* was found to be highly specific towards the mycobiont and its distribution was mainly determined by the fungal host species. By contrast, the cystobasidiomycete yeast was significantly less specialized towards the lichen host. Some degree of geographical and/or climatic effect on yeast community structure could be noted as contrasting communities were found between Estonia and Switzerland.

It is expected that highly dependent (including mutualistic) symbiotic relationships are more specialized with the network structured into compartments while neutral interactions show lower amounts of biotic specialization (Guimarães *et al.*, 2007). Contrasting interaction patterns, as seen from bipartite network analyses where photobiont interaction with the mycobiont was found to be significantly more segregated and specialized

Table 1 Photobiont and cystobasidiomycete yeast operational taxonomic unit (OTU) specialization (d') towards host lichen species (mycobiont) and the alternate symbiont (either yeast or photobiont).

Photobiont OTU	Mycobiont d'	Yeast d'	Yeast OTU	Mycobiont d'	Photobiont d'
PC02	0.75	0.44	YC01a	0.24	0.07
PC05	0.52	0.38	YC01b	0.31	0.14
PC06	0.70	0.33	YC04	0.17	0.14
PC07	1.00	0.34	YC07	0.43	0.13
PC11	0.72	0.58	YC08a	0.32	0.07
PC12	0.53	0.49	YC08b	0.01	0.08
PC13	0.35	0.10	YC08c	0.12	0.05
PC14	0.88	0.29	YC13	0.43	0.45
Average ± SD	0.68 ± 21	0.37 ± 0.15		0.26 ± 0.13	0.14 ± 0.13

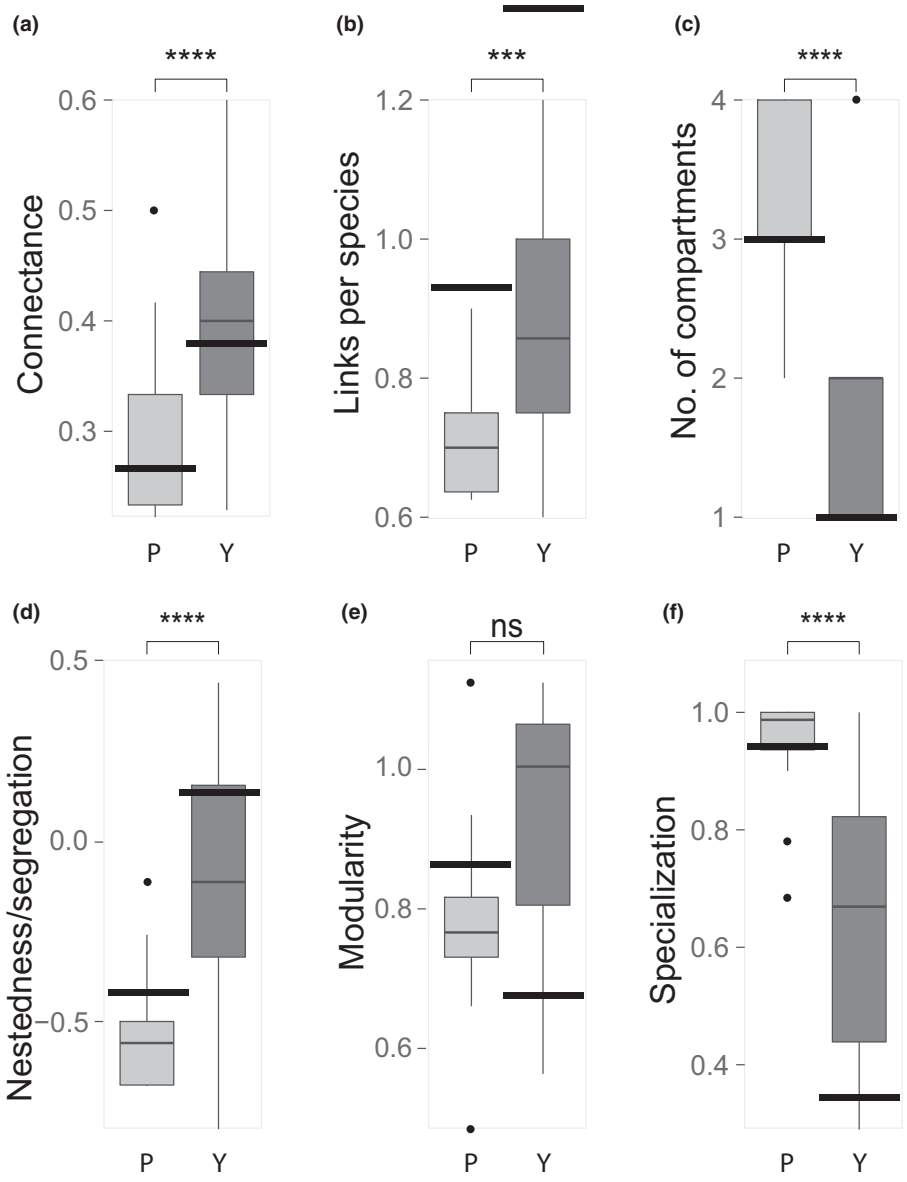

Fig. 2 Bar plots of the six studied interaction web parameters – (a) connectance, (b) links per species, (c) number of compartments, (d) nestedness/segregation (NOS), (e) modularity (Mod), and (f) specialization (H2) – indicating the averages and variation of calculated metrics between sites ($n = 25$). Box plot elements are defined as follows: centerline, median; box limits, upper and lower quartiles; whiskers, 1.5 × interquartile range; points, outliers. The bold black line marks the parameter value for the overall web, including interactions from all sites. Differences between the photobiont–host (P) and yeast–host (Y) webs were tested with Wilcoxon rank-sum test and significant differences in the means are indicated at the top of the chart: ****, $P < 0.0001$; ***, $P < 0.001$; ns (not significant), $P > 0.05$.

compared with the yeast network, could indicate that the interaction with yeast is less intimate and not as obligate at the fine phylogenetic scale.

The general mycobiont–photobiont associations revealed in this study are in accordance with previous findings (Beck *et al.*, 1998; Dahlkild *et al.*, 2001; Hauck *et al.*, 2007). The majority of detected photobiont taxa were highly specific (phylogenetic range of association) towards their host and only two taxa were found associated with more than one mycobiont. The mycobionts (with the exception of *P. sulcata*) were associated with two or three phylogenetically closely related photobiont lineages. This, however, does not necessarily conflict with strong specificity and intimacy at the physiological scale as it is likely that closely related photobiont lineages also portray similar physiological activities and thus there should not be a clear difference in lichen phenotype and fitness when switching to a phylogenetically close symbiont. Among the studied lichen species that coinhabit the same tree trunks, *P. furfuracea*, *H. physodes* and *H. tubulosa* shared a photobiont pool (partially also with *L. pulicaris*) and were never associated with *T. impressa* lineages from the nearby growing *Parmelia* or *Physcia*, while *P. sulcata* and *P. adscendens/tenella* were never found hosting the *T. simplex* photobionts. These results demonstrate that a strong phylogenetic specificity in lichen mutualistic symbiosis is already effective at a very fine microhabitat scale but

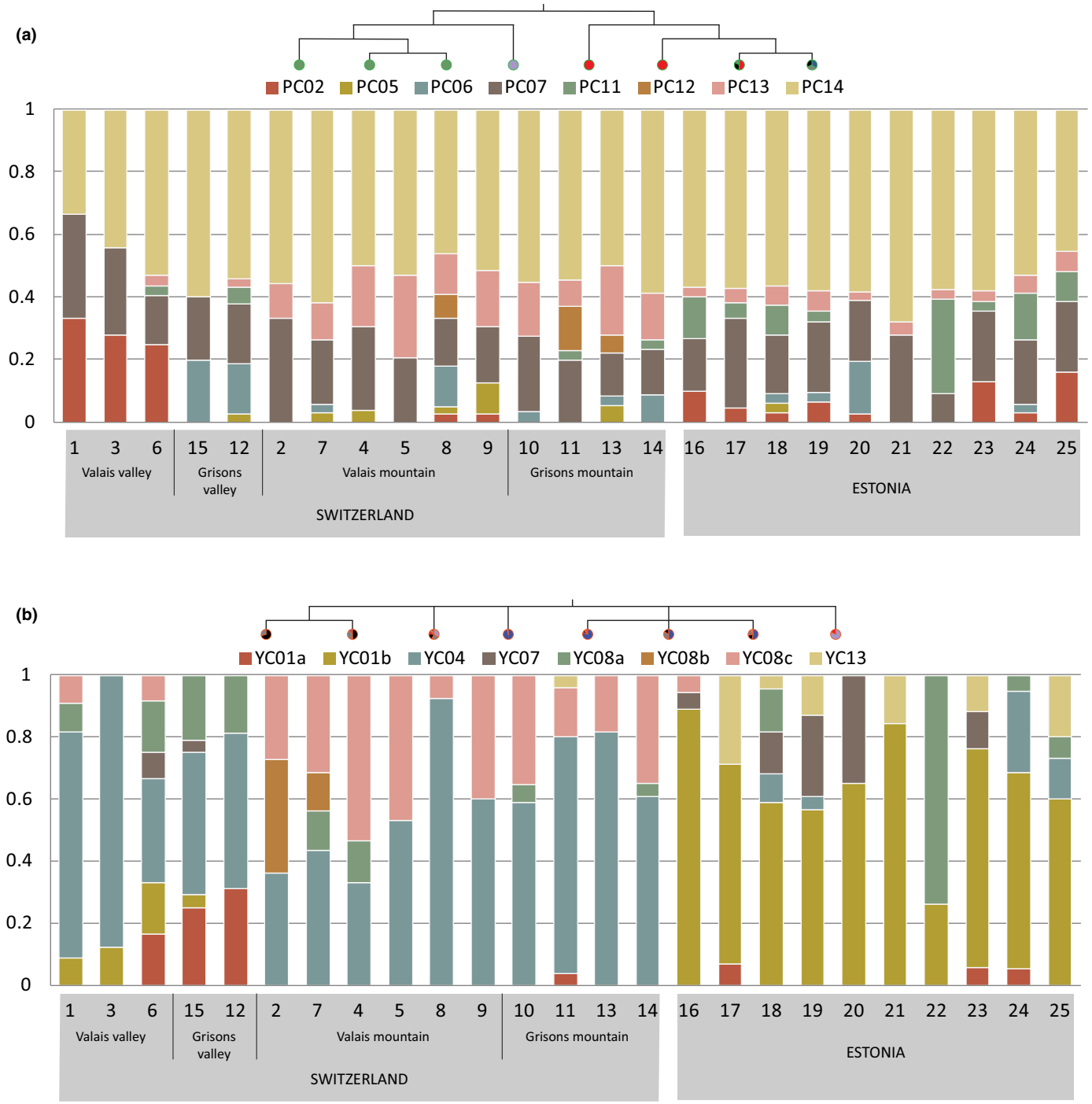

Fig. 3 (a, b) Proportional frequencies of most common (sampling frequency $n \geq 5$) operational taxonomic units (OTUs) of photobiont (a) and cystobasidiomycete yeast (b) across 25 sampling sites in Switzerland and Estonia. Sites are divided into bioclimatic groups according to Fig. 4. Color-referenced OTUs are given at the top of the bar plot and their phylogenetic relationships are indicated with the dendrogram inferred from BEAST internal transcribed spacer gene tree. Pie charts at the dendrogram tips refer to host associations as defined in Fig. 1.

the same patterns were found to extend over the sampling area to wider geographic scales.

Lecanora pulicaris showed the highest genetic and distributional differences between the photobionts – PC11 was the most frequently occurring photobiont for *L. pulicaris* in Estonia, while PC12 was only found in the Swiss Alps, whereas PC13 was found in both countries but occurred at greater frequency in Switzerland. It is possible that the mycobiont-driven selection for symbiont is determined by photobiont ecological specialization in combination with symbiont interaction efficiency (i.e. the fraction of carbon that the photobiont can deliver; Peksa & Škaloud, 2011; Werth & Sork, 2014; Rolshausen *et al.*, 2018). *Lecanora pulicaris* is also the only species studied here that is assumed to reproduce only sexually via aposymbiotic propagules (ascospores) and its horizontal

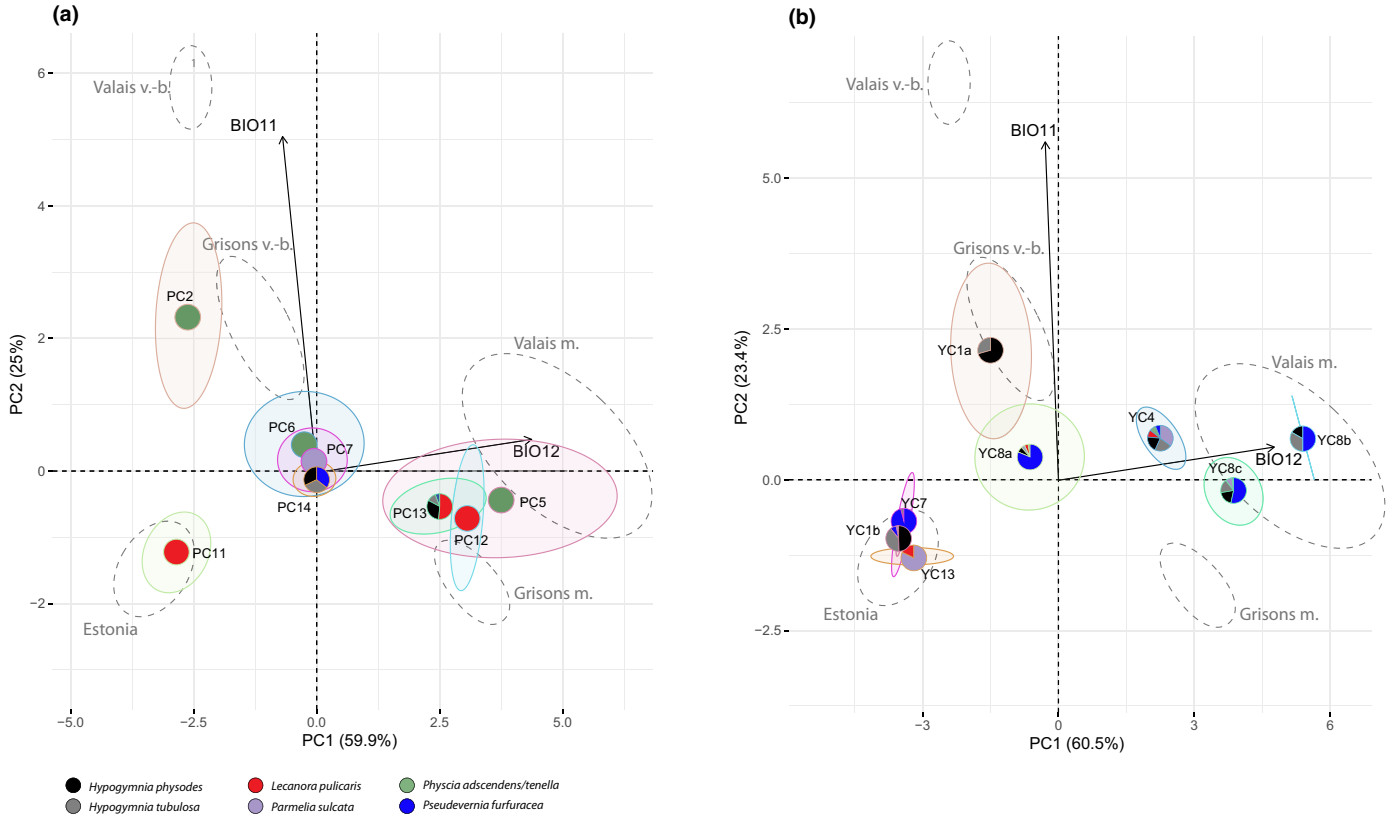

Fig. 4 Photobiont (a) and cystobasidiomycete yeast (b) operational taxonomic unit (OTU) principal component analysis plots with 95% confidence ellipses around group means (colored circles in the ellipse center), with the means being additionally color-coded by their host association. Only data for most common OTUs ($n \geq 5$) are shown. Dashed ellipses refer to the five bioclimatic groups of sites: Estonia (sites 16–25), Valais valley bottom ('Valais v.-b.'; sites 1, 3, 6), Valais mountain ('Valais m.'; sites 2, 4, 5, 7, 8, 9), Grisons valley bottom ('Grisons v.-b.'; sites 12, 15), and Grisons mountain ('Grisons m.'; sites 10, 11, 13, 14). In the PCA plot, only variables that are most correlated with the PCA dimensions are shown – mean yearly precipitation (BIO12) and average temperature of the coldest month (BIO11).

Table 2 Results of variance partitioning (based on redundancy analysis (RDA) and partial RDAs) showing the individual and combined contributions of explanatory variables for the photobiont and cystobasidiomycete yeast operational taxonomic unit distribution.

	Photobiont		Yeast	
Factor	df	Adj. r^2	df	Adj. r^2
Mycobiont species	5	**0.409**	2	**0.088**
Environment	22	**0.010**	19	−0.027
Photobiont/yeast	24	**0.041**	7	**0.092**
Host and environment	0	−0.006	0	−0.005
Environment and photobiont/yeast	0	−0.007	0	−0.006
Host and photobiont/yeast	0	0.140	0	−0.016
Host and environment and photobiont/yeast	0	−0.040	0	−0.001
Residuals		0.452		0.874

Adjusted square-*r* values in bold indicate for significant contribution. See text for a detailed explanation of variables.

symbiont transmission should favor an increase in the taxonomic range of compatible photobiont partners (Smith & Douglas, 1987; Steinová *et al.*, 2019). Geographical variation was also observed for *P. adscendens/tenella* photobionts. Here, the genetically more distant clade PC02 was predominantly found in Estonia and the Swiss Valais valley bottom. Whether such regional and/or climatic specialization of *Physcia* photobionts reflects the ecological preferences of a photobiont or the host phylogenetic or distributional constraints needs further investigation by first resolving the *P. adscendens* species complex.

None of the more frequently occurring (i.e. $n \geq 5$) cystobasidiomycete taxon was exclusive to one lichen species, indicating that there might not be a strict vertical transmission of the yeast. According to current knowledge, the photobiont and Cyphobasidiales yeast are described as living inside the lichen body mainly in a vegetative state (Nash III, 2010; Spribille *et al.*, 2016). In that case, both types of bionts within the lichen host are expected to be transmitted similarly with similar dispersal vectors. In asexually reproducing lichens, vertical transmission with lichen asexual propagules (isidia or soredia) should concurrently disperse all partners of the holobiont to a new location (Aschenbrenner *et al.*, 2014). The differences between symbiont distributions for vegetatively reproducing lichens should then result from any disparity in either the environmental factors or the biotic interactions. Although the dispersal mode is presumably the same (co-dispersion in asexual, symbiotic lichen propagule), the two symbionts showed different distribution patterns. Contrasting cystobasidiomycete yeast communities were

found between Estonia and Switzerland, with some taxa being common in Estonia and others in Switzerland, while the host specificity was maintained low in both countries. This does not correspond with Spribille *et al.* (2016), who reported a high degree of specificity over an even larger geographic distance (285 *Letharia vulpina* and 52 *Bryoria fremontii* samples from North America and Europe) than what was analyzed in our study. This could imply potential selectivity at the regional scale and would assume lower phylogenetic constraints compared with the photobiont.

Černajová & Škaloud (2019) speculated that cystobasidiomycete lineages might have a certain degree of specificity at higher taxonomic ranks and that Microsporomycetaceae could be specific to the genus *Cladonia* and Cyphobasidiales specific to Parmeliaceae. Based on our data, such conclusions cannot be unequivocally drawn, as Cyphobasidiales taxa (YC01–YC08) as well as yeasts from Microsporomycetaceae (YC18–YC21) were found to be associated with Parmeliaceae, Physciaceae and Lecanoraceae. It should, however, be noted that the cystobasidiomycete yeast taxa showed a variable degree of host specificity (e.g. the YC04 being associated almost equally with all six host species and YC01a and YC01b being predominantly associated with only *Hypogymnia* species) and also some preferences towards macroclimatic conditions. For example, YC01 was almost exclusively found at sites with lower precipitation, but climatic factors remained insignificant in variance partitioning analysis for both photobiont and yeast. As the current dataset is limited at the macroclimatic scale, strong conclusions about the role of climatic conditions on the distribution of cystobasidiomycete yeast cannot be made.

Spribille *et al.* (2016) found the yeast frequency to be correlated with concentrations of vulpinic acid, a specific lichen secondary metabolite. Our data included lichens with different medullary compounds (physodic and physodalic acids in *Hypogymnia* species, salazinic acid in *P. sulcata*, and olivetoric or physodic acid in *P. furfuracea*) but no variation in cortical substances (all species produce atranorin). We found no effect of medullary chemotype in structuring yeast community and thus direct production of these metabolites by the yeasts seems unlikely. The effect on photobiont in our data was minor but significant. With the exception of *P. furfuracea*, with its two distinct chemotypes, the majority of the studied species do not portray intraspecific chemical variation and thus the separation of metabolite effect from species or phylogenetic effect is not possible here. Whether yeast partial host specialization, as can be seen with YC01 and its preferential association with the two *Hypogymnia* species, implies an adaptation to lichen microhabitat conditions (e.g. adaptation to specific secondary metabolites or thallus cortex structure) or to a stronger degree of facilitation in the interaction remains an open question.

Partial specialization could also be the result of passive vertical codispersion with no facilitative effect between the partners. The generally lower host specificity compared with several known lichen mycoparasites (e.g. Tremellomycetes species; Lawrey & Diederich, 2003; Werth *et al.*, 2013; Millanes *et al.*, 2014) and with obligate photosynthetic partners (as shown in this paper) does indeed imply a lower interaction dependency, if not passive codispersion. The relationships between the partners do not necessarily need to stay constant during the life cycle. Indeed, as pointed out by Oberwinkler (2017), many mycoparasites are in their initial stage haploid and can form yeast colonies, while in the later state they can produce hyphae in special mycoparasite-initiated galls where ultimately sexual spores are produced. These galls have also been documented for Cyphobasidiales taxa (Millanes *et al.*, 2016), and thus the dimorphic life cycle of this fungus must be possible; however, none of these structures were observed in the studied samples.

Lichen-inhabiting basidiomycetes were previously mostly known from Tremellomycetes and Agaricomycetes (Diederich, 1996; Millanes *et al.*, 2011; Diederich *et al.*, 2018). Lichen-inhabiting Pucciniomycotina have been less studied and, until recently, only two taxa were known (i.e. *Cyphobasidium usneiicola* and *Cyphobasidium hypogymniicola*; Millanes *et al.*, 2016). The lichen-specific range of taxa in Pucciniomycotina was significantly expanded by Spribille *et al.* (2016), who introduced nine lichen-associated Cystobasidiomycetes clades, with seven clades within the order Cyphobasidiales. While the majority of the detected taxa within this study belong to Cyphobasidiales, we also found several clades with relatively high frequency outside of this potentially lichen-specific order. This indicates that lichen-associated cystobasidiomycetes (in either neutral or facilitative interaction) could be more diverse than previously considered, as also pointed out by Černajová & Škaloud (2019). As the general biology and wider distribution of these fungi is still poorly known, a more in-depth search from different hosts and their basidia-forming galls could help to better characterize their life cycle. It is possible that similar studies on other fungi (e.g. Abrothallales) could yield comparative results; however, many groups of lichenicolous fungi are highly specialized and each group would need to be separately studied.

At the same time, 100% PCR success confirms the presence of the Cyphobasidiales and/or related Cystobasidiomycetes fungal lineages within the studied lichens and implies that these fungi are potentially a highly diverse group of lichen-associated fungi, although perhaps not present or as dominantly present in all lichen species (as shown by Lendemer *et al.*, 2019). The comparatively low sequencing success of the yeast (60% vs 99% in photobiont), resulting from a mixed signal in Sanger sequencing chromatograms, contrasts with the results of photobionts, where in almost all samples only a single photobiont lineage was detected using the same DNA extraction. This could indicate the presence of multiple Cyphobasidiales (and related) fungal lineages within a lichen thallus. The presence of multiple yeast taxa within a lichen cortex was shown by Tuovinen *et al.* (2019). Sharing a habitat can be another indicator of a lower degree of functional specialization, as it is unlikely that they could fulfill the same symbiotic function.

In conclusion, Cyphobasidiales fungi have so far only been detected in lichens or some groups of lichens (as discussed in Spribille *et al.*, 2016; Černajová & Škaloud, 2019; Lendemer *et al.*, 2019, and also here). This could indicate their strong preference for lichens as a habitat, yet other organisms as hosts have not been specifically screened. At the same time, our results do not confirm their strong specialization to host species over wide geographical areas, as reported by Spribille *et al.* (2016). Rather, even at a

relatively limited geographic scale within Europe, we found distinct contrasting yeast communities and these yeasts were significantly less specialized to host species compared with the known obligate symbiont, the photobiont. This correlates with previously detected low specificity of lichen microbiomes (Fernández-Mendoza *et al.*, 2017). Further studies are needed to clarify the wider distribution of this fungal group and dependence on abiotic factors as well as the interaction specifics within lichen.

Acknowledgements

This project was funded by the Rectors' Conference of the Swiss Universities (CRUS) through the SCIEX-NMSch Scientific Exchange Programme (project SCIEX-Licode) and the Swiss National Science Foundation (projects 31003A-105830 and 31003A-127346 to CS). Authors from the Estonian institutions were supported by the Estonian Research Council through personal research funding projects PRG537 to ÜN, PUT1409 to LL and IUT20-28 to CGB, and by the European Commission through the European Regional Development Fund (Center of Excellence EcolChange).

Author contributions

KM and CS conceived and designed the research study; KM collected the specimens and carried out molecular laboratory works; CK conducted chemical and anatomical investigations of the specimens; KM, LL and CGB designed and performed the analyses. KM, LL, CGB, ÜN, CK, and CS all contributed to writing the paper.

ORCID

C. Guillermo Bueno https://orcid.org/0000-0002-7288-2271
Lauri Laanisto https://orcid.org/0000-0003-2215-7298
Kristiina Mark https://orcid.org/0000-0002-3229-3122
Ülo Niinemets https://orcid.org/0000-0002-3078-2192
Christoph Scheidegger https://orcid.org/0000-0003-3713-5331

References

Almeida-Neto M, Guimaraes P, Guimaraes PR Jr, Loyola RD, Ulrich W. 2008. A consistent metric for nestedness analysis in ecological systems: reconciling concept and measurement. *Oikos* **117**: 1227–1239.

Aschenbrenner IA, Cardinale M, Berg G, Grube M. 2014. Microbial cargo: do bacteria on symbiotic propagules reinforce the microbiome of lichens? *Environmental Microbiology* **16**: 3743–3752.

Bates ST, Berg-Lyons D, Lauber CL, Walters WA, Knight R, Fierer N. 2011. A preliminary survey of lichen associated eukaryotes using pyrosequencing. *Lichenologist* **44**: 137–146.

Beck A, Friedl T, Rambold G. 1998. Selectivity of photobiont choice in a defined lichen community: inferences from cultural and molecular studies. *New Phytologist* **139**: 709–720.

Bennett GM, Moran NA. 2015. Heritable symbiosis: the advantages and perils of an evolutionary rabbit hole. *Proceedings of the National Academy of Sciences, USA* **112**: 10169–10176.

Blüthgen N, Menzel F, Blüthgen N. 2006. Measuring specialization in species interaction networks. *BMC Ecology* **6**: 9.

Bosch TC, McFall-Ngai MJ. 2011. Metaorganisms as the new frontier. *Zoology* **114**: 185–190.

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 Biology* **123**: 625–637.

Cernava T, Erlacher A, Aschenbrenner IA, Krug L, Lassek C, Riedel K, Grube M, Berg G. 2017. Deciphering functional diversification within the lichen microbiota by meta-omics. *Microbiome* **5**: 82.

Chagnon PL, Magain N, Miadlikowska J, Lutzoni F. 2018. Strong specificity and network modularity at a very fine phylogenetic scale in the lichen genus *Peltigera*. *Oecologia* **187**: 767–782.

Chagnon P-L, U'Ren JM, Miadlikowska J, Lutzoni F, Elizabeth Arnold A. 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 SS. 2017. Partner abundance controls mutualism stability and the pace of morphological change over geologic time. *Proceedings of the National Academy of Sciences, USA* **114**: 3951–3956.

Culberson CF, Ammann K. 1979. Standardmethode zur dünnschichtchromatographie von flechtensubstanzen. *Herzogia* **5**: 24.

Dahlkild Å, Källersjö M, Lohtander K, Tehler A. 2001. Photobiont diversity in the Physciaceae (Lecanorales). *Bryologist* **104**: 527–537.

Delmas E, Besson M, Brice MH, Burkle LA, Dalla Riva GV, Fortin MJ, Gravel D, Guimarães PR Jr, Hembry DH, Newman EA. 2019. Analysing ecological networks of species interactions. *Biological Reviews* **94**: 16–36.

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

Diederich P, Lawrey JD, Ertz D. 2018. The 2018 classification and checklist of lichenicolous fungi, with 2000 non-lichenized, obligately lichenicolous taxa. *Bryologist* **121**: 340–426.

Divakar PK, Crespo A, Wedin M, Leavitt SD, Hawksworth DL, Myllys L, McCune B, Randlane T, Bjerke JW, Ohmura Y. 2015. Evolution of complex symbiotic relationships in a morphologically derived family of lichen-forming fungi. *New Phytologist* **208**: 1217–1226.

Dormann CF, Elith J, Bacher S, Buchmann C, Carl G, Carré G, Marquéz JRG, Gruber B, Lafourcade B, Leitão PJ. 2013. Collinearity: a review of methods to deal with it and a simulation study evaluating their performance. *Ecography* **36**: 27–46.

Dormann CF, Gruber B, Fründ J. 2008. Introducing the bipartite package: analysing ecological networks. *R News* **8**: 2413793.

Douglas AE, Werren JH. 2016. Holes in the hologenome: why host-microbe symbioses are not holobionts. *MBio* **7**: e02099.

Dress AW, Huson DH. 2004. Constructing splits graphs. *IEEE/ACM Transactions on Computational Biology and Bioinformatics* **1**: 109–115.

Drummond AJ, Suchard MA, Xie D, Rambaut A. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* **29**: 1969–1973.

Dupont YL, Hansen DM, Olesen JM. 2003. Structure of a plant–flower-visitor network in the high-altitude sub-alpine desert of Tenerife, Canary Islands. *Ecography* **26**: 301–310.

Ertz D, Guzow-Krzemińska B, Thor G, Łubek A, Kukwa M. 2018. Photobiont switching causes changes in the reproduction strategy and phenotypic dimorphism in the Arthoniomycetes. *Scientific Reports* **8**: 4952.

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

Fick SE, Hijmans RJ. 2017. WorldClim 2: new 1-km spatial resolution climate surfaces for global land areas. *International Journal of Climatology* **37**: 4302–4315.

Fortuna MA, Stouffer DB, Olesen JM, Jordano P, Mouillot D, Krasnov BR, Poulin R, Bascompte J. 2010. Nestedness versus modularity in ecological networks: two sides of the same coin? *Journal of Animal Ecology* **79**: 811–817.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113–118.

Grube M, Cardinale M, de Castro Jr JV, Müller H, Berg G. 2009. Species-specific structural and functional diversity of bacterial communities in lichen symbioses. *ISME Journal* **3**: 1105.

Grube M, Cernava T, Soh J, Fuchs S, Aschenbrenner I, Lassek C, Wegner U, Becher D, Riedel K, Sensen CW *et al.* 2014. Exploring functional contexts of symbiotic sustain within lichen-associated bacteria by comparative omics. *ISME Journal* **9**: 412.

Guimarães PR Jr, Rico-Gray V, Oliveira Paulo S, Izzo TJ, dos Reis SF, Thompson JN. 2007. Interaction intimacy affects structure and coevolutionary dynamics in mutualistic networks. *Current Biology* **17**: 1797–1803.

Hamming RW. 1950. Error detecting and error correcting codes. *Bell System Technical Journal* **29**: 147–160.

Hauck M, Helms G, Friedl T. 2007. Photobiont selectivity in the epiphytic lichens *Hypogymnia physodes* and *Lecanora conizaeoides*. *Lichenologist* **39**: 195–204.

Helms G, Friedl T, Rambold G, Mayrhofer H. 2001. Identification of photobionts from the lichen family Physciaceae using algal-specific ITS rDNA sequencing. *Lichenologist* **33**: 73–86.

Hodkinson BP, Gottel NR, Schadt CW, Lutzoni F. 2012. Photoautotrophic symbiont and geography are major factors affecting highly structured and diverse bacterial communities in the lichen microbiome. *Environmental Microbiology* **14**: 147–161.

Honegger R. 2009. Lichen-forming fungi and their photobionts. In: Deising HB, eds. *Plant relationships. The Mycota vol. 5*. Berlin, Germany: Springer, 307–333.

Honegger R. 2012. The Symbiotic phenotype of lichen-forming ascomycetes and their endo- and epibionts. In: Hock B, eds. *Fungal associations. The Mycota vol. 9*. Berlin, Germany: Springer, 287–339.

Huson DH. 1998. SplitsTree: analyzing and visualizing evolutionary data. *Bioinformatics* **14**: 68–73.

Huson DH, Bryant D. 2005. Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution* **23**: 254–267.

Katoh K, Misawa K, Ki Kuma, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**: 3059–3066.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C *et al.* 2012. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**: 1647–1649.

Kroken S, Taylor JW. 2000. Phylogenetic species, reproductive mode, and specificity of the green alga *Trebouxia* forming lichens with the fungal genus *Letharia*. *Bryologist* **103**: 645–660.

Lawrey JD, Diederich P. 2003. Lichenicolous fungi: interactions, evolution, and biodiversity. *Bryologist* **106**: 80–121.

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

Legendre P, Legendre LF. 2012. *Numerical ecology*. Oxford, UK: Elsevier.

Lendemer JC, Keepers KG, Tripp EA, Pogoda CS, McCain CM, Kane NC. 2019. A taxonomically broad metagenomic survey of 339 species spanning 57 families suggests cystobasidiomycete yeasts are not ubiquitous across all lichens. *American Journal of Botany* **106**: 1090–1095.

Liba CM, Ferrara FIS, Manfio GP, Fantinatti-Garboggini F, Albuquerque RC, Pavan C, Ramos PL, Moreira-Filho CA, Barbosa HR. 2006. Nitrogen-fixing chemo-organotrophic bacteria isolated from cyanobacteria-deprived lichens and their ability to solubilize phosphate and to release amino acids and phytohormones. *Journal of Applied Microbiology* **101**: 1076–1086.

Lohtander K, Källersjö M, Moberg R, Tehler A. 2000. The family Physciaceae in Fennoscandia: Phylogeny inferred from ITS sequences. *Mycologia* **92**: 728–735.

Madden T. 2002. The BLAST sequence analysis tool. In: McEntyre J, Ostell J, eds. *The NCBI handbook*. Bethesda, MD, USA: National Center for Biotechnology Information, Chapter 16: 1–15.

Magain N, Miadlikowska J, Goffinet B, Sérusiaux E, Lutzoni F. 2016. Macroevolution of specificity in cyanolichens of the genus *Peltigera* section *Polydactylon* (Lecanoromycetes, Ascomycota). *Systematic Biology* **66**: 74–99.

Margulis L, Fester R. 1991. *Symbiosis as a source of evolutionary innovation: speciation and morphogenesis*. Cambridge, MA, USA: MIT Press.

Marshall WA, Chalmers MO. 1997. Airborne dispersal of Antarctic terrestrial algae and cyanobacteria. *Ecography* **20**: 585–594.

Millanes AM, Diederich P, Ekman S, Wedin M. 2011. Phylogeny and character evolution in the jelly fungi (Tremellomycetes, Basidiomycota, Fungi). *Molecular Phylogenetics and Evolution* **61**: 12–28.

Millanes AM, Diederich P, Wedin M. 2016. *Cyphobasidium* gen. nov., a new lichen-inhabiting lineage in the *Cystobasidiomycetes* (*Pucciniomycotina*, *Basidiomycota*, Fungi). *Fungal Biology* **120**: 1468–1477.

Millanes AM, Truong C, Westberg M, Diederich P, Wedin M. 2014. Host switching promotes diversity in host-specialized mycoparasitic fungi: uncoupled evolution in the *Biatoropsis-Usnea* system. *Evolution* **68**: 1576–1593.

Miller MA, Pfeiffer W, Schwartz T.2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. *Gateway Computing Environments Workshop* (GCE): 1–8.

Moran NA, Tran P, Gerardo NM. 2005. Symbiosis and insect diversification: an ancient symbiont of sap-feeding insects from the bacterial phylum Bacteroidetes. *Applied and Environmental Microbiology* **71**: 8802–8810.

Moya P, Molins A, Martínez-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, Vancurova L, Škaloud P, Peksa O, Wedin M, Grube M. 2013. The symbiotic playground of lichen thalli–a highly flexible photobiont association in rock-inhabiting lichens. *FEMS Microbiology Ecology* **85**: 313–323.

Nash TH III. 2010. *Lichen biology*, 2nd edition reprint. New York, NY, USA: Cambridge University Press.

Nguyen N-P, Warnow T, Pop M, White B. 2016. A perspective on 16S rRNA operational taxonomic unit clustering using sequence similarity. *NPJ Biofilms and Microbiomes* **2**: 16004.

Oberwinkler F. 2017. Yeasts in Pucciniomycotina. *Mycological Progress* **16**: 831–856.

O'Brien HE, Miadlikowska J, Lutzoni F. 2013. Assessing population structure and host specialization in lichenized cyanobacteria. *New Phytologist* **198**: 557–566.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'hara R, Simpson GL, Solymos P, Stevens MHH, Wagner H. 2013. Package 'vegan'. Community ecology package, version 2: 1–295.

Pardo-De la Hoz CJ, Magain N, Lutzoni F, Goward T, Restrepo S, Miadlikowska J. 2018. Contrasting symbiotic patterns in two closely related lineages of trimembered lichens of the genus *Peltigera*. *Frontiers in Microbiology* **9**: 2770–2770.

Park CH, Kim KM, Elvebakk A, Kim OS, Jeong G, Hong SG. 2015. Algal and fungal diversity in Antarctic lichens. *Journal of Eukaryotic Microbiology* **62**: 196–205.

Partida-Martinez LP, Heil M. 2011. The microbe-free plant: fact or artifact? *Frontiers in Plant Science* **2**: 100.

Paul F, Otte J, Schmitt I, Dal Grande F. 2018. Comparing Sanger sequencing and high-throughput metabarcoding for inferring photobiont diversity in lichens. *Scientific Reports* **8**: 8624.

Peksa O, Škaloud P. 2011. Do photobionts influence the ecology of lichens? A case study of environmental preferences in symbiotic green alga *Asterochloris* (Trebouxiophyceae). *Molecular Ecology* **20**: 3936–3948.

Piercey-Normore MD, DePriest PT. 2001. Algal switching among lichen symbioses. *American Journal of Botany* **88**: 1490–1498.

Pires Mathias M, Guimarães Paulo R. 2013. Interaction intimacy organizes networks of antagonistic interactions in different ways. *Journal of the Royal Society Interface* **10**: 20120649.

Printzen C, Domaschke S, Fernández-Mendoza F, Pérez-Ortega S. 2013. Biogeography and ecology of *Cetraria aculeata*, a widely distributed lichen with a bipolar distribution. *MycoKeys* **6**: 33.

R Core Team.2013. *R: A language and environment for statistical computing*. [WWW document] URL www.r-project.org [accessed 8 February 2019].

Rafferty NE, CaraDonna PJ, Bronstein JL. 2015. Phenological shifts and the fate of mutualisms. *Oikos* **124**: 14–21.

Ragonnet-Cronin M, Hodcroft E, Hué S, Fearnhill E, Delpech V, Brown AJL, Lycett S. 2013. Automated analysis of phylogenetic clusters. *BMC Bioinformatics* **14**: 317.

Rambaut A. 2009. *FigTree v1.3.1. Program distributed by the author*. Edinburgh, UK: Institute of Evolutionary Biology, University of Edinburgh.

Rolshausen G, Dal Grande F, Sadowska-Deś AD, Otte J, Schmitt I. 2018. Quantifying the climatic niche of symbiont partners in a lichen symbiosis indicates mutualist-mediated niche expansions. *Ecography* **41**: 1380–1392.

Rosenberg E, Koren O, Reshef L, Efrony R, Zilber-Rosenberg I. 2007. The role of microorganisms in coral health, disease and evolution. *Nature Reviews Microbiology* **5**: 355.

Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, Sánchez-Gracia A. 2017. DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular Biology and Evolution* **34**: 3299–3302.

Sadowska-Deś AD, Dal Grande F, Lumbsch HT, Beck A, Otte J, Hur J-S, Kim JA, Schmitt I. 2014. Integrating coalescent and phylogenetic approaches to delimit species in the lichen photobiont *Trebouxia*. *Molecular Phylogenetics and Evolution* **76**: 202–210.

Scheidegger C. 1985. Systematische Studien zur Krustenflechte *Anzina carneonivea* (Trapeliaceae, Lecanorales). *Nova Hedwigia: Zeitschrift fur Kryptogamenkunde* **41**: 191–218.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Consortium FB. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences, USA* **109**: 6241–6246.

Singh G, Dal Grande F, Divakar PK, Otte J, Crespo A, Schmitt I. 2017. Fungal–algal association patterns in lichen symbiosis linked to macroclimate. *New Phytologist* **214**: 317–329.

Smith DC, Douglas AE. 1987. *The biology of symbiosis*. Baltimore, MD, USA: Edward Arnold (Publishers) Ltd.

Spribille T, Tuovinen V, Resl P, Vanderpool D, Wolinski H, Aime MC, Schneider K, Stabentheiner E, Toome-Heller M, Thor G *et al.* 2016. Basidiomycete yeasts in the cortex of ascomycete macrolichens. *Science* **353**: 488.

Steinová J, Škaloud P, Yahr R, Bestová H, Muggia L. 2019. Reproductive and dispersal strategies shape the diversity of mycobiont-photobiont association in *Cladonia* lichens. *Molecular Phylogenetics and Evolution* **134**: 226–237.

Strona G, Veech JA. 2015. A new measure of ecological network structure based on node overlap and segregation. *Methods in Ecology and Evolution* **6**: 907–915.

Thébault E, Fontaine C. 2010. Stability of ecological communities and the architecture of mutualistic and trophic networks. *Science* **329**: 853–856.

Tuovinen V, Ekman S, Thor G, Vanderpool D, Spribille T, Johannesson H. 2019. Two basidiomycete fungi in the cortex of wolf lichens. *Current Biology* **29**: 476–483.

Vázquez DP, Aizen MA. 2003. Null model analyses of specialization in plant–pollinator interactions. *Ecology* **84**: 2493–2501.

Werth S, Millanes AM, Wedin M, Scheidegger C. 2013. Lichenicolous fungi show population subdivision by host species but do not share population history with their hosts. *Fungal Biology* **117**: 71–84.

Werth S, Sork VL. 2014. Ecological specialization in *Trebouxia* (Trebouxiophyceae) photobionts of *Ramalina menziesii* (Ramalinaceae) across six range-covering ecoregions of western North America. *American Journal of Botany* **101**: 1127–1140.

White F, James PW. 1985. *A new guide to microchemical techniques for the identification of lichen substances*. London, UK: British Lichen Society.

White TJ, Bruns T, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR protocols: a guide to methods and applications*. New York, NY, USA: Academic Press, 315–322.

Wirtz N, Lumbsch HT, Green TA, Türk R, Pintado A, Sancho L, Schroeter B. 2003. Lichen fungi have low cyanobiont selectivity in maritime Antarctica. *New Phytologist* **160**: 177–183.

Wright ES. 2016. Using DECIPHER v2. 0 to analyze big biological sequence data in R. *R Journal* **8**: 352.

Yahr R, Vilgalys R, DePriest PT. 2006. Geographic variation in algal partners of *Cladonia subtenuis* (Cladoniaceae) highlights the dynamic nature of a lichen symbiosis. *New Phytologist* **171**: 847–860.

Zuur A, Ieno EN, Walker N, Saveliev AA, Smith GM. 2009. *Mixed effects models and extensions in ecology with R*. New York, NY, USA: Springer-Verlag, New York.

See also the Commentary on this article by Hawksworth & Grube, **227**: 1281–1283.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Locations of sampling sites in Estonia and in two valleys of Switzerland: Valais and Grisons. The sampling scheme used at each site is illustrated on the figure.

Fig. S2 Maximum likelihood ITS phylogeny of all collected *Lecanora* specimens together with ITS sequences of available, closely related taxa from GenBank.

Fig. S3 Principal component analysis (PCA) of climatic variables for the 25 collection sites extracted from the WorldClim v.2 datasets.

Fig. S4 Bayesian 50% consensus tree of available *Trebouxia* ITS sequences inferred by Beast v.1.8.4 together with OTU clustering results.

Fig. S5 Bayesian 50% consensus tree of available Cystobasidiomycetes ITS sequences inferred by Beast v.1.8.4 together with OTU clustering results.

Methods S1 Sampling efficiency, methods used for construction of extended ITS phylogenies for *Lecanora* (Fig. S2), *Trebouxia* (Fig. S4) and Cystobasidiomycete yeasts (Fig. S5), and for estimating explained variance by spatial levels and individual biotic and abiotic variables.

Table S1 Collection site locations, ecology and climatic values extracted from WorldClim v.2 30 s resolution datasets.

Table S2 Number of samples, ITS sequences, haplotypes and OTUs used.

Table S3 The full list of specimens together with haplotype and OTU assignments, host tree number, host tree species and lichen chemotype.

Table S4 GenBank accession codes for each haplotype, and associated species and specimens.

Table S5 Photobiont and cystobasidiomycete yeast interaction web parameter values.

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.