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Metagenomic data reveal diverse fungal and algal communities associated with the lichen symbiosis

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Abstract

Lichens have traditionally been considered the symbiotic phenotype from the interactions of a single fungal partner and one or few photosynthetic partners. However, lichen symbioses have been shown to be far more complex and may include a wider range of other interacting organisms, including non-photosynthetic bacteria, accessory fungi, and algae. In this study, we analyzed metagenomic shotgun sequences in an attempt to characterize lichen mycobiomes. Specifically, we inferred the range of fungi associated within lichen thalli from five groups of lichens – horsehair lichens (mycobiont = *Bryoria* spp.), shadow lichens (taxa in Physciaceae), rock posies (*Rhizoplaca* spp.), rock tripes (*Umbilicaria* spp.), and green rock shields (*Xanthoparmelia* spp.). Metagenomic reads from the multi-copy nuclear ribosomal internal transcribed spacer region, the standard DNA barcode region for fungi, were extracted, clustered, and used to infer taxonomic assignments. Our data revealed diverse lichen-associated mycobiomes. Many of the members of the lichen-associated mycobiomes that were identified here have not previously been found in association with lichens. Furthermore, closely related mycobionts tended to have more similar mycobiomes. We found little evidence supporting the ubiquitous presence of Cystobasidiales yeasts in macrolichens, although reads representing this putative symbiotic partner were found in samples of *Bryoria* lichens, albeit in low abundance. Our study further highlights the ecosystem-like features of lichens, with partners and interactions far from being completely understood. Future research is needed to more fully and accurately characterize lichen mycobiomes and how these fungi interact with the major lichen components, the photo- and mycobionts.

Keywords Cystobasidiomycetes · Endolichenic fungi · Genomics · Holobiont · ITS · Symbiosis

1 Introduction

Lichens have been iconic examples of symbiosis for the past 150 years (Honegger 2000). While a lichen was originally defined as a symbiotic relationship between a single fungus, the mycobiont, and a single or few species of green algae or cyanobacteria, the photobiont, studies have shown this is overly simplistic. It wasn't until the late twentieth century that

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in vitro studies began to look at other fungi as potentially lichen-associated organisms rather than mere contaminants (Petrini et al. 1990; Crittenden et al. 1995; Girlanda et al. 1997). It is now apparent that lichens are ecologically complex, internally consistent, and self-sustaining symbiotic phenotypes composed of evolutionarily diverse microbes (Goward 2008; Honegger 1993; Honegger 2001; Spribille et al. 2020).

Advances in sequencing technologies have allowed for deeper investigation into the diversity of the lichen symbiosis. In addition to the predominant myco- and photobionts, additional fungal and green algal/cyanobacterial species are often associated with a lichen thallus, in addition to non-photosynthetic bacteria (Grube et al. 2009; Lawrey and Diederich 2003; Muggia et al. 2013). Photobiont diversity can be shaped by reproductive and dispersal strategies of the mycobiont (Cao et al. 2015; Steinova et al. 2019), geography (Muggia et al. 2014; Werth and Sork 2014; Leavitt et al. 2015), growth substrate (Bačkor et al. 2010; Leavitt et al. 2013b; Muggia et al. 2014), and macroclimate (Lu et al. 2018; Singh et al. 2018). The diversity of photobionts has been only recently explored by environmental DNA metabarcoding approaches and has focused on species within the Mediterranean basin to date (Moya et al. 2017; Dal Grande et al. 2018). In contrast to high-throughput sequencing approaches, traditional and largely applied DNA barcoding using Sanger sequencing was able to detect only the principal photobiont in the thalli (Paul et al. 2018). Additionally, many studies show that lichens are surrounded by a consortium of bacteria (Bates et al. 2011) that may change with substrate, altitude, and geography (Cardinale et al. 2012; Hodkinson et al. 2012; Fernandez-Brime et al. 2019). Potential functions of bacterial microbiomes include providing the host with nutrients, as well as protective and growth-regulating functions (Cernava et al. 2017). Furthermore, carbon exchange between lichen green algae and non-photosynthetic bacteria has recently been suggested (Kono et al. 2017).

The lichen mycobiome – fungal communities superficially associated with the lichen thallus, e.g. on or near the lichen's surface/cortex, and within the thallus – can be made up of lichenicolous fungi (Lawrey and Diederich 2003) and endolichenic fungi (Arnold et al. 2009; U'Ren et al. 2010; Muggia et al. 2016). Lichenicolous fungi growing on a lichen thallus may or may not be parasitic, and are defined as "symptomatic" if they influence their host's morphology (Lawrey and Diederich 2003; U'Ren et al. 2010; Fleischhacker et al. 2015). The majority of lichenicolous fungi are classified within lichen-dominated groups (e.g., Lecanoromycetes), while endolichenic fungi are common among all major primary nonlichenized lineages, e.g., Sordariomycetes, Dothideomycetes, Leotiomycetes, and Pezizomycetes (Arnold et al. 2009; Hibbett et al. 2007). Further, endolichenic fungi are largely asymptomatic or cryptic with the host thallus. Many of the fungi associated with lichens appear to not be accidental colonizers of lichens (Arnold et al. 2009; U'Ren et al. 2010). While some studies have found patterns in the lichen-associated mycobiome – e.g., changing with altitude (Zhang et al. 2015; Wang et al. 2016) – others have found little specificity between the lichens and their associated mycobiome (Fleischhacker et al. 2015; Fernandez-Mendoza et al. 2017; Yu et al. 2018).

Recently, basidiomycete yeasts have been called into question as a potential symbiotic partner in the lichen symbiosis with the discovery of Cystobasidiomycetes (Basidiomycota, Pucciniomycotina) in the cortices of lichens (Spribille et al. 2016). The presence of this group of fungi was previously discovered in association with two genera in the lichen-forming family Parmeliaceae, *Hypogymnia,* and *Usnea* by Millanes et al. (2016) who clarified the phylogenetic position and the monophyly of two lichen-inhabiting species which were accommodated in the new genus *Cyphobasidium*. Later Černajová and Škaloud (2019) found Cystobasidiomycete yeasts in 95% of *Cladonia* specimens collected across Europe, though they were suggested to be either part of a superficial biofilm or living within the thallus without associating with the cortex itself. In contrast, Lendemer et al. (2019) found them in just nine of the 339 species investigated. The question remains of how abundant and specific cystobasidiomycetes are in lichen symbioses, as well as how consistent the mycobiome might be among different lichen-forming fungal species, e.g., do evolutionary constraints of the mycobiont influence the range and composition of associated fungi?

Intrathalline photobiont diversity – multiple photobiont species within a single lichen thallus – has previously been observed in a number of lichen symbioses (Muggia et al. 2013; Dal Grande et al. 2014; Moya et al. 2017; Škaloud et al. 2018). In some cases, algae with different physiological performances are ever-present in lichen thalli potentially facilitating the success of these lichens in a wide range of habitats and geographic areas and/or in changing environmental conditions. However, PCR amplification and Sanger sequencing has been shown to consistently fail to effectively generate DNA sequence data from lichen specimens when multiple *Trebouxia* lineages occur within a single lichen thallus (Paul et al. 2018), potentially biasing the perspective of lichen photobiont associations. The prevalence of intrathalline photobiont diversity in lichens remains unclear and thus impacts our understanding of its ecological and evolutionary significance.

As lichens are a model of symbiosis, there is a need to better characterize their microbial partners and associations. With the increasing availability of metagenomic short-read data from lichens, it may be possible to utilize these data to explore novel questions relating to lichen symbioses. Currently available data has been generated using a wide array

of methodological approaches, ranging from metagenomic and transcriptomic sequencing with reads obtained from samples from multiple species, each represented by a single thallus fragment (Leavitt et al. 2016; Spribille et al. 2016), to population-level samples with multiple thalli representing a single species pooled into replicate samples (Dal Grande et al. 2017; Dal Grande et al. 2018). Therefore, we used existing datasets of metagenomic shotgun sequences and implemented a bioinformatics pipeline to extract metagenomic reads representing the standard fungal DNA barcode region in an attempt to do the following: (i) characterize the lichen mycobiomes across multiple, phylogenetically distinct lichen groups, (ii) assess the prevalence of basidiomycete yeast, a putative symbiotic partner in some lichen symbioses, and (iii) investigate the potential for multiple species-level *Trebouxia* algal lineages within a single lichen thallus.

2 Materials and methods

2.1 Taxon sampling

Although the Code of Botanical Nomenclature anchors the name of the lichen to the Latin binomial of the mycobiont, whole lichens – the complete symbiotic phenotype or holobiont (all organisms found within a lichen thallus) – lack any formal taxonomic recognition (Goward 2008). Therefore, in this study when referring to the lichen holobiont, we use the appropriate taxonomic level of the mycobiont followed by 'lichen', e.g., '*Rhizoplaca* lichens' refers to lichen holobionts associating with mycobionts in the genus *Rhizoplaca* Zopf and not to the mycobiont alone. Our sampling focused on five morphologically distinct lichen groups: (i) *Rhizoplaca* lichens (rock posy lichens; Fig. 1a & b), (ii) *Xanthoparmelia* lichens (green rock shields; Fig. 1c & d), (iii) *Umbilicaria* lichens (rock tripe lichens; Fig. 1e), (iv) *Bryoria* lichens (horsehair lichens; Fig. 1f), and (iv) representatives from the mycobiont family Physciaceae (shadow lichens; Fig. 1g & h) (Table 1). *Rhizoplaca* lichens were represented by three distinct forms from the closely related *Rhizoplaca melanophthalma* group, all occurring in western North America: the vagrant/erratic forms representing *Rhizoplaca arbuscula* Rosentr., St. Clair & Leavitt (Fig. 1b; $n = 3$) and *R. melanophthalma* subsp. *crispa* Rosentr. & B.D. Ryan (n = 3), in addition to *R. melanophthalma* (DC.) Leuckert, which is attached to rocks (Fig. 1a; n = 3) (Leavitt et al. 2013a). *Xanthoparmelia* lichens were also represented by three distinct forms occurring in western North America: vagrant forms representing *Xanthoparmelia* aff. *chlorochroa* (Tuck.) Hale (Fig. 1d; $n = 3$), isidiate (vegetative reproductive propagules) forms (Fig. 1c; n = 3), and the sexually reproducing taxon *X. subcumberlandia* Elix & T.H. Nash (n = 3) (Leavitt et al. 2011). *Umbilicaria* lichens were represented by two species collected in Spain: *U. hispanica* (Frey) Davydov, Peršoh & Rambold (3 populations) and *U. pustulata* (L.) Hoffm. (Fig. 1e; 3 populations). For the *Umbilicaria* lichens, each sample represents metagenomic reads from a pooled population – 100 lichen thalli/population (Dal Grande et al. 2017) – rather than reads from an individual lichen thallus. *Bryoria* lichens were represented by two species: *Bryoria fremontii* (Tuck.) Brodo & D. Hawksw. (Fig. 1f; n = 3) and *B. tortuosa* (G. Merr.) Brodo & D. Hawksw. (n = 3) (Velmala et al. 2009). Lichens associating with the mycobiont family Physciaceae were represented by *Mobergia calculiformis* (W.A. Weber) H. Mayrhofer & Sheard (*Leavitt 16–697* [BRY-C]), *Physcia* aff. *biziana* (A. Massal.) Zahlbr. (*Leavitt 17–611* [BRY-C]), *Physciella* aff. *chloantha* (Ach.) Essl. (*Leavitt 17–586* [BRY-C]), *Oxnerella safavidiorum* S.Y. Kondr., B. Zarei-Darki, L. Lőkös & Hur (*Leavitt 16–665* [BRY-C]), and *Rinodina* (Ach.) Gray sp. (*Leavitt 16–665* [BRY-C]). For *Rhizoplaca* lichens, *Xanthoparmelia* lichens, and representatives of Physciaceae, specimens were collected in dry conditions, with subsamples for molecular study removed within 24 h of collection and frozen at −20 °C until DNA extraction. Sampling of *Bryoria* and *Umbilicaria* lichens were reported previously in Spribille et al. (2016) and Dal Grande et al. (2017, 2018), respectively.

2.2 Metagenomic sequencing

Metagenomic short reads used in this study originated from a range of sources and sequencing methods (Table 1). Metagenomic reads from *Rhizoplaca* lichens were initially reported in Leavitt et al. (2016, 2019) and are available in NCBI's Short Read Archive under project PRJNA576709. For newly generated metagenomic reads from *Xanthoparmelia* lichens and representatives of Physciaceae, total genomic DNA was extracted from a small portion of the lichen thallus (comprised of the mycobiont, photobiont, and other associated microbes) using the E.Z.N.A. Plant DNA DS Mini Kit (Omega Bio-Tek, Inc., Norcross, GA, USA) following the manufacturers' recommendations. Total genomic DNA was prepared following the standard Illumina whole genome sequencing (WGS) library preparation process using Adaptive Focused Acoustics for shearing (Covaris), followed by an AMPure cleanup step. The DNA was then processed with the NEBNext Ultra™ II End Repair/dA-Tailing Module end-repair and the NEBNext Ultra™ II Ligation Module (New England Biolabs) while using standard Illumina index primers. Libraries were pooled and sequenced with the HiSeq 2500 sequencer in high output mode at the DNA Sequencing Center, Brigham Young University, Provo, Utah, USA, using either 250 cycle paired-end reads or 300 cycle paired-end reads. Reads from *Xanthoparmelia* lichens and representatives from the mycobiont family Physciaceae are available in NCBI's Short Read Archive (Table 1). The reads from the

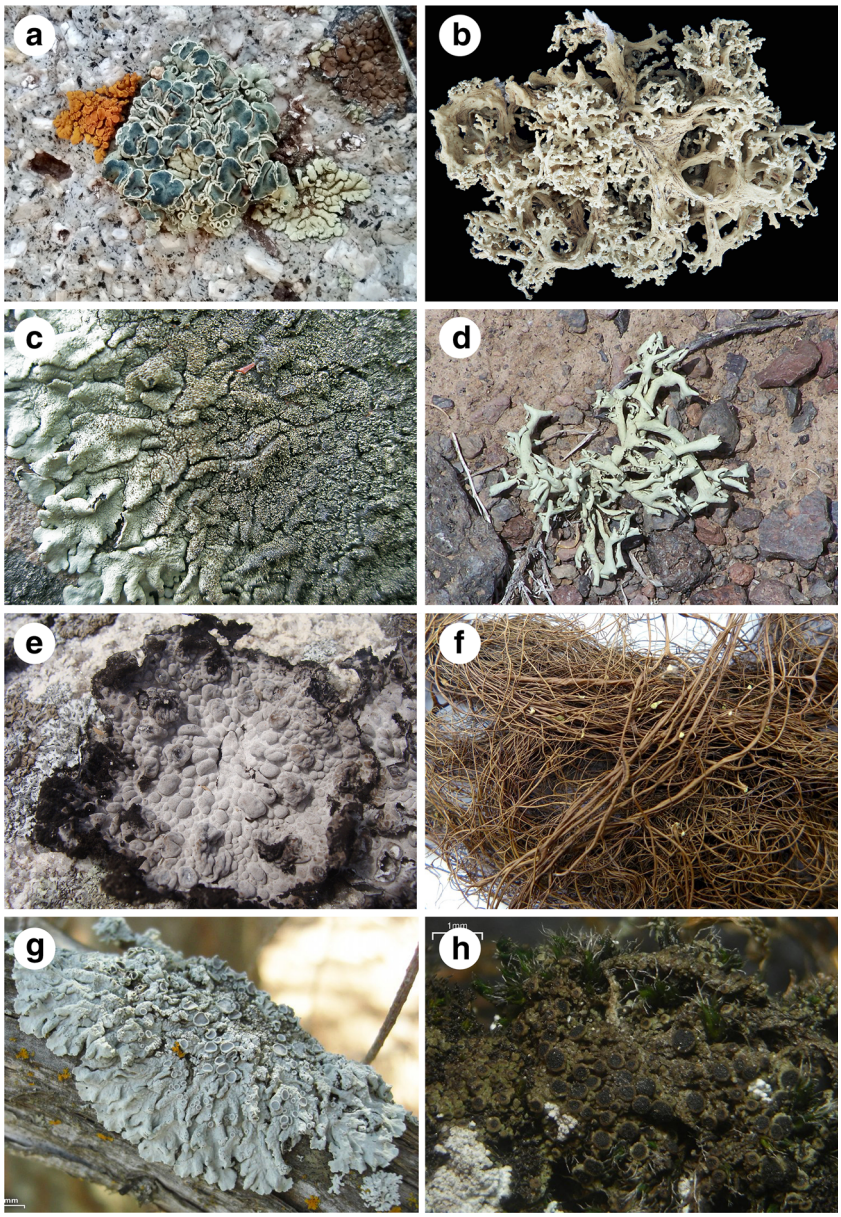


Fig. 1 Examples of lichens groups considered in this study, including *Rhizoplaca* lichens (**a** & **b**), *Xanthoparmelia* lichens (**c** & **d**), *Umbilicaria* lichens (**e**), *Bryoria* lichens (**f**), and Physciaceae lichens (**g** & **h**). **a**, *Rhizoplaca melanophthalma* – field image from La Sal Mountain Range, Utah, USA. **b**, *Rhizoplaca arbuscula* – collected from Lemhi Valley, Idaho, USA, voucher *Leavitt 18–1017* (BRY-C). **c**, *Xanthoparmelia* cf. *mexicana* – field image from Snake Range, Nevada, USA. **d**, *Xanthoparmelia* aff. *chlorochroa* – field image from Awapa Plateau, Utah, USA. **e**, *Umbilicaria pustulata* – field image from La Coruña, Galicia, España. (source: https://commons.wikimedia.org/wiki/File:Lasallia_pustulata.001_-_Islas_Cies.JPG [CC BY-SA 4.0]). **f**, *Bryoria fremontii* – from Oppland, Norway (source: https://www.flickr.com/photos/aburgh/27323080245 [CC BY-NC-SA 2.0]). **g**, *Physcia biziana* – field image from vicinity of Santa Fe, New Mexico, USA (Hollinger 2492). **h**, *Rinodina olivaceobrunnea* – field image from vicinity of John Day, Oregon, USA. (Hollinger 7073). Note: the name listed for each lichen is for the mycobiont (main fungal partner) and does not account for the range of potential other associated symbionts. Permission to use photographs in panels '**g**' and '**h**' was kindly provided by Jason Hollinger

Bryoria lichens are distinct because they are transcriptomic reads (Spribille et al. 2016), and we aimed to extract by-catch reads representing the internal transcribed spacer region (ITS). For the *Umbilicaria* lichens, each sample represents metagenomic reads from a pooled population (Pool-seq) – 100 lichen thalli/population (Dal Grande et al. 2017, 2018) – rather than reads from an individual lichen thallus.

2.3 Sequence analysis

All reads were filtered using TRIMMOMATIC v0.33 (Bolger et al. 2014) before mapping to remove low quality reads and/or included contamination from Illumina adaptors using the following parameters: ILLUMINACLIP; LEADING:3; TRAILING:3; SLIDINGWINDOW:4:15; and MINLEN:36. Previous studies have used assembled metagenomic contigs (Keepers et al. 2019) or mapped fungal reads to a fungal protein database (LaBonte et al. 2018) to provide crucial insight into fungal diversity in lichens and deciduous trees. Given the expected low coverage for fungi potentially co-occurring with a lichen thallus in short reads generated for this study, we chose to focus on the well-known repeat region which includes the standard fungal DNA barcode, the ITS region of the nuclear ribosomal DNA (nrDNA) (Schoch et al. 2012). Across fungi, nrDNA copy number has been shown to vary considerably, ranging from tens to over 1400 copies per genome (Lofgren et al. 2019; Bradshaw et al. 2020). Furthermore, a comparatively robust and well-curated ITS database exists for fungi (Nilsson et al. 2019).

Table 1 List of lichen specimens included in this study, including: non-scientific names referring to major lichen groups investigated here and the associated mycobiont and family affiliation; voucher number, NCBI Short Read Archive experiment accession number (SRX), or sample name from previous study; locality information or data source for each specimen; the total number of short reads (and read length) generated for each specimen; the number of reads mapped (and relative proportion) to the UNITE QIIME v.8 dynamic release ITS database for fungi; and the NCBI Short Read Archive project and accession numbers for each sample

Lichen	Mycobiont	DNA/SRX code	Locality	total # of reads (read length)	mapped reads	proportion ITS	SRA Project ID	SRA experiment accession number
green rock shields	*Xanthoparmelia* aff. *chlorochroa* (vagrant)	S. Leavitt 293f (BRY-C)	USA, NV, Elko Co.:	14,422,618 (125)	332,746	0.02307112	PRJNA646656	SAMN15548969
green rock shields	*Xanthoparmelia* aff. *chlorochroa* (vagrant)	S. Leavitt 818f (BRY-C)	USA, WY, Natroma Co.:	13,757,990 (125)	311,548	0.02264488	PRJNA646656	SAMN15548970
green rock shields	*Xanthoparmelia* aff. *chlorochroa* (vagrant)	S. Leavitt 16–524 (BRY-C)	USA, UT, Duchesne Co.: Ashley N.F., vicinity of Nutter's Canyon	28,898,632 (125)	527,247	0.0182447	PRJNA646656	SAMN15548971
green rock shields	*Xanthoparmelia* aff. *mexicana* (saxicolous/isidiate)	S. Leavitt 149f (BRY-C)	USA, AZ, Mojave Co.: Arizona Strip	14,226,759 (125)	123,489	0.00868005	PRJNA646656	SAMN15548972
green rock shields	*Xanthoparmelia* aff. *plittii* (saxicolous/isidiate)	S. Leavitt 576f (BRY-C)	USA, CA, San Diego Co.:	9,167,676 (125)	76,207	0.00831258	PRJNA646656	SAMN15548973
green rock shields	*Xanthoparmelia maricopensis* (saxicolous/isidiate)	J. Leavitt 6698 (BRY-C)	USA, Maricopa Co.: Crater Range	12,829,844 (150)	92,199	0.00718629	PRJNA646656	SAMN15548974
green rock shields	*Xanthoparmelia subcumberlandia* (saxicolous/fertile)	S. Leavitt 038f (BRY-C)	USA, UT, Wayne Co.: Boulder Mountain	4,544,904 (125)	46,983	0.01033751	PRJNA646656	SAMN15548975
green rock shields	*Xanthoparmelia subcumberlandia* (saxicolous/fertile)	S. Leavitt 072f (BRY-C)	USA, UT, Wayne Co.: Boulder Mountain	8,843,260 (125)	84,700	0.00957792	PRJNA646656	SAMN15548976
green rock shields	*Xanthoparmelia subcumberlandia* (saxicolous/fertile)	S. Leavitt 192f (BRY-C)	USA, CO, Dolores Co.:	10,677,112 (125)	232,652	0.02178979	PRJNA646656	SAMN15548977
horsehairs	*Bryoria fremontii*	SRX1846191	Spribille et al. 2016	33,462,916 (200 & 250)	469,130	0.0140194	PRJNA309871	SRX1846191
horsehairs	*Bryoria fremontii*	SRX1846192	Spribille et al. 2016	43,062,618 (200)	362,151	0.00840987	PRJNA309871	SRX1846192
horsehairs	*Bryoria fremontii*	SRX1846193	Spribille et al. 2016	20,317,220 (250)	96,649	0.004757	PRJNA309871	SRX1846193
horsehairs	*Bryoria tortuosa*	SRX1846179	Spribille et al. 2016	38,834,682 (200)	190,981	0.00491779	PRJNA309871	SRX1846179
horsehairs	*Bryoria tortuosa*	SRX1846180	Spribille et al. 2016	32,141,407 (200)	180,331	0.00561055	PRJNA309871	SRX1846180
horsehairs	*Bryoria tortuosa*	SRX1846182	Spribille et al. 2016	33,975,174 (250)	472,046	0.01389385	PRJNA309871	SRX1846182
rock posies	*Rhizoplaca arbuscula*	S. Leavitt 8678 (BRY-C)	USA, ID, Lemhi Co.: vicinity of Leadore	10,226,038 (100)	133,814	0.01308562	PRJNA576709	SRX6990531
rock posies	*Rhizoplaca arbuscula*	S. Leavitt 18–1005 (BRY-C)	USA, ID, Lemhi Co.: vicinity of Leadore	29,801,789 (50)	352,218	0.01181869	PRJNA646656	SAMN15548978
rock posies	*Rhizoplaca arbuscula*	S. Leavitt 18–1015 (BRY-C)	USA, ID, Lemhi Co.: vicinity of Leadore	7,749,422 (125)	136,148	0.01756879	PRJNA576709	SRX6990535
rock posies	*Rhizoplaca melanophthalma*	S. Leavitt 8801 (BRY-C)	USA, UT, Wayne Co.: Thousand Lakes Mountain	12,371,904 (100)	69,127	0.00558742	PRJNA576709	SRX6990558
rock posies	*Rhizoplaca melanophthalma*	S. Leavitt 8802 (BRY-C)		11,653,316 (100)	71,466	0.00613268	PRJNA576709	SRX6990560

Table 1 (continued)

Lichen	Mycobiont	DNA/SRX code	Locality	total # of reads (read length)	mapped reads	proportion ITS	SRA Project ID	SRA experiment accession number
			USA, UT, Wayne Co.: Thousand Lakes Mountain					
rock posies	*Rhizoplaca melanophthalma*	S. Leavitt 8810 (BRY-C)	USA, UT, Millard Co.: Fossil Mountain	11,816,706 (100)	48,753	0.00412577	PRJNA576709	SRX6990561
rock posies	*Rhizoplaca melanophthalma* spp. *crispa*	S. Leavitt 18–1002 (BRY-C)	USA, ID, Custer Co.: SE of Challis	6,688,542 (125)	82,788	0.01237759	PRJNA576709	SRX6990554
rock posies	*Rhizoplaca melanophthalma* spp. *crispa*	S. Leavitt 18–1003 (BRY-C)	USA, ID, Custer Co.: SE of Challis	7,540,500 (125)	110,708	0.01468179	PRJNA576709	SRX6990555
rock posies	*Rhizoplaca melanophthalma* spp. *crispa*	S. Leavitt 18–1004 (BRY-C)	USA, ID, Custer Co.: SE of Challis	29,385,326 (50)	177,762	0.00604935	PRJNA646656	SAMN15548979
rock tripes	*Umbilicaria hispanica*	H1 (Dal Grande et al. 2017)	Dal Grande et al. 2017	29,396,267 (145)	559,123	0.0190202	NA	NA
rock tripes	*Umbilicaria hispanica*	H3 (Dal Grande et al. 2017)	Dal Grande et al. 2017	27,171,115 (145)	524,642	0.01930881	NA	NA
rock tripes	*Umbilicaria hispanica*	H6 (Dal Grande et al. 2017)	Dal Grande et al. 2017	23,760,773 (145)	473,106	0.01991122	NA	NA
rock tripes	*Umbilicaria pustulata*	ESi Pool1 (Dal Grande et al. 2018)	Dal Grande et al. 2018	28,862,057 (100)	158,147	0.00547941	NA	NA
rock tripes	*Umbilicaria pustulata*	ESi Pool2 (Dal Grande et al. 2018)	Dal Grande et al. 2018	37,303,042 (100)	248,656	0.00666584	NA	NA
rock tripes	*Umbilicaria pustulata*	ESi Pool3 (Dal Grande et al. 2018)	Dal Grande et al. 2018	35,351,050 (100)	209,165	0.0059168	NA	NA
shadow lichens	*Mobergia calculiformis*	S. Leavitt 16–997 (BRY-C)	Mexico, Baja California Norte	25,328,630 (125)	893,862	0.03529058	PRJNA646656	SAMN15548980
shadow lichens	*Oxernella safavidiorum*	S. Leavitt 16–665 (BRY-C)	USA, UT, Millard Co.: Sawtooth Mountain	12,041,152 (125)	103,663	0.00860906	PRJNA646656	SAMN15548981
shadow lichens	*Physcia biziana*	S. Leavitt 17–611 (BRY-C)	USA, NV, White Pine County: Great Basin National Park	20,810,956 (125)	207,703	0.00998046	PRJNA646656	SAMN15548982
shadow lichens	*Physciella chloantha*	S. Leavitt 17–586 (BRY-C)	USA, NV, White Pine County: Great Basin National Park	22,390,882 (125)	206,766	0.00923438	PRJNA646656	SAMN15548983
shadow lichens	*Rinodina* sp.	S. Leavitt 17–566 (BRY-C)	USA, NV, White Pine County: Great Basin National Park	13,399,600 (125)	245,262	0.01830368	PRJNA646656	SAMN15548984

For reference ITS sequences, we used the UNITE QIIME v.8 dynamic release for fungi (Nilsson et al. 2019), filtered to include only sequences between 300 to 800 base pairs (reduced from 70,512 to 69,872 ITS sequences). Following recommendations by QIIME 2 developers, flanking regions, e.g., portions of the18S and/or 28S, with ITS sequences in the UNITE database were retained to reduce erroneous classifications when using the naïve Bayes classifier (https://doi.org/10.7287/peerj.preprints.27295v2). The UNITE ITS database was supplemented with all Cystobasidiomycetes ITS sequences reported in Spribille et al. (2016). All sampled lichens are reported to associate with members of the genus *Trebouxia* as the primary lichen photobiont. In addition to assessing fungal diversity in short reads generated from lichen thalli, we also included representative sequences for each of the *Trebouxia* operational taxonomic units (OTUs) circumscribed in Leavitt et al. (2015). Although lichens are known to associate with a broader range of algae than the core photobionts (Muggia et al. 2013), we did not assess accessory algae outside of *Trebouxia.*

For each metagenomic library, reads were mapped back to the composite ITS database using the Geneious read mapper in Geneious Prime (Kearse et al. 2012), implementing 'Medium-Low Sensitivity / Fast' sensitivity, iterated two times and saving all successfully mapped reads. Exploratory analyses with other read mapping approaches consistently recovered lower quantities of successfully mapped reads (data not shown). For each sample, metagenomic reads successfully mapped to the ITS references were imported into QIIME 2 (Bolyen et al. 2019). Reads were dereplicated using Vsearch 'dereplicate-sequences' (Rognes et al. 2016), implementing default settings. The dereplicated sequences were clustered into de novo OTUs at a 97% similarity in Vsearch using 'cluster-features-de-novo' (McDonald et al. 2012; Rognes et al. 2016). A naïve Bayes taxonomic classifier was trained using the same ITS reference library in QIIME 2 (Bokulich et al. 2018). The OTUs were then taxonomically classified with the trained naive Bayes trainer using the QIIME 2 'feature-classifier classify-sklearn' at a 0.95 confidence level to minimize false positives, with all other settings at default (McKinney 2010; Pedregosa et al. 2011; Bokulich et al. 2018).

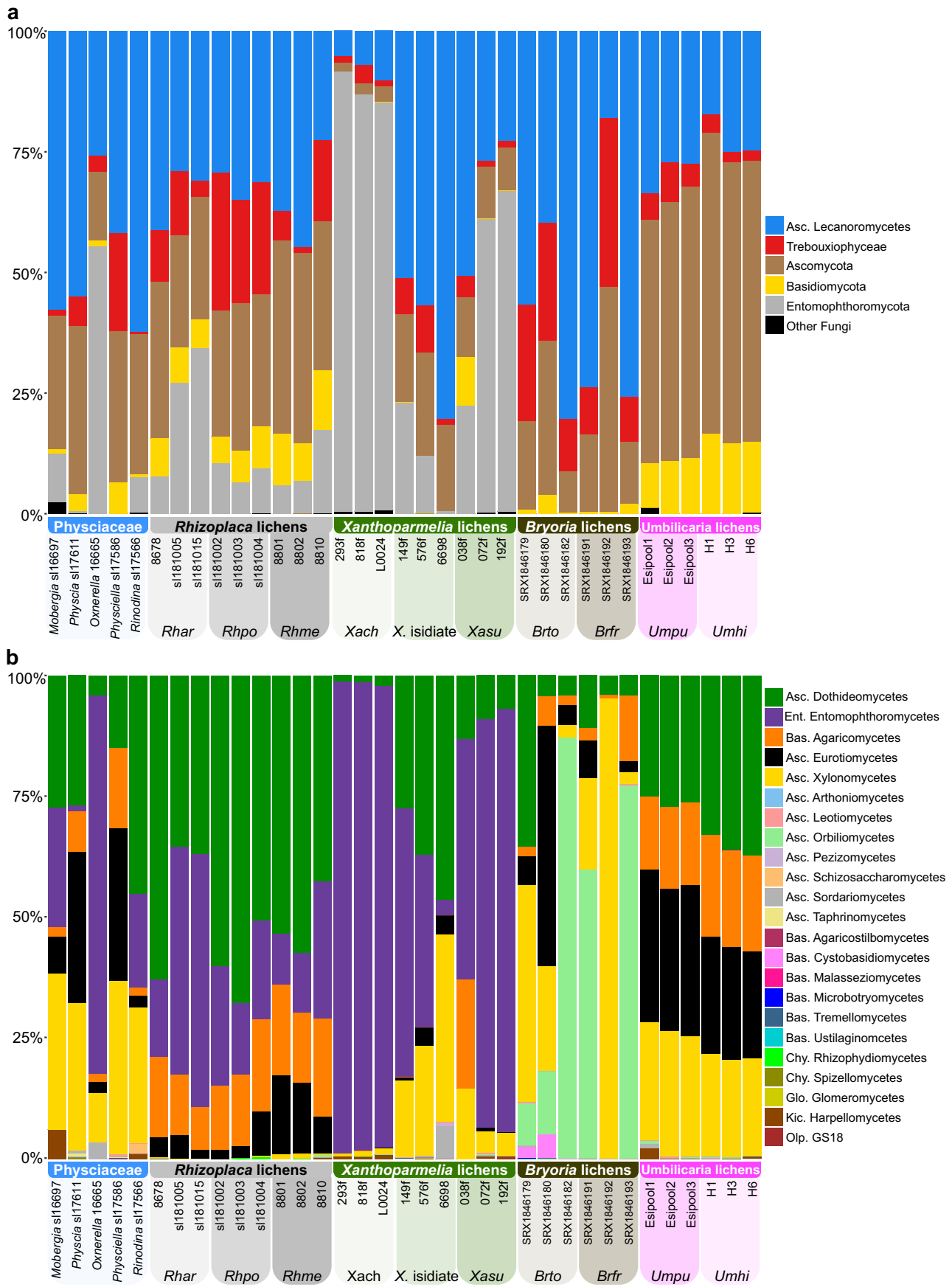
Of the estimated 2.2 to 3.8 million fungal species, only 3–8% are currently named (Hawksworth and Lücking 2017), and a much smaller portion are represented in available DNA reference libraries. Exploratory analyses of our lichen mycobiome data revealed poor taxonomic resolution below class levels for the majority of OTUs inferred here. Therefore, fungal OTUs that were classified at the class level were retained, and others with less taxonomic resolution were excluded. Classification of fungal OTUs generated from reads mapped to the reference ITS database was summarized using the QIIME 'Taxa Barplot' feature (Caporaso et al. 2010). Data were managed, analyzed, and visualized in R (R Core Team 2019) using ggplot2 (Wickham 2016) and tidyr (Wickham et al. 2019). To assess the similarity of lichen mycobiomes within and among phylogenetically distinct mycobionts, a principle component analysis (PCA) was performed on the class-level taxonomic classification using tidyr (Wickham et al. 2019), with the command 'prcomp'. While formal species-level taxonomy in the lichen photobiont *Trebouxia* remains woefully inadequate (Muggia et al. 2020), DNA sequence data representing a wide range of putative species-level lineages, with accompanying provisional names, is available (Leavitt et al. 2015). For *Trebouxia* (photobiont) OTUs, the classified reads were filtered at the 'species' level, based on the 69 putative species-level OTUs from Leavitt et al. (2015), using QIIME 'taxa filter-table' command to determine the range of *Trebouxia* diversity occurring within each sample. All code used in this experiment is provided as supplementary file S1.

3 Results

Between 0.41 and 3.68% of metagenomic reads from each sample were mapped back to the ITS reference library (Table 1). The primary lichen symbionts, the mycobiont and photobiont, accounted for ca. 50% of all ITS reads extracted from the metagenomic data on average (Fig. 2a). The relative abundance of ITS reads representing the mycobiont (inferred at the class level, e.g., Lecanoromycetes) was between 5.20% to 80.31% of ITS reads, with an average relative abundance of ca. 40%. The relative abundance of reads from the photobiont, *Trebouxia* spp., comprised between 0.68% to 35.09% of ITS reads, with an average relative abundance of ca. 10%.

Lichen-associated fungi made up a large fraction of metagenomic reads, representing a total of 22 fungal classes (Fig. 2b). Both in terms of abundance and diversity, Ascomycota OTUs were most frequently recovered and represented by 10 classes, excluding the mycobiont class Lecanoromycetes, followed by Basidiomycota represented by seven classes. Chytridiomycota (represented by two classes), Glomeromycota (one class), and Kickxellomycota (one class) were found in low abundance and diversity (Fig. 2). Overall, reads from Cystobasidiomycete yeasts were poorly represented in extracted ITS reads, found in only 5 of the 35 samples. Notably, ITS by-catch from the *Bryoria fremontii* transcriptomic data from which lichen-associated yeasts were reported in the cortex, resulted in the highest abundance of reads potentially representing cystobasidiomycete yeasts, with an average relative abundance of 0.7% of the ITS reads in the three *B. fremontii* samples. In the remaining two samples with evidence of Cystobasidiomycete yeasts, *Physcia biziana* and one sample of *Xanthoparmelia chlorochroa* (818F), had an average relative abundance of 0.03%.

Closely related mycobionts tended to have more similar mycobiomes (Fig. 3). The PCA revealed a general pattern of


a
100%
75%
50%
25%
0%
Asc. Lecanoromycetes
Trebouxiophyceae
Ascomycota
Basidiomycota
Entomophthoromycota
Other Fungi
Physciaceae
Rhizoplaca lichens
Xanthoparmelia lichens
Bryoria lichens
Umbilicaria lichens
Mobergia sl16697
Physcia sl17611
Oxnerella 16665
Physciella sl17586
Rinodina sl17566
8678
sl181005
sl181015
sl181002
sl181003
sl181004
8801
8802
8810
293f
818f
L0024
149f
576f
6698
038f
072f
192f
SRX1846179
SRX1846180
SRX1846182
SRX1846191
SRX1846192
SRX1846193
Esipool1
Esipool2
Esipool3
H1
H3
H6
Rhar
Rhpo
Rhme
Xach
X. isidiate
Xasu
Brto
Brfr
Umpu
Umhi
b
100%
75%
50%
25%
0%
Asc. Dothideomycetes
Ent. Entomophthoromycetes
Bas. Agaricomycetes
Asc. Eurotiomycetes
Asc. Xylonomycetes
Asc. Arthoniomycetes
Asc. Leotiomycetes
Asc. Orbiliomycetes
Asc. Pezizomycetes
Asc. Schizosaccharomycetes
Asc. Sordariomycetes
Asc. Taphrinomycetes
Bas. Agaricostilbomycetes
Bas. Cystobasidiomycetes
Bas. Malasseziomycetes
Bas. Microbotryomycetes
Bas. Tremellomycetes
Bas. Ustilaginomcetes
Chy. Rhizophydiomycetes
Chy. Spizellomycetes
Glo. Glomeromycetes
Kic. Harpellomycetes
Olp. GS18
Physciaceae
Rhizoplaca lichens
Xanthoparmelia lichens
Bryoria lichens
Umbilicaria lichens
Mobergia sl16697
Physcia sl17611
Oxnerella 16665
Physciella sl17586
Rinodina sl17566
8678
sl181005
sl181015
sl181002
sl181003
sl181004
8801
8802
8810
293f
818f
L0024
149f
576f
6698
038f
072f
192f
SRX1846179
SRX1846180
SRX1846182
SRX1846191
SRX1846192
SRX1846193
Esipool1
Esipool2
Esipool3
H1
H3
H6
Rhar
Rhpo
Rhme
Xach
X. isidiate
Xasu
Brto
Brfr
Umpu
Umhi

◀ **Fig. 2** Overview of lichen symbionts and associated fungi inferred from data from the internal transcribed spacer region extracted from metagenomic shotgun sequencing short reads sequenced from lichen thalli representing five different groups of lichens. **a**, proportion of reads assigned to the lichen symbionts – mycobiont (shown in blue) and photobiont (in red) – and other major fungal lineages. **b**, inferred membership of lichen mycobiomes (at class level); the main lichen symbionts are excluded – see panel '**a**' – and relative abundance of remaining fungal classes were adjusted proportionally. Abbreviations by names of fungal groups in panel '**b**' are: Asc. = Ascomycota, Bas. = Basidiomycota, Chy. = Chytridiomycota, Ent. = Entomophthoromycota, Glo. = Glomeromycota, and Kic. = Kickxellomycota. For *Rhizoplaca* lichens, '*Rhar*' = *R. arbuscula* (vagrant lichen), '*Rhpo*' = *R. melanophthalma* subsp. *crispa* (vagrant forms), and '*Rhme*' = *R. melanophthlama* (rock-dwelling, fertile forms); for *Xanthoparmelia* lichens, '*Xach*' = *X.* aff. *chlorochroa* (asexual, vagrant lichens), '*X.* isidiate' = three different isidiate, rock-dwelling forms (see Table 1), and '*Xasu*' = *X. subcumberlandia* (fertile, rock-dwelling forms); for *Bryoria* lichens, '*Brto*' = *B. tortuosa* and '*Brfr*' = *B. fremontii*; for *Umbilicaria* lichens, '*Uspu*' = *U. pustulata* and '*Ushi*' = *U. hispanica*. See Table 1 for a full list of sampled lichens

mycobiome similarity among samples representing mycobiont species, and relatively high levels of similarity among mycobiont congeners (Figs. 2b & 3). Differences in lichen mycobiomes are most distinct among different genera of lichen-forming fungi.

Evidence supporting intrathalline *Trebouxia* photobiont diversity was observed in 16 of the 29 samples (*Umbilicaria* samples not considered) (Fig. 4). Intrathalline photobiont diversity in *Umbilicaria pustulata* and *U. hispanica* was described in detail in Paul et al. (2018). Short read data from *Umbilicaria* lichen samples analyzed in this study were generated from 100 pooled individual thalli from a single population. Thalli from representatives of Physciaceae and *Rhizoplaca* lichens consistently contained a dominant *Trebouxia* lineage with >90% relative abundance. *Xanthoparmelia* lichens associated with a broader range of *Trebouxia* species, with evidence of multiple *Trebouxia* species occurring within an individual lichen thallus. Two of the six *Bryoria* samples also provided evidence of multiple *Trebouxia* species occurring within individual thalli (Fig. 4).

4 Discussion

The broad range of organisms involved in lichen symbioses has recently been highlighted, including diverse algae (Muggia et al. 2013; Moya et al. 2017), non-photosynthetic bacteria (Cardinale et al. 2006; Grube et al. 2009; Hodkinson and Lutzoni 2009), and diverse fungal lineages (Lawrey and Diederich 2003; Spribille et al. 2016; Tuovinen et al. 2019). Using data mining of fungal ITS reads from metagenomic shotgun sequences of lichen thalli, we provide a coarse snapshot of unexpectedly diverse lichen-associated mycobiomes (Fig. 2). The accessory fungi accounted for a significant proportion of ITS reads extracted from metagenomic shotgun sequencing data (Fig. 2b), spanning multiple phyla – dominated by Ascomycota and Basidiomycota but with representatives from Entomophthoromycota, Chytridiomycota, Glomeromycota, and Kickxellomycota. While a number of the class-level lineages inferred from metagenomic ITS reads are known to associate with lichens, e.g., Agaricomycetes, Dothideomycetes, Eurotiomycetes, and Sordariomycetes, other classes do not include fungi previously known to associate with lichens, e.g. Entomophthoromytes. In contrast to recent studies highlighting the role of two basidiomycete lineages in some lichen symbioses, *Tremella* (Tuovinen et al. 2019) and Cystobasidiomycete yeasts (Spribille et al. 2016), these were recovered only sporadically and in very low abundance in our samples. Nonetheless, these basidiomycete fungi have often been reported as lichenicolous, growing on a number of lichen hosts (Diederich 1996; Millanes et al. 2016). Below we discuss the potential implications of our findings and potential ways to move forward.

The relative importance of host versus environment in determining the diversity of the lichen mycobiome is poorly understood. However, lichen mycobiomes appear to comprise stable and transient guilds, which to some extent correlate with the ecological conditions of the lichen habitats. Fernandez-Mendoza et al. (2017) proposed three ecological components of lichen mycobiomes: (i) generalist taxa common to the environmental pool of bio- and sapro-trophic fungi, (ii) lichenicolous and endolichenic fungi specific to each genus/species, and (iii) species which disperse and possibly germinate on, among, and within lichen thalli, but do not play a definite ecological role in the lichen community. Our results indicate that closely related mycobionts tend to have more similar mycobiomes (Fig. 3), even in cases where distinct lichens commonly co-occur, e.g. *Xanthoparmelia* and *Rhizoplaca* lichens. Furthermore, umbilicate and vagrant forms of *Rhizoplaca* lichens shared similar fungal communities, despite the perceived ecological differences between growing on rocks versus occurring free on the soil. These data support the perspective that a significant component of the lichenicolous and endolichenic fungal community are specific to different mycobiont genera/species and that evolutionary constraints of the mycobiont may influence the range and composition of associated fungi.

Differences in the growth form of lichens likely create distinct microhabitats that may influence the intrathalline microbiome, particularly on the underside of the thallus or other specialized morphological features. While both *Rhizoplaca* and *Umbilicaria* lichens are umbilicate with a central holdfast, we found that mycobiome communities of umbilicate lichens occurring on rocks were quite distinct. Whether this is the result of the evolutionary constraint of the mycobiont host or broader biogeographic patterns of lichen associated fungi (*Rhizoplaca* lichen samples were all

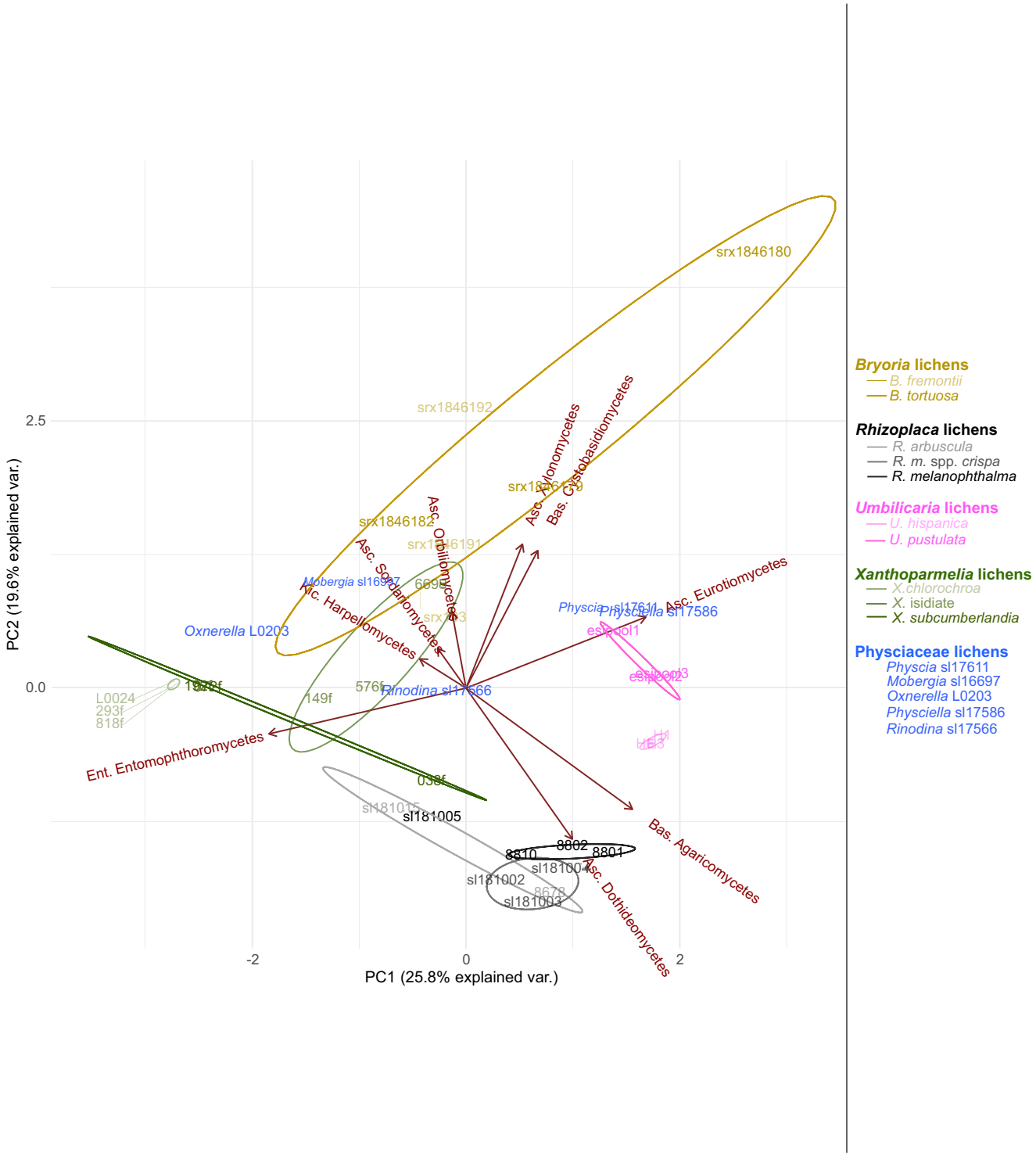


Fig. 3 Principal component analysis (PCA) of lichen mycobiome diversity in five distinct lichen groups, each represented by multiple species. Sample codes shown in PCA plot are linked to specimens listed in Table 1

collected from western North America, while *Umbilicaria* lichen samples originated from Europe) remains unknown. The influence of lichen secondary metabolites in shaping microbiome diversity also remains under-explored. Lichen secondary metabolites have broad antibacterial properties (Boustie and Grube 2005). Grube et al. (2015) hypothesized that the fungal partner plays an important role in regulating bacterial colonization of the thallus, and secondary

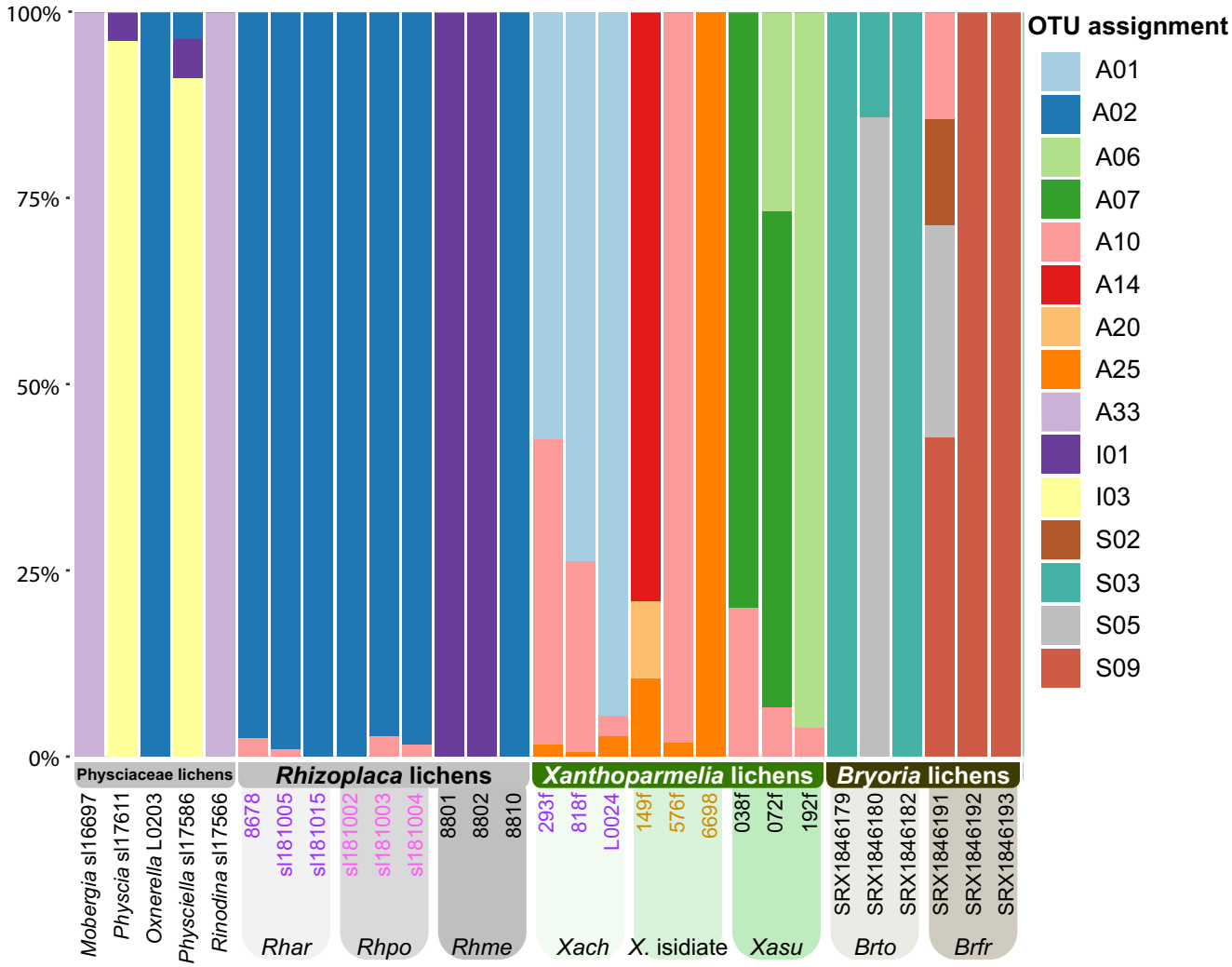


Fig. 4 Assessment of *Trebouxia* (photobiont) diversity for each metagenomic sample derived from a single lichen thallus. Distinct operational taxonomic units (OTUs) are color coded, and the proportional occurrence of each OTUs is reported for each metagenomic sample derived from a single thallus. For *Rhizoplaca* lichens, '*Rhar*' = *R. arbuscula* (vagrant lichen), '*Rhpo*' = *R. melanophthalma* subsp. *crispa* (vagrant forms), and '*Rhme*' = *R. melanophthlama* (rock-dwelling, fertile forms); for *Xanthoparmelia* lichens, '*Xach*' = *X.* aff. *chlorochroa* (asexual, vagrant lichens), '*X.* isidiate' = three different isidiate, rock-dwelling forms (see Table 1), and '*Xasu*' = *X. subcumberlandia* (fertile, rock-dwelling forms); and for *Bryoria* lichens, '*Brto*' = *B. tortuosa* and '*Brfr*' = *B. fremontii*. See Table 1 for a full list of sampled lichens. *Trebouxia* OTUs nomenclature follows Leavitt et al. (2015)

metabolites may also impact other components of the lichen microbiome. Each of the lichen groups sampled here can be characterized by the production of distinct secondary metabolites, and exploring the role of secondary metabolite variation in structuring mycobiome communities was beyond the scope of this study.

Broadly speaking, Sordariomycetes and Leotiomycetes are frequently recovered from lichens occurring in humid, temperate, boreal environments, and Antarctic environments, representing lineages closely related to plant endophytes (Arnold et al. 2009; U'Ren et al. 2010; Yu et al. 2018). In contrast, Dothideomycetes and Eurotiomycetes are more frequently associated with rock-inhabiting lichens (Muggia and Grube 2018). In rock-inhabiting lichens, the lichen-associated fungi are usually melanized fungi comprising unknown and known hyphomycetous lineages, which show close affinities to some symptomatic lichenicolous fungi, extremotolerant rock-inhabiting fungi from oligotrophic environments, and to plant and animal pathogenic black yeasts (Muggia et al. 2016; Muggia and Grube 2018). These fungi are widely known as black fungi because they accumulate melanins in their cell walls, which enable them to grow in oligotrophic environments and resist multiple abiotic stresses, such as high doses of radiation, desiccation, and temperature extremes (Gostinčar et al. 2009). Black fungi are, therefore, usually recognized as (poly)extremotolerant organisms.

In our study, different lichen-forming fungal genera tended to associate with distinct fungal communities (Fig. 2b & 3).

These relationships appear to be consistent across relatively broad geographic areas, at least for some lichens. Our results indicated that the mycobiomes of *Xanthoparmelia* lichen and *Rhizoplaca* lichen populations occurring across western North America were strikingly different (Fig. 2b). While disparate morphologies of *Rhizoplaca* lichens were shown to have relatively consistent mycobiomes, even in specimens collected across geographically distinct populations, differences in mycobiome communities of *Xanthoparmelia* lichens with different morphologies and reproductive strategies were observed (Fig. 2b). However, within *Xanthoparmelia* lichens, vagrant (obligately unattached specimens), rock-dwelling isidiate (reproducing via specialized asexual propagules), and rock-dwelling sexually reproducing forms tended to associate with distinct fungal communities, albeit inferred from limited sample sizes. Additional research will be required to more fully assess if distinct mycobiomes, or core subsets of the mycobiome, within lichen groups are maintained across geographic and ecological distances. If differing core mycobiome communities are found in association with distinct mycobionts, at what level does this specificity exist, e.g., mycobiont species, genera, etc.? Directed experimental design and broader sampling will be required to determine how lichen mycobiomes are structured at different evolutionary scales relative to the predominant mycobiont.

When investigating the potential for photobiont (*Trebouxia* spp.) diversity within a single lichen thallus, our results suggest that a single lichen thallus of some lichen groups, e.g., shadow lichens (members of the mycobiont family Physciaceae) and *Rhizoplaca* lichens, tend to associate with a single/one dominant *Trebouxia* lineage. For *Umbilicaria* lichens, Paul et al. (2018) observed a single pattern of a single dominant *Trebouxia* lineage per thallus. However, the metagenomic reads from *Umbilicaria* lichens used in the present study were generated from multiple lichen thalli pooled into a single population per site, and we were unable to corroborate these results reported. In contrast, it appears that *Xanthoparmelia* lichen (mycobiont = *Xanthoparmelia*) thalli consistently harbor multiple, distinct *Trebouxia* lineages. A previous study characterizing *Trebouxia* diversity associating with members of the mycobiont family Parmeliaceae also demonstrated distinct patterns of photobiont association between *Rhizoplaca* spp. and *Xanthoparmelia* spp., with *Xanthoparmelia* spp. associating with a much wider range of photobionts than *Rhizoplaca* spp. (Leavitt et al. 2015). Furthermore, these two mycobiont genera consistently associated with distinct *Trebouxia* lineages with very little overlap, and these results were corroborated by our findings (Fig. 4). By explicitly taking the potential for intrathalline photobiont diversity into consideration, we anticipate novel insight into different strategies of lichen symbiosis.

While our study provides novel insight into lichen symbioses and impetus for future research, there are a number of methodological limitations that potentially bias the results presented here. Metagenomic reads from lichen-forming fungi are expected to be dominated by reads from the major lichen symbionts, the myco- and photobionts (Pizarro 2019), and other eukaryotic microbial diversity associated with lichen thalli is likely found in much lower abundance in metagenomic short read data. Therefore, here we opted to target fungal reads from the multi-copy nuclear ribosomal cistron (nrDNA) in order to identify fungi that might be found in low relative abundance and likely overlooked using single copy regions and metagenomic binning approaches. Furthermore, while portions of the nrDNA are highly conserved across fungi, we focused on the ITS region due to the high variability and well-curated reference database (Schoch et al. 2012; Nilsson et al. 2019). However, nrDNA copy number varies by orders of magnitude across fungi, from tens to over 1400 copies per genome (Lofgren et al. 2019). Therefore, the relative abundance of fungal groups inferred in this study (e.g., Fig. 2b) does not accurately depict true relative abundance of lichen-associated fungi given the potential for a very wide range of nrDNA copy number of these fungi.

Another source of potential bias is from the bioinformatic pipeline implemented here. Even using relatively well-established pipelines for analyzing ITS amplicon-based metagenomic reads, bioinformatics analysis pipelines have been shown to vary greatly in their relative performance and accuracy in characterizing fungi from metagenomic data (Anslan et al. 2018). We would anticipate that the data mining approach implemented in this study may have introduced a number of unexpected and difficult to identify artifacts, ranging from potentially over- and under-representing different fungal lineages to erroneous taxonomic assignments. For example, in the present study, a significant proportion of reads from *Xanthoparmelia* lichens were assigned to the class Entomophthoromycetes, a lineage that has not previously been found in association with lichens. Whether the inferred prevalence of Entomophthoromycetes is biased by copy number variation of the nrDNA, an artifact of read mapping to the UNITE database, etc., or accurately represents a novel finding is unclear. Furthermore, only a small fraction of the estimated 2.2–3.8 million fungal species are represented in currently available curated databases. Therefore, in fungal metabarcoding studies, a large proportion of OTUs cannot be assigned to any meaningful taxonomic group, and these unclassifiable species hypotheses, or 'dark taxa', remain problematic in metagenomic studies of fungi (Nilsson et al. 2019).

How might differences in sample preparation, DNA extraction approaches, and library preparation influence the range of captured diversity? How comparable are by-catch rDNA reads from transcriptomic sequencing with metagenomic sequencing, and might one have an advantage for detecting organisms found in low abundance, e.g. Cystobasidiomycete yeasts? The impact of these potential methodological limitations is not

clear. Furthermore, important aspects of the experimental design are lost when data mining available metagenomic reads. In this study, the only metagenomic lichen samples originating outside of North America were represented by all the *Umbilicaria* specimens. While our results clearly indicate that the fungal communities associating with *Umbilicaria* lichens are distinct from those associating with other lichen groups analyzed here, we have no reference to assess if these differences are related to evolutionary constraints of the mycobiont or continental-scale biogeographic factors shaping fungal communities. Similarly, our sampling of Physciaceae lichens represented broader ecological and evolutionary diversity than the other sampled lichen groups, likely masking important patterns in mycobiome communities associated with members of the Physciaceae and making comparisons of Physciaceae lichens with other lichen groups less direct. Taken together, our results highlight, on one hand, the presence of a highly diverse, seemingly lichen host-specific mycobiome, and on the other hand, the risk of applying overly simplistic techniques – such as phylum rank classifications – to tackle the diversity of these lichen-associated fungal communities.

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References

Anslan S, Nilsson RH, Wurzbacher C, Baldrian P, Leho T, Bahram M (2018) Great differences in performance and outcome of high-throughput sequencing data analysis platforms for fungal metabarcoding. MycoKeys 39:29–40

Arnold AE, Miadlikowska J, Higgins KL, Sarvate SD, Gugger P, Way A, Hofstetter V, Kauff F, Lutzoni F (2009) A phylogenetic estimation of trophic transition networks for ascomycetous fungi: are lichens cradles of symbiotrophic fungal diversification? Syst Biol 58:283–297

Bačkor M, Peksa O, Škaloud P, Bačkorová M (2010) Photobiont diversity in lichens from metal-rich substrata based on ITS rDNA sequences. Ecotoxicol Environ Saf 73:603–612

Bates ST, Cropsey GW, Caporaso JG, Knight R, Fierer N (2011) Bacterial communities associated with the lichen symbiosis. Appl Environ Microbiol 77:1309–1314

Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, Huttley GA, Gregory Caporaso J (2018) Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6:90

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

Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope EK, da Silva R, Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK, Jiang L, Kaehler BD, Kang KB, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciolek T, Kreps J, Langille MGI, Lee J, Ley R, Liu YX, Loftfield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik AV, Metcalf JL, Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Nothias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Pruesse E, Rasmussen LB, Rivers A, Robeson MS II, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ul-Hasan S, van der Hooft JJJ, Vargas F, Vázquez-Baeza Y, Vogtmann E, von Hippel M, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CHD, Willis AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R, Caporaso JG (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 37:852–857

Boustie J, Grube M (2005) Lichens - a promising sources of bioactive secondary metabolites. Plant Gen Res 3:273–287

Bradshaw M, Grewe F, Thomas A, Harrison CH, Lindgren H, Muggia L, St Clair LL, Lumbsch HT, Leavitt SD (2020) Characterizing the ribosomal tandem repeat and its utility as a DNA barcode in lichen-forming fungi. BMC Evol Biol 20:2

Cao S, Zhang F, Liu C, Hao Z, Tian Y, Zhu L, Zhou Q (2015) Distribution patterns of haplotypes for symbionts from *Umbilicaria esculenta* and *U muehlenbergii* reflect the importance of reproductive strategy in shaping population genetic structure. BMC Microbiol 15:212

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336

Cardinale M, Puglia AM, Grube M (2006) Molecular analysis of lichen-associated bacterial communities. FEMS Microbiol Ecol 57:484–495

Cardinale M, Grube M, Castro JV Jr, Muller H, Berg G (2012) Bacterial taxa associated with the lung lichen *Lobaria pulmonaria* are differentially shaped by geography and habitat. FEMS Microbiol Lett 329:111–115

Černajová I, Škaloud P (2019) The first survey of Cystobasidiomycete yeasts in the lichen genus *Cladonia*; with the description of *Lichenozyma pisutiana* gen nov, sp nov. Fungal Biol 123:625–637

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

Crittenden PD, David JC, Hawksworth DL, Campbell FS (1995) Attempted isolation and success in the culturing of a broad spectrum of lichen-forming and lichenicolous fungi. New Phytol 130:267–297

Dal Grande F, Alors D, Divakar PK, Bálint M, Crespo A, Schmitt I (2014) Insights into intrathalline genetic diversity of the cosmopolitan lichen symbiotic green alga *Trebouxia decolorans* Ahmadjian using microsatellite markers. Mol Phylogenet Evol 72:54–60

Dal Grande F, Sharma R, Meiser A, Rolshausen G, Büdel B, Mishra B, Thines M, Otte J, Pfenninger M, Schmitt I (2017) Adaptive

differentiation coincides with local bioclimatic conditions along an elevational cline in populations of a lichen-forming fungus. BMC Evol Biol 17:93

Dal Grande F, Rolshausen G, Divakar PK, Crespo A, Otte J, Schleuning M, Schmitt I (2018) Environment and host identity structure communities of green algal symbionts in lichens. New Phytol 217:277–289

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

Fernández-Brime S, Muggia L, Maier S, Grube M, Wedin M (2019) Bacterial communities in an optional lichen symbiosis are determined by substrate, not algal photobionts. FEMS Microbiol Ecol 95(3):fiz012. https://doi.org/10.1093/femsec/fiz012

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

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

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

Gostinčar C, Grube M, De Hoog S, Zalar P, Gunde-Cimerman N (2009) Extremotolerance in fungi: evolution on the edge. FEMS Microbiol Ecol 71:2–11

Goward T (2008) Nameless little things. Evansia 25:54–56

Grube M, Cardinale M, de Castro Jr JV, Muller H, Berg G (2009) Species-specific structural and functional diversity of bacterial communities in lichen symbioses. ISME J 3:1105–1115

Grube M, Cernava T, Soh J, Fuchs S, Aschenbrenner I, Lassek C, Wegner U, Becher D, Riedel K, Sensen CW, Berg G (2015) Exploring functional contexts of symbiotic sustain within lichen-associated bacteria by comparative omics. ISME J 9:412–424

Hawksworth DL, Lücking R (2017) Fungal diversity revisited: 22 to 38 million species. Microbiol Spectr 5

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson O, Huhndorf S, James T, Kirk PM, Lücking R, Lumbsch T, Lutzoni F, Matheny PB, McLaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai Y-C, Gams W, Geiser DM, Griffith GW, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde K, Koljalg U, Kurtzman CP, Larsson KH, Lichtward R, Longcore J, Miadlikowska J, Miller A, Monclavo J-M, Mozley-Standridge S, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schuessler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White M, Winka K, Yao Y-J, Zhang N (2007) A higher-level phylogenetic classification of the Fungi. Mycol Res 111:509–547

Hodkinson B, Lutzoni F (2009) A microbiotic survey of lichen-associated bacteria reveals a new lineage from the Rhizobiales. Symbiosis 49: 163–180

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. Environ Microbiol 14:147–161

Honegger R (2000) Simon Schwendener (1829-1919) and the dual hypothesis of lichens. Bryologist 103:307–313

Honegger R (2001) The symbiotic phenotype of lichen-forming ascomycetes. In: Fungal Associations. Springer, Berlin, Heidelberg, pp 165–188

Honegger R (1993) Developmental biology of lichens. New Phytol 125: 659–677

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A (2012) Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649

Keepers KG, Pogoda CS, White KH, Anderson Stewart CR, Hoffman JR, Ruiz AM, Mccain CM, Lendemer JC, Kane NC, Tripp EA (2019) Whole genome shotgun sequencing detects greater lichen fungal diversity than amplicon-based methods in environmental samples. Front Ecol Evol:7

Kono M, Tanabe H, Ohmura Y, Satta Y, Terai Y (2017) Physical contact and carbon transfer between a lichen-forming *Trebouxia* alga and a novel *Alphaproteobacterium*. Microbiology 163:678–691

LaBonte NR, Jacobs J, Ebrahimi A, Lawson S, Woeste K (2018) Data mining for discovery of endophytic and epiphytic fungal diversity in short-read genomic data from deciduous trees. Fungal Ecol 35:1–9

Lawrey JD, Diederich P (2003) Lichenicolous fungi: interactions, evolution, and biodiversity. Bryologist 106:80–120

Leavitt SD, Johnson LA, Goward T, St Clair LL (2011) Species delimitation in taxonomically difficult lichen-forming fungi: an example from morphologically and chemically diverse *Xanthoparmelia* (Parmeliaceae) in North America. Mol Phylogenet Evol 60:317–332

Leavitt SD, Fernández-Mendoza F, Pérez-Ortega S, Sohrabi M, Divakar PK, Vondrák J, Lumbsch HT, St. Clair LL (2013a) Local representation of global diversity in a cosmopolitan lichen-forming fungal species complex (*Rhizoplaca*, Ascomycota). J Biogeogr 40:1792–1806

Leavitt SD, Nelsen MP, Lumbsch HT, Johnson LA, St Clair LL (2013b) Symbiont flexibility in subalpine rock shield lichen communities in the southwestern USA. Bryologist 116:149–161

Leavitt SD, Kraichak E, Nelsen MP, Altermann S, Divakar PK, Alors D, Esslinger TL, Crespo A, Lumbsch HT (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). Mol Ecol 24:3779–3797

Leavitt SD, Grewe F, Widhelm T, Muggia L, Wray B, Lumbsch HT (2016) Resolving evolutionary relationships in lichen-forming fungi using diverse phylogenomic datasets and analytical approaches. Sci Rep 6:22262

Leavitt SD, Keuler R, Newberry CC, Rosentreter R, St Clair LL (2019) Shotgun sequencing decades-old lichen specimens to resolve phylogenomic placement of type material. Plant Fungal Syst 64: 237–247

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. Am J Bot 106:1090–1095

Lofgren LA, Uehling JK, Branco S, Bruns TD, Martin F, Kennedy PG (2019) Genome-based estimates of fungal rDNA copy number variation across phylogenetic scales and ecological lifestyles. Mol Ecol 28:721–730

Lu J, Magain N, Miadlikowska J, Coyle JR, Truong C, Lutzoni F (2018) Bioclimatic factors at an intrabiome scale are more limiting than cyanobiont availability for the lichen-forming genus *Peltigera*. Am J Bot 105:1198–1211

McDonald D, Clemente JC, Kuczynski J, Rideout JR, Stombaugh J, Wendel D, Wilke A, Huse S, Hufnagle J, Meyer F, Knight R, Caporaso JG (2012) The biological observation matrix (BIOM) format or: how I learned to stop worrying and love the ome-ome. Gigascience 1:7

McKinney W (2010) Data structures for statistical computing in Python

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

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, Grube M (2018) Fungal diversity in lichens: from extremotolerance to interactions with algae. Life 8:15

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 Microbiol Ecol 85:313–323

Muggia L, Pérez-Ortega S, Kopun T, Zellnig G, Grube M (2014) Photobiont selectivity leads to ecological tolerance and evolutionary divergence in a polymorphic complex of lichenized fungi. Ann Bot 114:463–475

Muggia L, Nelsen MP, Kirika PM, Barreno E, Beck A, Lindgren H, Lumbsch HT, Leavitt SD, Trebouxia working group (2020) Formally described species woefully underrepresent phylogenetic diversity in the common lichen photobiont genus Trebouxia (Trebouxiophyceae, Chlorophyta): An impetus for developing an integrated taxonomy. Mol Phylogenet Evol 106821. https://doi.org/10.1016/j.ympev.2020.106821

Muggia L, Fleischhacker A, Kopun T, Grube M (2016) Extremotolerant fungi from alpine rock lichens and their phylogenetic relationships. Fungal Divers 76:119–142

Nilsson RH, Larsson K-H, Taylor AFS, Bengtsson-Palme J, Jeppesen TS, Schigel D, Kennedy P, Picard K, Glöckner FO, Tedersoo L, Saar I, Kõljalg U, Abarenkov K (2019) The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. Nucleic Acids Res 47:D259–D264

Paul F, Otte J, Schmitt I, Dal Grande F (2018) Comparing sanger sequencing and high-throughput metabarcoding for inferring photobiont diversity in lichens. Sci Rep 8:8624

Pedregosa F, Varoquaux G, Gramfort A, Michel V, Thirion B, Grisel O, Blondel M, Prettenhofer P, Weiss R, Dubourg V, Vanderplas J, Passos A, Cournapeau D, Brucher M, Perrot M, Duchesnay É (2011) Scikit-learn: machine learning in Python. J Mach Learn Res 12:2825–2830

Petrini O, Hake U, Dreyfuss MM (1990) An analysis of fungal communities isolated from fruticose lichens. Mycologia 82:444–451

Pizarro D (2019) Metagenomic sequencing with new bioinformatics approaches to understand the evolution of lichen forming fungi (dissertation). Complutense University of Madrid Complutense University of Madrid, Madrid

R Core Team (2019) The R project for statistical computing. http://www.r-project.org/

Rognes T, Flouri T, Nichols B, Quince C, Mahe F (2016) VSEARCH: a versatile open source tool for metagenomics. PeerJ 4:e2584

Schoch CL, Seifert KA, Huhndorf S et al (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. Proc Natl Acad Sci 109:6241–6246. https://doi.org/10.1073/pnas1117018109

Singh G, Dal Grande F, Schnitzler J, Pfenninger M, Schmitt I (2018) Different diversification histories in tropical and temperate lineages in the ascomycete subfamily Protoparmelioideae (Parmeliaceae). Mycokeys 36:1–19

Škaloud P, Moya P, Molins A, Peksa O, Santos-Guerra A, Barreno E (2018) Untangling the hidden intrathalline microalgal diversity in *Parmotrema pseudotinctorum*: *Trebouxia crespoana* sp nov. Lichenologist 50:357–369

Spribille T, Tuovinen V, Resl P, Vanderpool D, Wolinski H, Aime MC, Schneider K, Stabentheiner E, Toome-Heller M, Thor G, Mayrhofer H, Johannesson H, McCutcheon JP (2016) Basidiomycete yeasts in the cortex of ascomycete macrolichens. Science 353:488–492

Spribille T, Tagirdzhanova G, Goyette S, Tuovinen V, Case R, Zandberg WF (2020) 3D biofilms: in search of the polysaccharides holding together lichen symbioses. FEMS Microbiol Lett 367:fnaa023.a

Steinova J, Skaloud P, Yahr R, Bestova H, Muggi L (2019) Reproductive and dispersal strategies shape the diversity of mycobiont-photobiont association in *Cladonia* lichens. Mol Phylogenet Evol 134:226–237

Tuovinen V, Ekman S, Thor G, Vanderpool D, Spribille T, Johannesson H (2019) Two basidiomycete fungi in the cortex of wolf lichens. Curr Biol 29:476–483e475

U'Ren JM, Lutzoni F, Miadlikowska J, Arnold AE (2010) Community analysis reveals close affinities between endophytic and endolichenic fungi in mosses and lichens. Microb Ecol 60:340–353

Velmala S, Myllys L, Halonen P, Goward T, Ahti T (2009) Molecular data show that *Bryoria fremontii* and *B tortuosa* (Parmeliaceae) are conspecific. Lichenologist 41:231–242

Wang Y, Zheng Y, Wang X, Wei X, Wei J (2016) Lichen-associated fungal community in *Hypogymnia hypotrypa* (Parmeliaceae, Ascomycota) affected by geographic distribution and altitude. Front Microbiol 7:1231

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

Wickham H (2016) ggplot2: elegant graphics for data analysis. Springer-Verlag, New York

Wickham H, Averick M, Bryan J, Chang W, McGowan L, François R, Grolemund G, Hayes A, Henry L, Hester J (2019) Welcome to the Tidyverse. J Open Source Software 4:1686

Yu NH, Park SY, Kim JA, Park CH, Jeong MH, Oh SO, Hong SG, Talavera M, Divakar PK, Hur JS (2018) Endophytic and endolichenic fungal diversity in maritime Antarctica based on cultured material and their evolutionary position among Dikarya. Fungal Syst Evol 2:263–272

Zhang T, Wei XL, Zhang YQ, Liu HY, Yu LY (2015) Diversity and distribution of lichen-associated fungi in the Ny-Alesund region (Svalbard, high Arctic) as revealed by 454 pyrosequencing. Sci Rep 5:14850