# Lichens as tools to monitor disturbances in the La Sal Mountains

## Final Report Submitted to Canyonlands Natural History Association

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Principal investigator: Steven Leavitt, Brigham Young University
steve_leavitt@byu.edu

Agency: U.S. Forest Service (Barb Smith)
barb.smith@usda.gov

# HIGHLIGHTS

- Funding from Canyonland Natural History Association resulted in two peer-reviewed publications, with a third currently under review
    - ZHANG, Y., CLANCY, J., JENSEN, J., MCMULLIN, R. T., WANG, L., & LEAVITT, S. D. (2022). PROVIDING SCALE TO A KNOWN TAXONOMIC UNKNOWN—AT LEAST A 70-FOLD INCREASE IN SPECIES DIVERSITY IN A COSMOPOLITAN NOMINAL TAXON OF LICHEN-FORMING FUNGI. JOURNAL OF FUNGI, 8(5), 490.
    - LEAVITT, S. D., HOLLINGER, J., SUMMERHAYS, S., MUNGER, I., ALLEN, J., & SMITH, B. (2021). ALPINE LICHEN DIVERSITY IN AN ISOLATED SKY ISLAND IN THE COLORADO PLATEAU, USA—INSIGHT FROM AN INTEGRATIVE BIODIVERSITY INVENTORY. ECOLOGY AND EVOLUTION, 11(16), 11090-11101.
    - ROBISON, A., BAUGH, M., MUGGIA, L., & LEAVITT, S. D. (UNDER REVIEW) FRUTICOSE LICHEN COMMUNITIES AT THE EDGE: DISTRIBUTION AND DIVERSITY IN A DESERT SKY ISLAND ON THE COLORADO PLATEAU. CONSERVATION.

- Supported undergraduate research training – 7students
    - THREE CONFERENCE PRESENTATIONS
    - TWO PEER-REVIEWED RESEARCH ARTICLES, PLUS A THIRD UNDER REVIEW (LISTED ABOVE)

- Project resulted in collaborations with researchers from Canada (Dr. Troy McMullin, Canadian Museum of Nature), China (Dr. Yanyun Zhang, Anhui Normal University), and Italy (Dr. Lucia Muggia, University of Trieste)

- Documented three lichen-draped conifer sites in the subalpine forest in the La Sal Mountains

Lichen-draped conifer in the "Moonlight Meadows" area near Geyser Pass (headwaters of Mill Creek).

# PROJECT GOALS & DELIVERABLES

**Fire-damage boreal oakmoss lichen in the "Moonlight Meadows" area of the headwaters of Mill Creek headwaters.** The "Pack Creek Fire" caused had a significant impact near lichen-draped conifer sites in 2021, although the these sites remained largely unburned.

(1) **DOCUMENTING THE RANGE AND DISTRIBUTION OF CONIFER STANDS WITH ABUNDANT FRUTICOSE LICHENS AND CHARACTERIZING THE ASSOCIATED LICHENS AND ENVIRONMENTAL FACTORS FACILITATING THESE VULNERABLE COMMUNITIES.**

We documented three, spatially restricted lichen-draped conifer sites in subalpine *Picea engelmanii* forests. A full description of this study was submitted on 31 August 2022 to the journal *Conservation*. We will provide the final version of the manuscript once it is accepted for publication and made available. In the meantime, the submitted manuscript is available as Appendix 1. Supplementary files associated with this study can be found here. We note that the Pack Creek Fire in June 2021 destroyed over half of the dataloggers placed to characterized abiotic factors influencing these communities, and that component of the student was abandoned.

> Publication (in review): Robison, A., Baugh, M., Muggia, L., & LEAVITT, S. D. (under review) Fruticose lichen communities at the edge: distribution and diversity in a desert sky island on the Colorado Plateau. Conservation.

**Highlights from research – see Appendix 1 for full details**

- La Sal Mountains harbor the most well-developed fruticose lichen communities in the Colorado Plateau and Great Basin
  - only known to occur at three spatially restricted sites
  - a total of 30 lichens occur in these communities, seven representing three fruticose genera
    - We report a number of likely new records for the Colorado Plateau. For example, the fungus *Schizoxylon albescens* Gilenstam, Döring & Wedin documented here, which can occur both as a lichen and a saprobe, represents one of the first reports for North America. *Tetramelas pulverulentus* (Anzi) A. Nordin & Tibell and *Bibbya vermifera* (Nyl.) Kistenich, Timdal, Bendiksby & S.Ekman are also reported for likely the first time

on the Colorado Plateau. Our study also provides the first evidence of *Myriolecis juniperina* in subalpine forests. However, limitations in presently available DNA reference libraries, in addition to inherent limitations to DNA-based specimen identification, highlight that the occurrence of these taxa must be interpreted with caution. Final determinations for the unexpected or unusual lichens must be confirmed with physical voucher specimens.

        - Haplotype diversity in fruticose lichens suggest variable dispersal strategies and likely variable response to ecological disturbances.
            - The *Ramalina sinensis* populations in the La Sals represent a lineage genetically distinct from other *R. sinensis* populations worldwide
            - *Evernia divaricata* likely colonized the La Sal Mountains multiple times independently, with haplotypes from the La Sals intermixed with haplotypes from specimens collected worldwide.
            - *Usnea cavernosa*, the most abundant lichen in the lichen-draped conifer sites, likely has high dispersal capacity as evidenced by DNA sequences that are highly similar to other populations in the Intermountain West and Europe.
    - We document a diverse fungal community found in association with the foliose lichen communities
        - 160 genetic clusters from Ascomycetes representing 22 orders spanning seven classes
        - Reads from basidiomycetes were less common in our data (44 genetic clusters) but represented expected fungal lineages occurring with lichens, including Tremellomycetes and Cystobasidiomycetes
    - also document nearly 160 genetic clusters representing Ascomycetes

- Our results provide a baseline for ongoing monitoring and help to raise awareness of unique lichen communities and other biodiversity in the region.

(2) **Documenting populations of three, rare alpine lichens in the La Sal Mountains – "mountain sausage lichen" (*Broado oroarctica*), "varnished tube lichen" (*Hypogymnia austerodes*) and "fine rockwool" (*Pseudephebe pubescens*).** "

Despite extensive field work in 2021 and 2022, only a single new population of *Pseudephebe pubescens* was observed (near the summit of Mount Tukuhnikivats, along the eastern ridgeline. Neither *Hypogymnia austerodes* or *Brodoa oroarctica* have been observed since our surveys begin in 2017 and these lichens may now be extirpated or occur at very low frequencies.

The results of our surveys have been reported in a peer-reviewed research article published in the journal *Ecology and Evolution*. The article is linked in the citation below and also provided as Appendix 2.

- Leavitt, S. D., Hollinger, J., Summerhays, S., Munger, I., Allen, J., & Smith, B. (2021). Alpine lichen diversity in an isolated sky island in the Colorado Plateau, USA—Insight from an integrative biodiversity inventory. Ecology and evolution, 11(16), 11090-11101.

**Highlights from research – see Appendix 2 for full details**

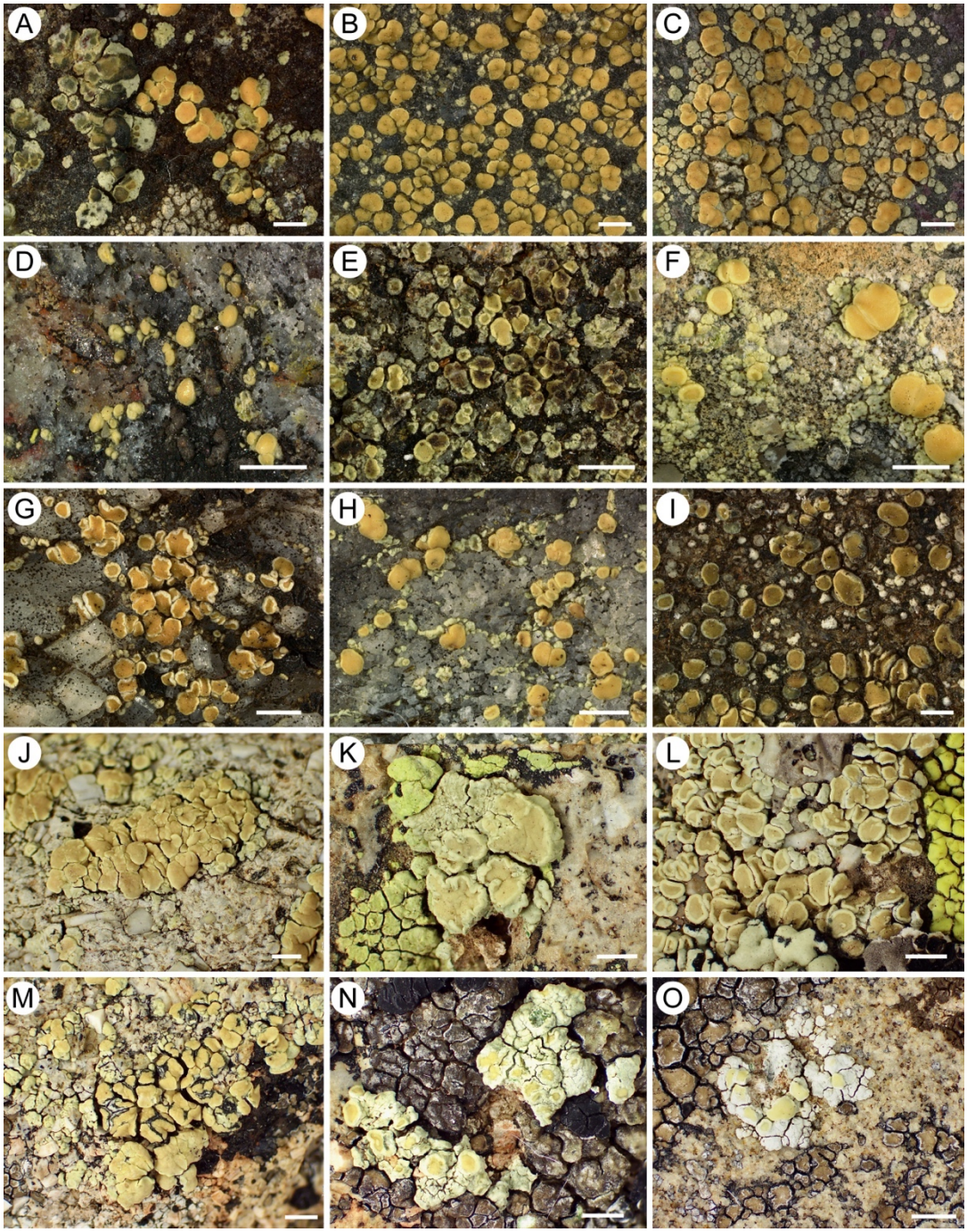
- We documented the most diverse alpine lichen community known to date from the southern Rocky Mountains, with up to 240 candidate species/species-level lineages of lichen-forming fungi.

- 139 species were inferred using integrative taxonomy, plus an additional 52 candidate species within 29 different putative species complexes.

- By integrating vouchered specimens, DNA sequence data, and photographic documentation, we provide an important baseline of lichen-forming fungal diversity for the limited alpine habitat in the Colorado Plateau.

- These data provide an important resource for subsequent research in the ecology and evolution of lichens alpine habitats, including DNA barcodes for most putative species/species-level lineages occurring in the La Sal Mountains, and vouchered collections representing any potentially undescribed species that can be used for future taxonomic studies.

(3) **Discovering hidden diversity in "granite-speck rim-lichens" (*Lecanora polytropa*) populations in the La Sals.** Previous CNHA-funding research revealed a number of lichen groups with incredible, previously overlooked diversity. Among these, the "granite-speck rim-lichens", previously thought the be a single species, likely comprises multiple distinct species.




**Variation in "granite-speck rim-lichens" throughout North America.** Our study revealed that this one taxon likely represents at least 70 species, 14 of which occur in alpine habitats in the La Sals.

This project resulted in a fruitful collaboration with colleagues in China, Canada, and Spain, in addition to mentoring multiple undergraduate students. The results of the first portion of this study were published in the *Journal of Fungi*, a peer-reviewed research journal.

The article is linked in the citation below and also provided as Appendix 3.

- Zhang, Y., Clancy, J., Jensen, J., McMullin, R. T., Wang, L., & Leavitt, S. D. (2022). Providing Scale to a Known Taxonomic Unknown—At Least a 70-Fold Increase in Species Diversity in a Cosmopolitan Nominal Taxon of Lichen-Forming Fungi. Journal of Fungi, 8(5), 490.
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**Highlights from research – see Appendix 3 for full details**

- species delimitations models revealed up to 103 species in the *L. polytropa* clade,
  - 75 corresponded to the nominal taxon *L. polytropa*.
- Inferences from phylogenomic alignments generally supported that these represent evolutionarily independent lineages or species.
- Less than 10% of the candidate species were comprised of specimens from multiple continents.
- High levels of candidate species were recovered at local scales but generally with limited overlap across regions.
- *Lecanora polytropa* likely ranks as one of the largest species complexes of lichen-forming fungi known to date.

The research has also spawned several other associated projects, including work looking infer glacial refugia sites in Spain, Alaska, and the Intermountain West. Furthermore, we are currently working to write formal descriptions for some of the undescribed species-level lineages occurring in the La Sal Mountains. However, the type specimen for *Lecanora polytropa* was collected in the Rocky Mountains, and we need to match the type species to the appropriate candidate species before describing new species. Working with Dr. Troy McMullin (Canadian Museum of Nature), we hope to complete the initial taxonomic revision within the next year.

This project has now expanded to look at diversity in another common lichen occurring in the La Sals – *Lecidea atrobrunnea.* We have found unexpected high levels of diversity in this nominal taxon too, and will be submitting our finds for publication (recognizing support from CNHA) in October 2022.

*Lecidea atrobrunnea*, a montane and alpine specialist. This nominal taxon also hides multiple undescribed species-level lineages that ultimately will merit formal recognition.

**(4) OUTREACH**

Moab Festival of Science 2021. Our work in the La Sals was showcased at two "lichen hikes" during the 2021 Moab Festival of Science. We will be participating again in 2022, recognizing the support from CNHA in our lichen research near Moab.

Guided excursion

To showcase the diversity of lichen communities in the La Sals, I would be happy to lead an excursion for board members of CNHA, Forest Service personnel, and other interested parties. Also, I would welcome any opportunity to share the results of this study in public lectures organized by CNHA or the Forest Service.

**Fire damage to unique lichen-draped conifer sites near Geyser Pass in the La Sal Mountains.** Support from CNHA facilitated the establishment of baseline data crucial for long-term monitoring.

# APPENDIX 1:

# FRUTICOSE LICHEN COMMUNITIES AT THE EDGE: DISTRIBUTION AND DIVERSITY IN A DESERT SKY ISLAND ON THE COLORADO PLATEAU

*conservation*

MDPI

*Article*

# Fruticose lichen communities at the edge: distribution and diversity in a desert sky island on the Colorado Plateau

**Abigail Robison[1], Mikele Baugh[2], Lucia Muggia[3], and Steven D. Leavitt [4,*]**

[1] Brigham Young University, Department of Biology, Provo, UT, USA 84602; abigail.robison@gmail.com
[2] Brigham Young University, Department of Biology, Provo, UT, USA 84602; mikele.baugh@gmail.com
[3] University of Trieste, Department of Life Sciences, via Licio Giorgieri 10, 34127 Trieste, Italy; lucia_muggia@hotmail.com
*,[4] Brigham Young University, Department of Biology & M.L. Bean Life Science Museum, Provo, UT, USA 84602; Correspondence: steve_leavitt@byu.edu; Tel.: 1-801-422-4879

**Abstract:** Subalpine habitats in sky islands in the Southwestern USA are currently facing large-scale transformations due to interactions among climate change, habitat alteration, increased fire frequency, etc. Lichens have widely been used as bioindicators of environmental change. On the Colorado Plateau, fruticose lichens occur in patchy, disconnected populations, including unique lichen-draped conifer sites in subalpine forests in the La Sal Mountains in southeastern Utah. Here, we aim to document the distribution and fungal diversity within these lichen communities. We find that lichen-draped conifer sites are restricted to only three known, small areas in *Picea englemannii* forests above 3000 m.ASL, two of which have recently been impacted by wildfire. We document 30 different species of lichen-forming fungi in these communities, several which represent the first reports from the Colorado Plateau. We also characterize mycobiont haplotype diversity for the fruticose lichen populations. We also report a range of diverse fungi associated with these lichens, including genetic clusters representing 22 orders spanning seven classes of Ascomycetes and more limited clusters representing Basidiomycetes. Our results provide a baseline for ongoing monitoring and help to raise awareness of unique lichen communities and other biodiversity in the region.

**Keywords:** amplicon sequencing, biodiversity; biomonitoring; ecological sampling; epiphyte; Illumina; internal transcribed spacer region (ITS); inventory; ITS2; subalpine; semi-arid

**Citation:** Lastname, F.; Lastname, F.; Lastname, F. Title. *Conservation* **2022**, *2*, Firstpage–Lastpage. https://doi.org/10.3390/xxxxx

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## 1. Introduction

Some habitats in temperate forests are currently being driven toward large-scale transformations due to interactions among climate change, habitat alteration, severe wildfires, insects and pathogens, and other disturbances [1]. The nature of these ecological disturbances has serious ramifications for regional biodiversity and ecosystem health, as these and other factors directly and abruptly affect forest vegetation [2]. Subalpine habitats in sky islands in the Southwestern USA are particularly vulnerable to these contemporary disturbances, with biological communities likely becoming more isolated and potentially negatively impacting regional biodiversity [2,3]. With increasing temperatures, aridification, and changes in precipitation patterns in the Southwestern USA, subalpine habitats in sky islands are experiencing increases in drought, fire frequency, and fire severity [4,5]. In North American subalpine forest, fire return intervals are becoming shorter, negatively influencing forests' ability to recover after fire [6]. Extensive disturbances in these forest may lead to new vegetations states with novel responses to climate [2]

Species adapted to high altitude/latitude habitats may be particularly vulnerable to extirpation if changing habitat conditions outpace the rate of dispersal [7]. Peripheral and isolated populations of subalpine species rank as potential indicators for monitoring

change in the Southwestern USA, as they may be affected before other more common, connected communities [8]. For many vulnerable species/organismal groups, limited understanding of the spatial and temporal components of a species' life history characteristics may stimy researchers' ability to utilize these in monitoring and conservation research or may lead to possible misinterpretation of processes that the indicator species aims to unravel [9,10].

Lichens have long been utilized to monitor ecological disturbances [11-16] and more recently to inform conservation decisions [17,18]. Lichens that have patchy distributions may be more vulnerable to extinction/extirpations, particularly epiphytes of mountain forests [19,20]. Connected, large populations/communities facilitate recolonization when one patch is at risk [21], and disconnected and smaller populations/communities may be particularly vulnerable to disturbances [22]. Even where species have broadly distributed and well-connected populations, peripheral or patchy populations can be more vulnerable than those at the center of a larger metapopulation [23]. Peripheral populations may promote range expansion, but when suitable habitat occurs in isolated patches, such as sky islands, expansion into new suitable habitat may not be possible. Patchy distributions coupled with new vegetations states because of contemporary disturbances may fundamentally alter the occurrence of epiphytic lichens [2,24]

Fruticose lichens are present in patchy, disconnected populations across the Colorado Plateau in the Southwestern USA (Fig. 1). While these fruticose lichens are found in temperate forests worldwide, boreal and temperate elements of western North America are genetic "hot spots" for some epiphytic lichens [25,26]. While most fruticose lichen populations are quite small and spatially restricted on the Colorado Plateau, robust lichen communities with high population density and biomass have been observed (Fig. 2). During excursions in the La Sal Mountain Range ("La Sals"), a sky island on the Colorado Plateau, in southeastern Utah, USA, we observed spatially restricted robust lichen communities draping conifers in subalpine forests (Fig. 2). The lichen-draped conifer sites are dominated by the lichens *Evernia divaricata* (L.) Ach., *Ramalina sinensis* Jatta, and various *Usnea* species. Presently, little is known about these sites, including the extent of lichen-draped conifers sites, the amount of genetic variation, the range of associated lichens, and factors influencing their origin and persistence. Given the apparent limited, restricted distributions, the sites likely merit careful conservation consideration.

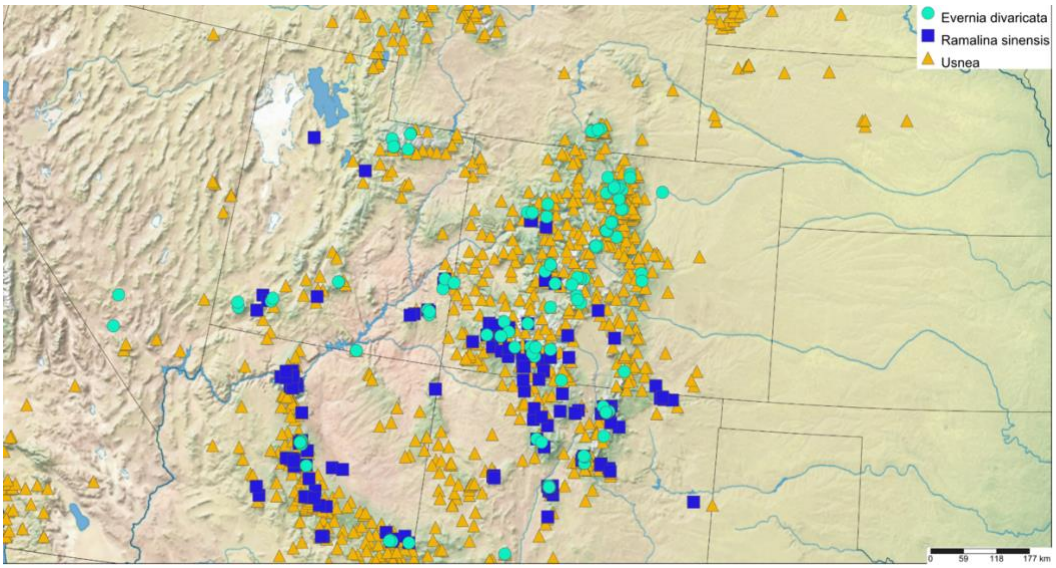

Fig. 1. Distribution of fruticose lichen *Evernia divaricata*, *Ramalina sinensis*, and *Usnea* species across the Intermountain West region of the USA. The La Sal Mountain are located in southeastern Utah near the state border with Colorado.

The purpose of this study is to (1) characterize for the first time the extent of lichen-draped conifer sites in subalpine forest in an isolated sky island on the Colorado Plateau – the La Sals, (2) inventory the range of lichen diversity in these communities, (3) assess genetic diversity within the fruticose lichen populations of *E. divaricata*, *R. sinensis*, and *U.* spp., and (4) characterize the lichen-associated fungal community. This information will be important for establishing a biomonitoring baseline and assessing conservation needs in unique subalpine communities in a region that is vulnerable to large-scale ecological transformations.

## 2. Materials and Methods

*Site selection, field methods, and bulk sampling*

For this study, subalpine forests in the La Sal Mountains were opportunistically surveyed between July 2018 – July 2022 to identify the number and extent of spatially restricted robust lichen communities draping conifers (Fig. 2). Survey locations were identified, in part, by using Google Earth Pro to identify suitable habitat, considering road and trail access points, in addition to proximity to water sources, e.g., streams or other wetland sites. Here, "lichen-draped conifer sites" were qualitatively characterized by the presence of at least two of the three common fruticose lichens – *Evernia divaricata*, *Ramalina sinensis*, and *Usnea* spp. – and a density similar to those shown in Fig. 2, e.g., sporadic occurrences of limited numbers of fruticose lichen thalli on a limb/bole were not considered to represent lichen-draped conifer sites.

**Fig. 2 (previous page)**. Lichen-draped conifer sites in the La Sal Mountains. The top row includes photos from site 'Geyser Pass 1', 'Geyser Pass 2', and 'Medicine Lake' (left to right). The bottom panel depicts one of the 15 lichen covered branches sampled for DNA metabarcoding.

In addition to identifying lichen-draped conifer sites in the La Sal Mountains, we also attempted to characterize the full range of lichen-forming fungi at one of the most superficially diverse sites. Within the "Geyser Pass 2" site – see Table 1, lichen samples were collected in July 2021, employing an "intuitive meander" method. The overarching aim was to collect representative bulk samples to comprehensively represent the epiphytic lichen diversity in these locally unique communities. Fifteen lichen-covered branches, ca. 0.5 m long (Fig. 2), were collected from 15 trees separated by at least three meters. Lichens occurring on rock and soil were not collected. Furthermore, we did not target lichens occurring on conifers beyond the dense, fruticose-dominated lichen communities, e.g., lichens occurring near the base of the tree or those on branches with limited or no fruticose lichens. In addition the main lichen-forming fungi, lichens also harbor complex fungal communities – the 'mycobiome' [27-29], and these may play important roles in facilitating the development of fruticose lichen thalli [30-32]. Therefore, we attempted to characterize the mycobiome and other associated fungi from the same samples. Fungal diversity was inferred using a DNA metabarcoding approach [28] – see below.

**Table 1**. The three lichen-draped conifer sites identified in the La Sal Mountains, a sky island on the Colorado Plateau.

| Name | Estimated area | Coordinates | Altitude |
|---|---|---|---|
| Geyser Pass 1 | 10,000 $m^2$ | 38.4864, -109.2491 | 3050 m.ASL |
| Geyser Pass 2 | 19,000 $m^2$ | 38.4821, -109.2368 | 3200 m.ASL |
| Medicine Lake | 65,000 $m^2$ | 38.4118, -109.2458 | 3040 m.ASL |

Samples were collected, dried in the field, returned to the herbarium, and stored at -80° C until the bulk sampling and DNA extraction steps. In the herbarium, fruticose lichens representing *E. divaricata*, *R. sinensis*, and *U.* species were carefully removed from the 15 sampled branches and roughly sorted by morphology. Using sterilized tweezers, small portions of thallus material from the fruticose lichens were carefully removed and placed directly into a sterile Nasco Whirl-Pak 18 oz. collecting bag (Nasco, Fort Atkinson, WI, USA). We attempted to sample similarly sized pieces of the apical part of thallus material from each fruticose lichen collected for the fruticose bulk sample. We also prepared a separate bulk sample representing smaller crustose and foliose lichens occurring on the bark of the sampled branches. Using a 10× hand lens, small, similarly sized portions of lichen thalli were sampled from all potentially different lichens using sterilized tweezers to pick or scrape material for bulk, metagenomic analyses of crustose and foliose lichens occurring with the fruticose lichens.

Twenty *Usnea* thalli representing the range of observed morphological diversity (Fig. 3) were selected to investigate the secondary metabolite variation using thin layer chromatography (TLC), following standard methods with solvent system 'G' (Culberson 1972; Orange et al. 2001), and these were identified using traditional phenotype-based approaches.

*Molecular Laboratory Methods*

To help ensure that metagenomic samples representing small crustose and foliose lichens weren't overwhelmed by DNA from the larger fruticose lichens, DNA was extracted from the fruticose and crustose/foliose lichen community samples separately. Community samples were homogenized using sterilized mortar and pestles; and DNA was extracted from homogenized material from each sample using the PowerMax Soil DNA Isolation Kit (Qiagen). To characterize the range of fungal diversity in the bulk samples, we amplified a portion of the internal transcribed spacer region – the standard barcoding region for fungi [3] – from each meta-community DNA extraction. Specifically, the hypervariable ITS2 region was amplified using polymerase chain reaction PCR with primers ITS3F (GCATCGATGAAGAACGCAGC) with ITS4R (TCCTCCGCTTATTGATATGC). PCR products were sequenced at RTL Genomics (Lubbock, TX), using the Illumina MiSeq 2x300 paired-end MiSeq platform.


**Fig. 3.** Photos of representative *Usnea* specimens collected at "Geyser Pass 1" site. *Usnea cavernosa* (panel 'A'), was the most common *Usnea* species sampled in the study. Scale bar = 3 cm (photo credit: S. Leavitt)

*Short-read data analyses*

FROGS v3.2 (Find, Rapidly OTUs with Galaxy Solution) was used to analyze ITS2 amplicon metabarcoding data [33,34]. FROGS v3.2 is a standardized pipeline containing a set of tools used to process amplicon reads produced from Illumina sequencing. We followed the protocol outlined in [34]. In short, paired-end reads for each sequence in the data were merged, primers were trimmed, and unmatched sequences were discarded in the FROGS v3.2 preprocessing step. Merged reads were then filtered using the FROGS v3.2 swarm clustering tool; and the clusters were formed using the aggregation distance clustering set to 1, as per the guidelines for v3.2. Chimeric sequences were then removed using the FROGS v3.2 chimera removal tool implementing default parameters. The FROGS v3.2 filtering tool was then used to remove low abundance clusters by setting the minimum proportion of sequences to keep OTUs to 0.000005. All remaining clusters were filtered using the ITSx tool to ensure that clusters met requirements for the ITS2 region in preparation for the taxonomic affiliation step. Initial taxonomic assignment of the clusters was completed by comparing the clusters passing filters to the UNITE 8.3 database using the RDP probabilistic classifier [35] and BLAST comparisons [36]. All analyses were performed on the Migale Galaxy Server. Taxonomic assignments of lichen-forming fungi inferred from the FROGS pipeline, were refined using sequence comparisons from the BOLD Project LIMWSL – "Lichens of the Intermountain West". Non-lichen-forming fungi were considered at the taxonomic levels of class and order. In cases were the RDP and BLAST-based taxonomic assignments differed at the class and order levels, the taxonomic assignment was considered "unknown". We note that using sequence similarity to infer taxonomic identity and other issues with publicly available sequences come with significant caveats [36,37].

We also attempted to characterize haplotype diversity in fruticose lichens, e.g., *Evernia divaricata*, *Ramalina sinensis*, and *Usnea* spp., at the "Geyser Pass 2" site. To find

unique haplotypes within each of the three fruticose lichens, short reads were mapped to the clusters representing (1) *E. divaricata*, (2) *R. sinensis*, and (3) *Usnea* spp., separately using Geneious Prime 2022.1.1, implementing the Geneious Prime 'Map to Reference' option set to "Low Sensitivity / Fastest", iterated two times and saving the mapped reads ("used reads"). The used reads were then clustered using the CD-HIT web server [38], and clustering reads at 100% similarity. Only clusters represented by ten more identical reads were considered. For both *E. divaricata* and *R. sinensis*, all ITS sequences presently available on GenBank were downloaded and combined with the taxon-specific haplotypes. The *Usnea* haplotypes were combined with ITS sequences compiled in [39]. Multiple sequences alignments were made using the program MAFFT v7 [40,41] implemented the G-INS-i alignment algorithm and '1PAM / K=2' scoring matrix, with an offset value of 0.1, the 'unalignlevel' = 0.2, and the remaining parameters were set to default values. A maximum-likelihood (ML) tree was inferred from each ITS alignment using IQ-TREE [42] to characterize the range of haplotype diversity within each taxon.

## 3. Results

Our surveys of forests in the La Sal Mountains on the Colorado Plateau revealed the widespread occurrence the fruticose lichens *Ramalina sinensis* and *Usnea* spp., with *Evernia divaricara* restricted to more limited habitat in subalpine forests (also rarely occurring on alpine turf). Despite the widespread occurrence of fruticose lichens in the La Sals, lichen-draped conifers were found at only three sites, two at headwater drainages of Mill Creek, near Geyser Pass, and one near Medicine Lakes (Table 1). The complete area for each identified site with extensive lichen-draped conifers is provided as supplementary file S1 (polygon area). Habitat surrounding the two lichen-draped conifer sites near Geyser Pass was nearly completely burned in the "Pack Creek Fire" in 2021 (https://utahfireinfo.gov/2021/06/26/pack-creek-fire-june-26-update/), although the lichen-draped conifers sites remained largely intact. Pre-fire surveys did not reveal extensive lichen-draped conifer communities in the Geyser Pass/Mill Creek headwaters area before the Pack Creek Fire.

From the two bulk samples collected at the "Geyser Pass 2" site, ca. 3 g. of bulk lichen material from fruticose lichens and ca. 2 g. of bulk lichen material from crustose and foliose lichens on bark was used for DNA extraction. Illumina ITS2 amplicon sequencing resulted in 197,080 and 214,206 reads per in the crustose/foliose and fruticose samples, respectively. Short reads are available in NCBI's Short Read Archive under PRJNA875162. Reports of the FROGS pipeline, e.g., preprocessing, chimera removal, OTU filter, and ITSx, are available in supplementary files S2-S6. In summary, 2.6% of sequences, representing 27.7% of clusters, were excluded as chimeric sequences (supplementary file S3). Of the remaining clusters, 82.1% (1,371) were excluded, not meeting the minimum proportion threshold, e.g., low abundance clusters; the remaining 299 clusters comprised 97.8% of the sequences passing the chimera filter (supplementary file S4). From these, 33 additional clusters were excluded, not passing the ITSx filter, resulting in a total of 266 clusters retained for taxonomic assignment (supplementary file S5).

Across all samples, the 266 clusters were assigned to 20 classes of Fungi, 43 orders, 78 families, 111 genera, and 125 species using the FROGS affiliation pipeline based on the UNITE 8.3 fungal database (supplementary file S6 & S7). Relatively high levels of non-lichen-forming Ascomycota clusters were inferred here – 156 clusters, with more modest numbers of clusters representing Basidiomycota – 44 clusters and a single cluster representing Chytridiomycota (Fig. 4; supplementary files S7). Only 17.3% of the 266 clusters were inferred to be derived from lichen-forming fungi, although most short reads were derived from lichen-forming fungi (Fig. 4). The 46 clusters inferred to represent lichen-forming fungi in the FROGS affiliation step represented 30 species/candidate species in eight families (Table 2).

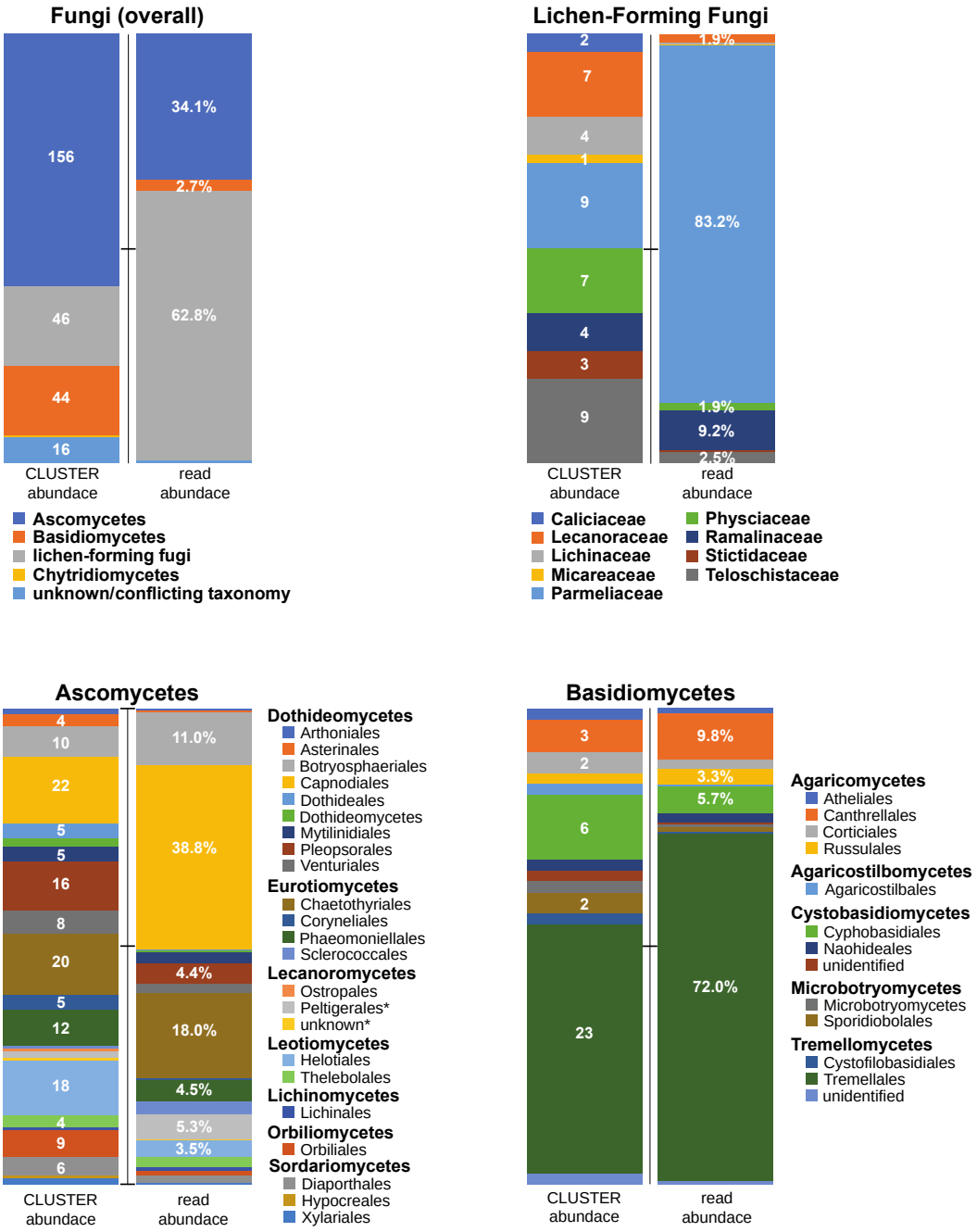


**Fig. 4.** Fungal diversity inferred from DNA metabarcoding at "Geyser Pass 2" sites using the ITS2 barcoding marker. Panels compare taxonomic identity of genetic clusters (left) and proportion of short read data assigned to each taxonomic group (right). Top left panel represents an overview of taxonomic assignments from short read data; top right panel represents lichen-forming fungi; bottom panel panels represent other lichen-associated ascomycetes (bottom left) and basidiomycetes (bottom right) represent in bulk samples.

**Table 2**. List of lichen-forming fungi occurring at a lichen-draped conifer site – "Geyser Pass 2" in the La Sal Mountains, Utah, USA. Genetic clusters were inferred from DNA metabarcoding of the ITS2 marker using the FROGS pipeline [34]. Fruticose lichens are shown in bold text; and the number of haplotypes, rather than genetic clusters, is reported for *Usnea* species.

| Taxon | #genetic clusters | Notes |
|---|---|---|

| | | |
|---|---|---|
| *Amandinea* aff. *punctata* (Hoffm.) Coppins & Scheid. | 2 | cosmopolitan lichen likely comprising multiple, distinct species-level mycobiont lineages [43]; common on Colorado Plateau |
| *Bibbya vermifera* (Nyl.) Kistenich et al. | 1 | uncommon in North America, and this is likely the first report from the Colorado Plateau |
| *Caloplaca* sp. | 1 | ITS2 sequence from La Sals was 99.4% similar to unidentified *Caloplaca* from central Europe |
| *Micarea* sp. | 1 | ITS2 sequence from La Sals was 91.4% similar to *Micarea* sequences on GenBank |
| ***Evernia divaricata* (L.) Ach.** | 2 | occurs worldwide on conifers in montane to subalpine forests; red-listed in all European countries where it occurs [25] |
| *Lecidella euphorea* (Flörke) Hertel | 3 | occurs worldwide and likely comprises multiple, distinct species-level mycobiont lineages [44]; common on Colorado Plateau |
| *Lecidella* sp. | 1 | ITS2 sequence from the La Sals was recovered in the "*Lecidella elaeochroma* clade" [44] |
| *Melanohalea exasperatula* (De Not.) O. Blanco et al. | 2 | widespread across Europe and western North America [45]; common on Colorado Plateau |
| *Melanohalea subolivacea* (Nyl.) O. Blanco et al. | 2 | widespread across western North America [45]; common on Colorado Plateau |
| *Myriolecis juniperina* (Śliwa) Śliwa, Zhao Xin & Lumbsch | 1 | occurs on the Colorado Plateau at mid elevations – this is the first known report from subalpine forests |
| *Myriolecis* sp. | 1 | ITS2 sequence from the La Sals was recovered with a provisionally named species *M.* "*altaterrae*" *nom. prov.* |
| *Myriolecis wetmorei* (Śliwa) Śliwa, Zhao Xin & Lumbsch | 1 | occurs at higher elevations throughout western North America (and Armenia); common on Colorado Plateau |
| *Parvoplaca* sp. | 1 | ITS2 sequence was 97.4% similar to *P. nigroblastidiata* from Turkey |
| *Phaeophyscia* sp. | 1 | *Phaeophyscia* species commonly occur in montane habitats throughout western North America |
| *Phylliscum* aff. *demangeonii* (Moug. & Mont.) Nyl. | 4 | ID uncertain: ITS2 sequence was 97.8% similar to ITS sequence from type (NR_120130); however, BLAST searches also shown high similarity to uncultured *Rhinocladiella* (Eurotiomycetes) |
| *Physcia adscendens* (Fr.) H. Olivier | 1 | widely distributed in temperate and boreal areas in all continents; common on Colorado Plateau |
| *Polycauliona* sp. | 1 | NA. *P. candelaria* occurs scattered throughout the Intermountain West, but the ITS2 sequence from the La Sals was highly dissimilar to *P. candelaria* sequences on GenBank (ca. 92% similarity) |
| ***Ramalina sinensis* Jatta** | 3 | cosmopolitan in temperate regions, and common in montane habitats on the Colorado Plateau |
| *Rinodina* sp. 1 | 1 | NA – voucher specimen required for identification |
| *Rinodina* sp. 2 | 2 | NA – voucher specimen required for identification |
| *Rinodina* sp. 3 | 1 | NA– voucher specimen required for identification |
| *Schizoxylon albescens* Gilenstam, Döring & Wedin | 1 | occurs both as lichen and as saprobe [46]; not previously reported in North America |
| *Stictidaceae* sp. | 2 | ITS2 sequence from La Sals was 93.6% similar to *Stictis brunnescens*. If these clusters truly represent a species in *Stictis*, they are one of the first members of the genus reported for western North America |
| *Tetramelas pulverulentus* (Anzi) A. Nordin & Tibell | 1 | endoparasite within members of the Physciaceae; likely first report from the Colorado Plateau |
| ***Usnea* aff. *barbata* (L.) F.H. Wigg.** | 2 (haplotypes) | widespread across western North America; rarely collected on Colorado Plateau. |
| ***Usnea cavernosa* Tuck.** | 98 (haplotypes) | Eurasia and North American distribution; occurring sporadically on the Colorado Plateau |
| ***Usnea lapponica*** | 1 (haplotypes) | likely cosmopolitan, occurring throughout the Intermountain West |
| ***Usnea perplexans* Stirt.** | 2 (haplotypes) | likely cosmopolitan, occurring throughout the Intermountain West |
| ***Usnea* sp.** | 1 (haplotypes) | NA |

| | | |
|---|---|---|
| *Xanthomendoza montana* (L. Lindblom) Søchting et al. | 6 | widespread across western North America [47]; common on Colorado Plateau |

From the short read data, we inferred eight haplotypes in *E. divaricata*, 13 in *Ramalina sinensis*, and 104 representing *Usnea* species, the vast majority represented *U. cavernosa* (Table 3; supplementary file S8).

Of the 20 *Usnea* sampled for TLC, 17 produced usnic and salazinic acids, with three specimens producing usnic acid alone. The most abundant *Usnea* species in the lichen-draped conifer sites was *U. cavernosa*, accompanied by *U. barbata*, *U. lapponica*, *U. perplexens*, and an unidentified *Usnea* species (Fig. 3).

Non-lichen-forming Ascomycetes comprised 22 orders spanning seven classes (Fig. 4). Dothideomycetes were the most diverse class, with Capnodiales, Pleosporales, and Botryoshaeriales representing the highest number of genetic clusters within this class. Euriotiomycetes were also well represented in the short read data, with Chaetothyriales and Phaeomonielleles the most diverse orders in the class. Over two thirds of the reads inferred to originate from ascomycetes represented only three orders – Capnodiales, Chaetothyriales, and Botryoshaeriales (Fig. 4).

Basidiomycete lineages were represented in 2.7% of all reads, with the vast majority inferred to originate from Tremellales (Tremellomycetes), representing 23 clusters (Fig. 4). Agaricomycetes were also well represented in the short read data, with the order Cantharellales comprising nearly 10% of all basidiomycete reads. Six clusters were inferred to belong to Cyphobasidiales (Cystobasidiomycetes) (Fig. 4), four of which have previously been shown to have close associations with lichens.

**Table 3**. Genetic variation in fruticose lichens occurring at a lichen-draped conifer site – "Geyser Pass 2" in the La Sal Mountains, Utah, USA.

| Taxon | # of hapoltypes | Notes |
|---|---|---|
| *Evernia divaricata* | 8 | Haplotypes from the La Sals were distributed across multiple, weakly supported clades in the ITS topology. |
| *Ramalina sinensis* | 13 | Haplotypes from the La Sals were recovered within a single clade comprised exclusively of closely related haplotypes from the La Sals and sister to a clade comprised of two specimens from Arizona and New Mexico. |
| *Usnea spp.* | 104 total<br>*U.* aff. *barbata* [2]<br>*U. cavernosa* [98]<br>*U. lapponica* [1]<br>*U. perplexans* [2]<br>*U.* sp. [1] | The *U. cavernosa* haplotypes were recovered within a clade comprised of closely related sequences from Idaho (USA) and Switzerland. The phylogenetic position of specimens identified as *U. barbata*, *U. perplexans*, and *U.* sp. were unresolved; the *U. lapponica* haplotype was recovered within a clade of other *U. lapponica* sequences from Austria, Estonia, Canada, India, Spain, Switzerland, and the USA. |

## 4. Discussion

Here we document for the first time unique and vulnerable fruticose lichen communities occurring in subalpine forests in a desert sky island on the Colorado Plateau (Fig. 2) [25]. Lichen-draped conifer sites were found at only three locations in subalpine *Picea engelmanii* forests in the La Sal Mountains in southeastern Utah (Table 1). The fruticose lichens occurring in the La Sals represent isolated populations of lichens that are found in montane forest around the world, particularly in the Northern Hemisphere. Fruticose lichen communities in the La Sals occur at the western edge of their distribution moving into the arid canyonlands region of Utah (Fig. 1); and documenting the diversity, distribution and extent of these lichen-draped conifer sites provides the first step in

conserving and monitoring these unique communities. We inventoried the range of lichen-forming fungi co-occurring with these fruticose lichens within one lichen-draped conifer site in the La Sals, highlighting several unexpected lichens. Furthermore, by also characterizing the range of lichen-associated fungi (non-lichen-formers), we hope to provide a broader understanding of the range of biodiversity associated with these sites. Below we discuss the implications of our findings.

The impact of climate change, land use, and frequency of wildfires on the Colorado Plateau will continue to have major impacts on biological communities [4,48,49], including lichens [50]. The lichen-draped conifer sites in the La Sal Mountains are unique, and similar communities of locally abundant fruticose lichens occurring in similar densities have not been observed in the Colorado Plateau or Great Basin in western North America (S. Leavitt, *personal observation*). The factors that have facilitated the successful development of these communities in the La Sals remains uncertain. Warm summer monsoonal climates have been shown to support the greatest number of epiphyte species in the southwestern USA [50], and we speculate that summer monsoonal precipitation is also crucial in the establishment of the fruticose lichen communities in the La Sals. The pronounced summer monsoonal patterns also support fruticose lichen communities in montane habitats in Arizona, Colorado, and New Mexico (Fig. 1), and similar lichen-draped conifer sites may occur sporadically in those regions as well. However, we have not observed levels of high density and biomass comparable to that in the La Sals (S. Leavitt, *personal observation*). Given the patchy nature of these communities across subalpine forests, other factors, in addition to monsoonal precipitation, likely play important roles in determining the extent of these communities.

Historically, fires, bark beetle outbreaks, land use strategies, and wind damage have impacted subalpine forests across western North America [51]. Increasing warm, dry conditions are presently increasing the rate of fires in subalpine habitats [4]. Furthermore, land management, forest structure, stand age, light availability, soil moisture, fire frequency, etc. have also been documented to influence epiphytic lichen communities [52]. Strikingly, a large wildfire in 2021 burned a significant proportion of subalpine forests in the La Sals. While habitat surrounding the lichen-draped conifer sites near Geyser Pass were heavily impacted by this fire, lichen-draped conifer stands remained largely intact. We speculate that soil moisture likely played a crucial role in reducing the impact of the recent fire. The correlation between the unburned conifer stands with high fruticose lichen biomass, in conjunction with perennial water availability, suggests a potential connection to soil moisture.

Haplotype diversity in *E. divaricata*, *R. sinensis*, and *U.* spp. provides some evidence to speculate on the origin of these populations. For example, ITS hapotypes of *E. divaricata* were distributed across multiple, weakly supported clades in the ITS topology with all available GenBank sequence (results not shown), suggesting multiple independent dispersal events into the La Sals. In contrast, *R. sinensis* haplotypes from the La Sals were recovered within a single monophyletic clade comprised of closely related haplotypes and distinct from all other sequences currently available on GenBank, except for an ITS sequence generated from a specimen collected in Utah. The phylogenetic substructure in the *R. sinensis* ITS tree corresponding to distinct geographic regions worldwide suggest dispersal limitations among geographically distinct populations (results not shown). The geographic extent of the genetically distinct population occurring in the La Sals merits additional attention. Haplotypes representing *U. cavernosa* from the La Sals were highly similar to each other and other sequences generated from specimens collected in the Intermountain West and Switzerland (results not shown). These results provide some evidence that *U. cavernosa* has broad dispersal capacity with little population substructure. However, the other *Usnea* haplotypes were relatively distinct from those presently available on GenBank, and we do not speculate on the origin of these species. Ultimately, broader sampling and model-based migration models will be essential to characterize

dispersal of fruticose lichens into sky islands in western North America, e.g., [53,54] and the mechanisms proposed here are intended only as speculative hypotheses. The interplay of dispersal capacity with biotic and abiotic factors influencing the establishment and persistence of these unique fruticose lichen communities will require additional research.

While the fruticose lichens recorded in these sites also occur in other montane habitats throughout western North America, several unexpected lichens were also inferred from our DNA metabarcoding data to co-occur in these communities (Table 2). For example, the fungus *Schizoxylon albescens* Gilenstam, Dörıng & Wedin documented here, which can occur both as a lichen and a saprobe, represents one of the first reports for North America. Clusters inferred to represent *Tetramelas pulverulentus* (Anzi) A. Nordin & Tibell and *Bibbya vermifera* (Nyl.) Kistenich, Timdal, Bendiksby & S.Ekman are also reported for likely the first time on the Colorado Plateau. Our data also provide the first evidence of *Myriolecis juniperina* in subalpine forests. However, limitations in presently available DNA reference libraries, in addition to inherent limitations to DNA-based specimen identification, highlight that the occurrence of these taxa must be interpreted with caution [55]. Final determinations for the unexpected or unusual lichens must be confirmed with physical voucher specimens.

Our study also provides an important, albeit incomplete, perspective into the range of lichen associated fungi at a community level (Fig. 4). The lichen-associated fungi inferred in our study largely match what has been found in different lichens in previous work, with most reads and cluster diversity inferred to represent members of Dothideomycetes and Eurotiomycetes [56-58]. Interestingly, Leotiomycetes and Sordariomycetes, which are commonly associated with fruticose and foliose lichens, were found in lower abundance in our samples and diversity than Dothideomycetes and Eurotiomycetes which are typically more common in crustose lichens [56-58].

Reads from basidiomycetes were less common in our data but represented expected fungal lineages occurring with lichens, including Tremellomycetes and Cystobasidiomycetes [27,30,32]. The function of the basidiomycete yeast in various lichen symbioses is still being investigated, but some studies indicate that they produce polysaccharides and secondary metabolites that contribute to the structure of the thalli and perhaps even affect some chemical and biological properties or nutrient acquisition [59,60]. Some species in *Cyphobasidium* have been hypothesized to be parasitic on species of *Usnea* [61]. In other cases, endolichenic fungi may facilitate protection from predation, photoprotection, enhanced desiccation tolerance, and reduced depression of photosynthesis during saturation because the thallus has hydrophobicity or maintains non-saturated spaces [31,59]. While specific roles of lichen-associated fungi remain largely unknown for fruticose lichens occurring in the La Sals, our data provide an initial perspective into the range of lichen-associated fungal diversity which can be reconsidered as our understanding of the specific roles of endolichenic fungi improves.

The fungal diversity inferred in this study likely extends beyond strictly endolichenic fungi to those that occur superficially or ephemerally on lichens. Similarly, fungi occurring in close proximity to lichens or with tree bark were likely inadvertently collected and represent a small fraction of the reads. Furthermore, it is unlikely that we fully characterized the range of fungal diversity within the lichens. Fungal diversity is not uniformly distributed across lichen thalli, and here, we targeted apical regions of fruticose thalli. Older portions of the lichen, such as the holdfast, likely harbor distinct fungi, and these may not be represented in our data [62].

Similar to inferences of taxonomic diversity of lichen-forming fungi, the taxonomic assignment of lichen-associated fungi inferred here is subject to change. One advantage to DNA metabarcoding approaches is that the data generated for this study are reusable and interoperable [63]. Our short read data is findable and can be combined with similar data in future studies to improve taxonomic assignments or subsequent comparisons across space and time.

**Fig. 5.** Fire damage to fruticose lichen communities near the headwaters of Mill Creek in the La Sal Mountains in southern Utah, USA.

The limited lichen-draped conifer communities in the La Sal Mountains face increasing threats, particularly fire, increasing aridity [49], changing temperature and precipitation patterns [64], and changes in land use strategies. In fact, during the course of this study, two of the three known lichen-draped conifer sites were damaged by wildfire (Fig. 5). The impact of increasing temperatures and changing precipitation patterns on these lichen communities is harder to predict. Although winter precipitation is forecast to decrease, current models suggest increasing average annual precipitation, with more precipitation in the late summer months [64]. In Hungary, *E. divaricata* populations have been shown to be increasing in recent decades as the result of changing climate [65]. However, populations in the subalpine habitats in the southwestern USA may follow a different trajectory [49], and developing models predicting changes in fruticose lichen populations should be a top priority to more effectively monitor ecological health in subalpine forests. Finally, raising awareness of these rare lichen communities may help guide land use strategies to help ensure the persistence of unique lichen communities and other biodiversity in the region.

**Supplementary Materials:** The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Supplementary file S1: The complete area for each identified site with extensive lichen-draped conifers provided as polygon area. Supplementary file S2: The FROGS v3.2 preprocessing report, including a summary of filtered reads and details on merged sequences, Supplementary file S3: the FROGS v3.2 chimera removal report, including the proportion of clusters and sequences that were removed, in addition to chimera detection by sample, Supplementary file S4: the FROGS v3.2 OTU filter report, including the proportion of low abundance clusters and sequences that were removed, Supplementary file S5: the FROGS v3.2 ITSx summary report, including the proportion of low abundance clusters and sequences that were removed, in addition to the OTUs removed by sample, Supplementary file S6: the FROGS v3.2 taxonomic assignment summary report, including the taxonomy distribution across samples, Supplementary file S7: the complete list of the ITS2 clusters generated using FROGS v3.2 and passing quality filters, separated in clusters representing lichen-forming fungi, lichen-associated ascomycetes, lichen-associated basidiomycetes, unknown fungi or those with conflicting taxonomic assignments, lichen-associated chytridiomycetes, and the results from thin layer chromatography of selected *Usnea* specimens, Supplementary file S8: fruticose lichen haplotypes (*Evernia divaricata*, *Ramalina sinensis*, and *Usnea* species) aligned with available sequences from GenBank (*E. divaricata* and *R. sinensis*) or a custom ITS datasets (*U.* spp).

**Author Contributions:** Conceptualization, A.R., M.B., and S.L.; methodology, A.R., M.B., L.M., and S.L.; validation, M.G. and S.L.; formal analysis, A.R., M.B., and S.L.; investigation, A.R., M.B., L.M., and S.L.; resources, S.L.; data curation, S.L.; writing—original draft preparation, A.R. and M.B.; writing—review and editing, A.R., M.B., L.M., and S.L.; visualization, A.R., M.B., and S.L.; supervision, S.L.; project administration, S.L.; funding acquisition, S.L. All authors have read and agreed to the published version of the manuscript.

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# Appendix 2:

## Alpine lichen diversity in an isolated sky island in the Colorado Plateau, USA—Insight from an integrative biodiversity inventory.

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ORIGINAL RESEARCH

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# Alpine lichen diversity in an isolated sky island in the Colorado Plateau, USA—Insight from an integrative biodiversity inventory

Steven D. Leavitt[1] | Jason Hollinger[2] | Sara Summerhays[3] | Isaac Munger[3] | Jonah Allen[3] | Barb Smith[4]

[1]M.L. Bean Life Science Museum & Department of Biology, Brigham Young University, Provo, Utah, USA

[2]Herbarium, Department of Biology, Western Carolina University, Cullowhee, North Carolina, USA

[3]Department of Biology, Brigham Young University, Provo, Utah, USA

[4]Wildlife Biologist/Botanist, Moab District, Manti–La Sal National Forest, Moab, Utah, USA

**Correspondence**
Steven D. Leavitt, M.L. Bean Life Science Museum & Department of Biology, Brigham Young University, Provo, UT 84602, USA.
Email: steve_leavitt@byu.edu

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## Abstract

Lichens are major components of high altitude/latitude ecosystems. However, accurately characterizing their biodiversity is challenging because these regions and habitats are often underexplored, there are numerous poorly known taxonomic groups, and morphological variation in extreme environments can yield conflicting interpretations. Using an iterative taxonomic approach based on over 800 specimens and incorporating both traditional morphology-based identifications and information from the standard fungal DNA barcoding marker, we compiled a voucher-based inventory of biodiversity of lichen-forming fungi in a geographically limited and vulnerable alpine community in an isolated sky island in the Colorado Plateau, USA—the La Sal Mountains. We used the newly proposed Assemble Species by Automatic Partitioning (ASAP) approach to empirically delimit candidate species-level lineages from family-level multiple sequence alignments. Specimens comprising DNA-based candidate species were evaluated using traditional taxonomically diagnostic phenotypic characters to identify specimens to integrative species hypotheses and link these, where possible, to currently described species. Despite the limited alpine habitat (ca. 3,250 ha), we document the most diverse alpine lichen community known to date from the southern Rocky Mountains, with up to 240 candidate species/species-level lineages of lichen-forming fungi. 139 species were inferred using integrative taxonomy, plus an additional 52 candidate species within 29 different putative species complexes. Over 68% of sequences could not be assigned to species-level rank with statistical confidence, corroborating the limited utility of current sequence repositories for species-level DNA barcoding of lichen-forming fungi. By integrating vouchered specimens, DNA sequence data, and photographic documentation, we provide an important baseline of lichen-forming fungal diversity for the limited alpine habitat in the Colorado Plateau. These data provide an important resource for subsequent research in the ecology and evolution of lichens alpine habitats, including DNA barcodes for most putative species/species-level lineages occurring in the La

Sal Mountains, and vouchered collections representing any potentially undescribed species that can be used for future taxonomic studies.

**KEYWORDS**
ASAP, DNA barcoding, La Sal Mountains, Rocky Mountains, vouchered collections

## 1 | INTRODUCTION

Accurately and efficiently characterizing species-level biodiversity is fundamental to investigating a wide range of biological topics, including conservation biology, ecology, evolution, and the impact of climate change (Heywood & Watson, 1995). However, biodiversity assessments are often difficult, particularly for organismal groups with significant proportions of undescribed diversity and in geographic regions that have received limited attention. Sample identification may result in ambiguous specimen assignments using either traditional morphology-based approaches or DNA sequence-based approaches (Lücking et al., 2020; Naciri & Linder, 2015). Morphology-based identifications may not be repeatable, even in cases where identifications are performed by specialists (Carvalho et al., 2011, 2015; Vondrák et al., 2016), and are further confounded by the potential for difficult-to-identify specimens, for example, immature or environmentally modified specimens lacking diagnostic features. DNA barcoding represents a transformative and reliable framework for organizing specimens for systematic research and documenting diversity (DeSalle & Goldstein, 2019), in addition to providing genetic data that can be used to infer evolutionary relationships. However, sequence-based approaches for specimen identification have significant limitations, including incomplete DNA reference libraries, potential to over-split species-level lineages, failing to diagnose closely related species, and ambiguous specimen assignments (Leaché et al., 2018; Leavitt et al., 2016; Lücking et al., 2020; Moritz & Cicero, 2004; Naciri & Linder, 2015).

Integrating various lines of evidence in biodiversity inventories, for example, traditional taxonomic approaches with DNA sequence data, can provide improved perspectives into biodiversity surveys (Cao et al., 2016; Sheth et al., 2017). A phylogenetically informed reinterpretation of morphological characters can strengthen taxonomic conclusions and provide direction for nominal taxa in need of revision to more accurately portray evolutionary histories (Hutsemékers et al., 2012). In many groups, presently described species represent only a fraction of the estimated overall diversity (Guiry, 2012; Hawksworth & Lücking, 2017; Pons et al., 2006). Given the limited taxonomic expertise for many groups and the meticulous nature of classical monographic research, integrative approaches can help remedy the current "taxonomic impediment" problem (Dayrat, 2005; Vinarski, 2020) by facilitating the discovery and taxonomic description of novel taxa (Cao et al., 2016).

Alpine, arctic, and Antarctic habitats worldwide support specialized biological communities that are adapted to harsh environmental conditions (Billings, 1974; Chapin & Körner, 1994), and important components of alpine-adapted communities are often poorly known (Pereira et al., 2012). In these ecosystems, abiotic factors, especially climate, dominate biotic interactions, make them particularly vulnerable to changing climate (Cannone et al., 2007). Detecting changes in occurrence, distribution, or abundance of alpine species is based on knowledge of which species occur in specific locations, information that is conspicuously absent for many organismal groups. In the intermountain region of western North America, expansions and contractions of species' ranges have proceeded through local movements along elevation gradients to and from scattered high-elevation patches of habitat throughout the Pleistocene (Guralnick, 2007; Jiménez-Moreno & Anderson, 2013), including a number of alpine sky islands in the Southern Rocky Mountain Region of the United States. The Southern Rocky Mountains are centered on the ranges of Colorado, extending northwards to the Medicine Bow Mountains in southeast Wyoming and south to the Sangre de Cristo Range in north central New Mexico. It also includes two isolated ranges—the La Sal Mountains in southeastern Utah and the San Francisco Peaks in northern-central Arizona. Here, isolated alpine habitats are geographically subdivided among different mountain ranges, for example, "sky islands," harboring unique biodiversity due to a variety of factors spanning multiple spatial and temporal scales (Knowles, 2000; Marx et al., 2017). Climate-driven distributional shifts lead to complex patterns of diversification and demography in alpine specialists (Galbreath et al., 2009). In the alpine zone of the Southern Rocky Mountains, vascular plants have been systematically inventoried over the past three decades, demonstrating rich plant communities harboring substantial endemism, ca. 10% (Fowler et al., 2014). However, only a limited number of studies investigating lichen diversity in the same region are available (Table 1).

In many alpine habitats, lichen communities are diverse and found in high relative abundance (Bruun et al., 2006; Imshaug, 1957). Alpine lichens, including those occurring on rock, soil, and/or alpine turf, play important ecological roles, ranging from nutrient cycling to habitat and food sources to soil stabilization (Asplund & Wardle, 2017). Despite the high diversity and abundance of lichens in alpine ecosystems, many of these ecosystems are sensitive to environmental disturbances, including climatic shifts and changes in land management strategies (Cornelissen et al., 2001; Geml et al., 2010; Kranner et al., 2008; St. Clair et al., 2007). Therefore, monitoring alpine lichen communities can provide crucial insight into the biological impacts of climate change in some of the most vulnerable ecosystems. However, the diversity and distributions of many components of alpine lichen communities have been incompletely characterized, emphasizing the need to efficiently generate crucial baseline assessments. Recent, collaborative taxonomic efforts have further highlighted the incredible lichen diversity in high altitude/latitude

**TABLE 1** Summary of current understanding of alpine lichen diversity in the Southern Rocky Mountains, USA

| Sampling area | # of species | Source |
|---|---|---|
| Bald Mountain, Uinta Mountains (Utah) | 65 | St. Clair et al. (2007) |
| Beartooth Plateau, Beartooth Mountains (Montana and Wyoming) | 80 | Eversman (1995) |
| Mount Audubon, Front Range (Colorado) | 86 | Egan (1970, 1971) |
| Niwot Ridge Long-Term Ecological Research Site, Front Range (Colorado) | 92 | Flock (1978) |
| Sierra Blanca Peak, Sierra Blanco Range (New Mexico) | 76 | Egan (1971) |
| La Cal Basin, Sangre de Cristo Mountains (New Mexico) | 89 | Egan (1971) |
| Lake Peak, Sangre de Cristo Mountains (New Mexico) | 76 | Egan (1971) |
| Wheeler Peak, Snake Range (Nevada) | 58 | Noell and Hollinger (2015) |
| La Sal Mountains | 189+ | This study |

ecosystems (McCune et al., 2020; Nimis et al., 2018; Spribille et al., 2010, 2020). These studies also reveal that a significant proportion of this diversity has not yet received formal taxonomic recognition. For example, recent lichen diversity inventories in two Alaskan national parks revealed that ca. 10% of the sampled lichens could not be assigned to a known species (Spribille et al., 2010, 2020).

Effective strategies for using molecular sequence data to aid in the identification of fungi continue to be developed (Abarenkov et al., 2018; Lücking et al., 2020). Coupling these strategies with phenotype-based data will likely facilitate more effective cataloging the global fungal diversity and provide novel insight into ecological and evolutionary processes (Sattler et al., 2007; Struck et al., 2018). Dispersal of alpine and arctic lichens has received considerable attention in recent years. Frequent long-distance dispersal has been documented for a number of alpine and arctic lichens (Fernández-Mendoza & Printzen, 2013; Garrido-Benavent et al., 2021; Geml et al., 2010; Onuţ-Brännström et al., 2017) and a "mountain hopping" mechanism of dispersal explains, in part, the broad distribution of many alpine lichens (Garrido-Benavent & Pérez-Ortega, 2017). Therefore, the Rocky Mountains in North America play a fundamental role in understanding the processes that influence the distribution of alpine lichen communities (Garrido-Benavent & Pérez-Ortega, 2017; Hale et al., 2019; Weber, 2003). To investigate how this might look in practice, here we attempt to characterize lichen-forming fungal species diversity in the alpine zone of an isolated sky island in the Southern Rocky Mountains using an integrative taxonomic approach—the La Sal Mountains (hereafter the "La Sals") in the Colorado Plateau in eastern Utah. This insular range is surrounded by semiarid, low-elevation, canyon dissected terrain (Figure 1), and the high peaks and ridgelines of the La Sals support one of the few true alpine lichen communities in the Colorado Plateau, including ca. 320 ha of vegetated alpine turf. The alpine habitat is known to support a number of sensitive vascular plants, including the endemic La Sal daisy (*Erigeron mancus* Rydb.; Fowler & Smith, 2010). Limited, informal surveys in alpine habitats in the La Sals have suggested the potential for a robust alpine lichen community. However, the origin and establishment of this alpine lichen community is not currently well understood for this unique ecosystem in the Colorado Plateau. Furthermore, specific responses of lichens to ongoing climate change and changes in land management strategies in the alpine habitat in the La Sals remain unknown. Crucial insight might be gained into these processes by more fully characterizing the alpine lichen community in the La Sals.

For this study, our aim was to characterize lichen-forming fungal diversity occurring in alpine habitat on the insular La Sal Mountains in the Colorado Plateau. We ask (a) how might incorporating DNA sequence data in general surveys influence our perspective of species-level diversity? (b) Can these data expedite identifying challenging species complexes that require additional taxonomic work? And (c) how well do current DNA sequence repositories used for DNA barcoding reflect actual diversity? To address these questions, we used an integrative taxonomic approach based on recently collected vouchered specimens, incorporating both traditional morphology-based identifications and information from the standard fungal DNA barcoding. Our findings provide a baseline for future studies investigating ecology and evolution in alpine lichens in western North America.

## 2 | MATERIALS AND METHODS

### 2.1 | Study site, sampling, and morphology-based identifications

The La Sals comprise three distinct groups—the "North," "Middle," and "South" groups (File S1), which were formed in the Laramide orogeny during the Oligocene when intrusive, dioritic magmas uplifted overlying sedimentary rocks (Hunt & Waters, 1958; Nelson et al., 1992). Our sampling focused exclusively on habitats above


**FIGURE 1** Alpine habitat in the La Sal Range. (a) "Middle Group," view of Mount Tukuhnikivatz (3,805 m.a.s.l.) from near the summit of Mount Peale (3,879 m.a.s.l.); (b) view from the head of Dark Canyon (3,500 m.a.s.l.), a subalpine basin in the "Middle Group"; (c and d) distinct rock-dwelling lichen communities; (e and f) distinct soil-dwelling lichen communities

timberline in the "North" and "Middle" groups—ca. 3,250 ha above 3,350 m above sea level (m.a.s.l.). Sites were selected to represent the range of geological and ecological features found in these alpine habitats (Figure 1; File S1). Most of the alpine habitat is dominated rock/talus, with only 320 ha of vegetated alpine habitat. In addition to alpine peaks and ridgelines, we collected in two alpine basins, Beaver Basin in the "North Group" and Dark Canyon in the "Middle Group" (Figure 1b). We used an opportunistic taxonomic sampling approach, for example, "intuitive meander" (Whiteaker et al., 1998), aiming to generate a comprehensive but qualitative overview of diversity in the alpine region of the La Sals. Vouchered collections were made during the summers of 2018 and 2019 on all available substrates, including soil, mosses, vascular plants (including limited lignum found above the current timberline), and rock surfaces. Lichens from timberline krummholz—stunted conifers occurring near tree line—were not sampled. Visual assessments of lichens were made in the field with a 10× hand lens, and representatives of the range of observed variation were collected and returned to the laboratory for indentification. If specimens were observed representing lichens that we had collected previously, they were not necessarily collected at additional sites.

Lichen specimens collected during the fieldwork phase of this project are deposited in the Herbarium of Non-Vascular Cryptogams (BRY-C) at Brigham Young University, Provo, Utah, USA. Photographs of new collections were made using an Olympus DP-22 camera attached to an Olympus SZH-10 stereomicroscope. Compilations of stacked images were done with Zerene Stacker 1.04 (Richland, Washington, USA). We used the Consortium of North American Lichen Herbaria (https://lichenportal.org) to identify historic lichen records representing specimens from the alpine regions of the La Sals.

## 2.2 | DNA extraction, amplification, and sequencing

We attempted to generate molecular sequence data for the mycobiont from all vouchered collections made in 2018/2019. Many vouchers included additional, accessory lichens; and efforts were made to sample material from these accessory lichens, along with the targeted lichen. From selected specimens, a small portion of the thallus free of visible contamination was excised, and total genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, USA). We amplified the fungal internal transcribed spacer region (ITS) using primers ITS1 (Gardes & Bruns, 1993) with ITS4 (White et al., 1990). Polymerase chain reaction (PCR) amplifications were performed using Ready-To-Go PCR

Beads (GE Healthcare, Pittsburgh, PA, United States), with cycling parameters following a 66–56°C touchdown reaction (Lindblom & Ekman, 2006). PCR products were visualized on 1% agarose gel and enzymatically cleaned using ExoSAP-IT Express (USB, Cleveland, OH, United States). Complementary strands were sequenced using the same primers used for amplifications, and sequencing reactions were performed using BigDye 3.1 (Applied Biosystems, Foster City, CA, United States). Products were run on an ABI 3730 automated sequencer (Applied Biosystems) at the DNA Sequencing Center at Brigham Young University, Provo, UT, United States. In cases where the initial PCR and/or sequencing reactions failed to yield high-quality reads or resulted in unexpected/questionable sequences, PCR and sequencing reactions were attempted up to three times per problematic sample.

### 2.3 | DNA-based inference of mycobiont candidate species and phylogenetic reconstructions

All sequences generated for the study were subjected to an initial "blastn" search against GenBank's nucleotide collection (Altschul et al., 1990) to confirm family-level membership. Exploratory multiple sequence alignments (MSAs) of all ITS sequences generated for this study resulted in unreliable alignments due to the high variability of the ITS region at deeper fungal taxonomic levels. Therefore, all subsequent MSAs generated here were performed at the mycobiont family-level. Family-level MSAs were generated using the program MAFFT v7 (Katoh & Toh, 2008; Rozewicki et al., 2017). We implemented the G-INS-i alignment algorithm and "1PAM/K = 2" scoring matrix, with an offset value of 0.1, the "unalignlevel" = 0.4, and the remaining parameters were set to default values.

To circumscribe candidate mycobiont species from the family-level ITS MSAs, we used Assemble Species by Automatic Partitioning (ASAP; Puillandre et al.). ASAP is a recently developed method that circumscribes species partitions using an implementation of a hierarchal clustering algorithm based on pairwise genetic distances from single-locus sequence alignments (Puillandre et al., 2021). The pairwise genetic distances are used to build a list of partitions ranked by a score, which is computed using the probabilities of groups to be panmictic species. ASAP, therefore, provides an objective approach to circumscribe relevant species hypotheses as a first step in the process of integrative taxonomy.

Each family-level ITS MSA was analyzed under a maximum likelihood (ML) criterion as implemented in IQ-TREE v2 (Nguyen et al., 2014), with 1,000 ultrafast bootstrap replicates (Hoang et al., 2017), with the best-fitting substitution model for the entire ITS region selected using ModelFinder (Kalyaanamoorthy et al., 2017). Trees were visualized using FigTree v1.4.4 (Rambaut, 2008). Species partitions inferred from the family-level ASAP analyses were compared to phylogenetic reconstructions to determine reasonable DNA-based candidate species (DNA-CS) using the criterion of reciprocal monophyly in DNA-CSs, in addition to qualitative assessments of branch lengths and lichen morphology (see below).

### 2.4 | Integrative taxonomy

Incorporating phenotypic data in the assessment of DNA-CSs through integrative taxonomy provides critical information for evaluating species-level diversity in lichen-forming fungi (Lücking et al., 2020). Phenotypic traits of all vouchered specimens were examined in light of the DNA-CSs delimited using ASAP and the phylogenetic inferences. Diagnostic features from relevant taxonomic keys and monographs, as well as a variety of online resources, were considered. As needed, thin-layer chromatography (Culberson, 1969; Orange et al., 2001) was used to aid with specimen identifications.

To characterize how DNA-CSs compared with phenotypically circumscribed species and currently available sequence data in GenBank, each DNA-CS was categorized within the following categories: "match"—ITS sequences ≥98% similar to sequences from the same taxon currently available in GenBank; "species complex"—ITS sequences ≥97% similar to morphologically similar species but represented by multiple candidate species; "affinity"—sequences representing candidate species not recovered as monophyletic, represented by multiple candidate species; "mismatch"—ITS sequences <95% similar to sequences from the same taxon currently available in GenBank; and "no comparison"—sequences from identified species were not available in GenBank. We note that currently available ITS sequence data in GenBank represents only a small portion of extant fungal species, and many of the sequences are incorrectly identified to species level (Nilsson et al., 2006). Furthermore, pairwise similarity-based approaches, such as BLAST, can provide misleading perspectives into taxonomic assignment and relationships (Lücking et al., 2020). Hence, we used the Protax-fungi pipeline for taxonomic placement using ITS sequences, as implemented on the PlutoF platform and using the UNITE database (Abarenkov et al., 2010, 2018). Protax-fungi provides statistical assessment of taxonomic assignment precision, from species to phylum ranks, here choosing a plausible classification value of 0.05.

## 3 | RESULTS

From a total of 446 vouchered collections housed at BRY-C, DNA was extracted from 805 distinct lichen thalli (many vouchers included multiple, distinct lichens), and we successfully amplified ITS sequence data from 734 thalli (Table S1; GenBank accession numbers MZ243469–MZ244202). All supplementary files, alignments, and photographs of sampled thalli are available in Dryad: https://doi.org/10.5061/dryad.9ghx3ffh4.

### 3.1 | Genetic diversity of lichen-forming fungi and DNA-based species delimitation

A total of 24 families of lichen-forming fungi were represented by ITS sequence data, with the most frequently collected families including the following: Lecanoraceae ($n$ = 238), Physciaceae ($n$ = 71), Megasporaceae ($n$ = 67), Teloschistaceae ($n$ = 66), and Candelariaceae

($n = 57$) (Table S1). The family-level ASAP species delimitation analyses resulted in a total of 244 species partitions, with an average of 2.8 sequences per species partition and ranging from one to seven sequences per species partition (Table 2). High genetic diversity was observed in many traditional, phenotype-based species, often with multiple ASAP species partitions inferred from nominal species, and these putative species complexes represented 96 of 244 ASAP species partitions (Appendix S1). Family-level phylogenetic reconstructions also revealed high genetic diversity; and, in general, ASAP species partitions corresponded with distinct, reciprocally monophyletic clades (File S3). Comparing ASAP species partitions with family-level phylogenetic reconstructions reduced the DNA-CSs from 244 to 222 (Appendix S1; File S3), combining several closely related and/or nonmonophyletic ASAP species partitions, particularly in the families Candelariaceae, Lecanoraceae, and Teloschistaceae (Table 2).

### 3.2 | Integrative taxonomy

Our integrative specimen identification approach (phenotype + sequence data) resulted in a total of 189 species (Table 2). In many cases, morphologically similar specimens were recovered in divergent, well-supported phylogenetic lineages; and from an integrative perspective, these were combined into a single species (e.g., taxa in Candelariaceae and Megasporaceae; File S3). In other cases, traditionally accepted species known to display considerable morphological variable were recovered in divergent, well-supported phylogenetic lineages, and these were also combined into a single "integrative" species.

Over half (54%) of all candidate species inferred in this study were ≥98% similar to sequences representing the same taxa and presently available on GenBank (searched 15 December 2020). Over a third of all candidate species appear to belong to species complexes of morphologically similar taxa with at least 97% genetic similarity, representing 29 putative species complexes (Appendix S1; File S3). In contrast, nearly a third (31%) of all candidate species had no match on GenBank (sequences from identified species not presently available in GenBank) or their sequences from identified species were highly dissimilar from sequences from the same taxon on GenBank. Of the newly generated sequences that were <95% similar to sequences presently available in public databases, most belonged to members of Lecanoraceae, Megasporaceae, Psoraceae, Rhizocarpaceae, and Verrucariaceae (Table S1). Approximately 5% of new sequences were <90% similar sequences presently available on GenBank. In the Protax-fungi analysis, over 68% of sequences could not be assigned to species-level rank with statistical confidence, and nearly 8% of sequences were not assigned any taxonomic rank (File S4).

### 3.3 | Historic collections and taxa lacking DNA sequencing data

Ten lichens collected in alpine regions of the La Sals between 1954 through the 1980s were not observed during the 2018/2019 fieldwork, including two conspicuous and notable macrolichens, *Brodoa oroarctica* (Krog) Goward and *Hypogymnia austerodes* (Nyl.) Räsänen (Appendix S1). We failed to obtain DNA sequence data from a limited number of potentially unique lichens observed during the 2018/2019 fieldwork. A single specimen representing *Rhizocarpon disporum* (Nägeli ex Hepp) Müll. Arg. was photographed above Beaver Basin in 2019 but not collected. Additional species collected for which all sequencing attempts failed include the following: *Athallia saxifragarum* (Poelt) Arup, Frödén & Søchting (*Leavitt 18-651*), *Buellia* De Not. sp. (*Leavitt 18-426*), *Rinodina imshaugii* Sheard (*Leavitt 18-574*), and *R. olivaceobrunnea* C. W. Dodge & Baker (*Leavitt 18-626*). Similarly, sequencing efforts for a number of cyanobacteria-associated lichens, largely in Collemataceae, frequently resulted in unusable chromatograms. Finally, six lichenicolous fungi were documented but are not represented by DNA sequence data (Appendix S1).

## 4 | DISCUSSION

Despite the limited alpine habitat on the insular La Sals in the Colorado Plateau, USA, we document the most diverse alpine lichen communities known to date from the southern Rocky Mountains (Table 1), with at least 189 documented species of lichen-forming fungi (Appendix S1). The actual number of species in the alpine habitat in the La Sals is likely higher. Our integrative data (DNA sequence data + phenotype-based inference) suggest that a substantial number of nominal lichens occurring in the La Sals are comprised of multiple candidate species-level lineages (DNA-CS; Appendix S1; File S3). Including DNA-CS within these species complexes leads to a greater than 25% increase in species-level diversity, with at least 52 additional candidate species (Appendix S1). Furthermore, additional surveys, including alternative sampling strategies, would likely result in additional species not documented here (e.g., Vondrák et al., 2016). A preliminary checklist, with accompanying taxonomic notes, is reported in companion paper (Leavitt et al., in preparation), and below, we discuss the implications of our integrative inventory approach in better understanding alpine lichen diversity.

Despite the important perspective gained from integrative inventories, our results highlight several potential challenges that may continue to impede inventories of understudied organisms. While our study captured some of the highest levels of alpine lichen diversity in the western continental United States, the combination of traditional voucher-based approaches with DNA barcode sequencing individual lichen thalli was relatively labor- and cost-intensive. Furthermore, the taxonomic status for a significant proportion of this diversity remains ambiguous despite integrating morphological features with sequence data. Subtle or difficult to discern diagnostic traits, conflicting interpretations of morphological variation, including the recognition of morphologically cryptic species-level lineages, and phenotypic convergence may potentially confound inferences from taxonomic inventories (Argüello et al., 2007; Crespo & Pérez-Ortega, 2009; Printzen, 2009). Accurate and precise identifications

**TABLE 2** Summary of lichen-forming fungal species diversity collected in the La Sal Mountains in eastern Utah, USA

| Family | # ASAP species | Candidate species | Integrative species (species complexes) |
|---|---|---|---|
| Acarosporaceae (16) | 10 (1.6) | 12 | 11 |
| Caliciaceae (6) | 3 (2) | 3 | 3 |
| Candelariaceae (57) | 23 (2.5) | 18 | 8 |
| Cladoniaceae (24) | 9 (2.6) | 9 | 5 |
| Collemataceae (2) | 2 (1) | 2 | 2 |
| Gyalectaceae (1) | 1 (1) | 1 | 1 |
| Lecanoraceae (238) | 76 (3.3) | 69 | 43 |
| Lecideaceae (2) | 1 (2) | 1 | 1 |
| Megasporaceae (67) | 18 (3.7) | 22 | 14 |
| Parmeliaceae (22) | 3 (7) | 5 | 5 |
| Peltigeraceae (19) | 6 (3.2) | 6 | 6 |
| Pertusariaceae (3) | 1 (3.0) | 1 | 1 |
| Physciaceae (71) | 17 (4.2) | 18 | 18 |
| Psoraceae (21) | 3 (7) | 3 | 3 |
| "Pseudoaspicilia" (1*) | 1 (1) | 1 | 1 |
| Ramalinaceae (1*) | 1(1) | 1 | 1 |
| Rhizocarpaceae (36) | 6 (6) | 6 | 6 |
| Sporastatiaceae (7) | 4 (1.75) | 1 | 1 |
| Stereocaulaceae (10) | 4 (2.5) | 4 | 4 |
| Teloschistaceae (66) | 26 (2.5) | 19 | 19 |
| Tephromelataceae (12) | 2 (6) | 2 | 1 |
| Thelotremataceae (1) | 1 (1) | 1 | 1 |
| Umbilicariaceae (17) | 15 (1.4) | 4 | 4 |
| Verrucariaceae (38) | 11 (3.5) | 13 | 12 |
| Species from recent collections w/o sequence data | NA | 5 | 5 |
| Lichenicolous fungi w/o sequence data | NA | 3 | 3 |
| Additional species from historic collections | NA | 10 | 10 |
| 24 families | 244 total ASAP species (2.8) | 240 total candidate species | 189 total integrative species |

*Note:* The first column lists the family and number of sampled thalli represented by ITS sequence data in parentheses; the second column reports the number of species (average number of sequences/species) inferred using the newly proposed Assemble Species by Automatic Partitioning (ASAP) approach to empirically delimit candidate species-level lineages from family-level multiple sequence alignments; the third column reports the number of candidate species based on combining information from the ASAP partitions and phylogenetic reconstructions; and the fourth column reports integrative species, combining information from the DNA-based candidate species with morphological data.

of lichen fungi from biodiversity inventories is often labor-intensive, often requiring a team of taxonomic experts to appropriately interpret variation (Lücking et al., 2020; McCune et al., 2020; Spribille et al., 2020).

Similarly, DNA barcoding approaches are confounded by inherent limitations with the standard fungal DNA barcoding marker, the ITS, coupled with the lack of comprehensive DNA reference libraries for effective taxonomic assignment (Lücking et al., 2020; Nilsson et al., 2019). While the standard barcode marker for fungi can diagnose distinct species-level lineages in many cases (Schoch et al., 2012), variation in rDNA can in some species complexes provides biased perspectives, potentially over-splitting natural species-level groups. Intraspecific and intragenomic variation in this repeat region is not well known in fungi (Lofgren et al., 2019). In some lichen-forming fungi, for example, the *Rhizoplaca melanophthalma* aggregate, minimal intragenomic variation was observed (Bradshaw

et al., 2020) and the ITS region successfully diagnoses the majority of species in this complex (Leavitt et al., 2013). However, based on genomic data from members of the *R. melanophthalma* aggregate, some highly divergent ITS sequences inferred as distinct candidate species in single-locus species delimitation analyses likely belong to a single species (Bradshaw et al., 2020; Keuler et al., 2020). Inferences from single-locus species delimitation analyses, such as those performed in this study, are inherently limited (Fujita et al., 2012), and most DNA-based species hypotheses will likely need to be investigated on a case-by-case basis.

Limitations with currently available reference libraries for sequence comparison were manifested in the low proportion successful taxonomic assignment of DNA-CS at the species level (File S4), with over 68% of sequences that could not be assigned to species-level rank with statistical confidence. Confounding the poor success in DNA-based taxonomic assignment, nearly a third of all morphologically identified species were not represented by sequence data in public repositories, and in other cases, sequence data from unidentifiable alpine lichens did not closely match to currently available sequence data (Appendix S1). The results support the perspective that substantially increasing the number of sequences based on verified material will be essential for efficient DNA barcode identification (Lücking et al., 2020; Nilsson et al., 2019). In general, for biodiversity surveys where comprehensive taxonomic treatments are impractical, best practices for reporting uncertain identifications are not well established. However, with recent improvements in how unclassifiable species hypotheses are handled in the UNITE database, these "dark" taxa can now be integrated with the taxonomic backbone of the Global Biodiversity Information Facility and an unlimited number of parallel taxonomic classification systems are supported (Nilsson et al., 2019). Without high-quality sequence databases that are thoroughly curated by taxonomists and systematists, integrative biodiversity inventories of lichen-forming fungi will remain labor- and cost-intensive (Begerow et al., 2010).

The results of this study expand novel sequence data into publicly available repositories, providing the first ITS sequences for many species and candidate species-level lineages. Furthermore, we link these sequence data to digital imagery from vouchered specimens (File S1), in addition to the physical vouchered collections (Appendix S1). We documented unexpected ITS sequence variation in multiple nominal species occurring in the La Sals (Appendix S1; File S3), and the species complexes inferred here provide important direction for identifying lineages that require additional research. By providing publicly available ITS sequence data for the majority of specimens collected for this study, our results can be easily integrated in future research using the formal barcoding marker for fungi (Schoch et al., 2012). Rather than relying exclusively on phenotype-based identifications that may be biased in comparison with other studies (Brunialti et al., 2012, 2019; Giordani et al., 2009), these ITS data can be directly integrated into a wide range of future studies using the standard DNA barcoding marker for fungi. Our molecular-based approach for initial species delimitation using ASAP provided only an initial perspective into diversity, providing important direction for future taxonomic research. With ongoing taxonomic revisions of lichen-forming fungi, including the description of new taxa, the sequence data generated here will facilitate more accurate reassignment of specimens from the La Sals to the appropriate taxonomic group.

While the high levels of diversity documented in this study are striking, we predict that other alpine habits in the southern Rocky Mountains may have similar levels of diversity. Small crustose lichens on rocks and soil are common in most alpine regions but are easily overlooked in vegetation surveys (Ahti & Oksanen, 1990). Even when recognized, these crustose lichens may not be documented because of difficulties with identification due to a lack of diagnostic features or environmental modifications in extreme habitats (Kappen, 1973; McMullin et al., 2020). Here, we aimed to overcome these challenges by integrating vouchered specimens (permanently housed in BRY-C), photographic documentation (File S2), and DNA sequence data (deposited in GenBank) to create a robust, transparent inventory of lichen-forming fungal diversity for the limited and vulnerable alpine habitat in the Colorado Plateau, USA. These data provide an important resource for subsequent biodiversity research in alpine habitats, including DNA barcodes for most putative species occurring in the La Sals, crucial information on species distributions, and vouchered collections representing any potentially undescribed species that can be used for phenotypic comparisons.

The opportunistic sampling approach implemented in this study was intended to capture the broad range of species diversity in alpine habitat on the La Sals, rather than provide quantitative insight into distribution patterns or site-specific species richness (McCune & Lesica, 1992; McMullin et al., 2010; Newmaster et al., 2005). Although different habitats throughout the alpine zone in the La Sals, for example, talus slopes, alpine turf, and late snowmelt areas, support distinct lichen communities, we cannot make robust comparisons among these different communities given the limitations with present sampling. Future ecological sampling approaches will be essential to characterizing factors influencing lichen community assembly and monitoring disturbances and ecological changes. For example, mountain goats (*Oreamnos americanus*) were released in 2013 in the La Sal Mountains with notable, site-specific impact on alpine communities, including lichens (Leavitt & Smith, 2020). The long-term impact of the large ungulates on alpine communities will require long-term monitoring. Coupling environmental sampling approaches with the DNA reference sequences provided here will facilitate more efficient sampling strategies for DNA-based monitoring of ecological changes in alpine lichen communities in the La Sals. Both amplicon-based and whole-genome shotgun metagenomic approaches using environmental samples have been shown to capture higher levels of diversity than traditional inventory strategies (Keepers et al., 2019; Wright et al., 2019), the trade-offs among cost, speed, accuracy, and precision of metagenomic approaches must be carefully considered (Lücking et al., 2020). Metagenomic approaches may also reflect the dispersal of propagules of lichen fungi and not necessarily mature, established lichens, inflating species richness (Keepers et al., 2019; Tripp et al., 2019). Incorporating field

observations, vouchered specimens, and phenotype-based data is essential for utilizing metagenomic methods for characterizing fungal diversity.

The origin and stability of members of the alpine lichen community in the isolated La Sal Range remain in question. With limited alpine habitat in the La Sals, major climatic fluctuations during the Pleistocene and geographic distance from other more extensive alpine habitats, one might predict that fluctuating conditions might lead to depauperate alpine lichen communities (Jiménez-Moreno & Anderson, 2013; Louderback et al., 2015). However, we observed the opposite pattern—species-rich and genetically diverse alpine lichen communities in the La Sals (Appendix S1 and File S3). High levels of intraspecific genetic diversity were common across most families of lichen-forming fungi occurring in the La Sals (File S3), often with nominal species represented by multiple DNA-CSs. These results indicate that a large number of lichens were able to successfully disperse, establish, and persist in the isolated alpine habitat in Colorado Plateau. Did members of these communities persist in situ in refugia (Holderegger & Thiel-Egenter, 2009)? The steep-sloped ridgelines and conical, eroded peaks in the La Sals may have existed as nunataks (Richmond, 1962), providing suitable habitat for long-term persistence even during Pleistocene glacial cycles and resulting in the high diversity observed in this study. Contemporary climate change is now having cascading effects on ecosystems, affecting community structure, biotic interactions, and biogeochemistry (Abbott & Jones, 2015; Ernakovich et al., 2014). These sky islands offer considerable potential for investigating not only how different evolutionary and ecological processes structure biological communities but also the impact of modern habitat changes (Czortek et al., 2018). What is the role and frequency of contemporary or recent dispersal events in driving alpine lichen community structure? Similarly, what are the roles of environmental filters and other historical and stochastic factors driving lichen community structure in alpine sky islands (Marx et al., 2017)? In contrast to other alpine regions in the Rocky Mountains where macrolichens comprise an important component of alpine lichen communities (Imshaug, 1957), alpine-specific macrolichens, for example, cetrarioid species, *Thamnolia subuliformis* (Ehrh.) W. L. Culb., etc., were notably absent from alpine habitats in the La Sals, except for a single observation of *Evernia divaricata* (L.) Ach. Our hope is that results from this study will provide further impetus to explore questions relating to the origin and stability of alpine lichen communities.

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## CONFLICT OF INTEREST

The authors declare no conflicting interests.

## AUTHOR CONTRIBUTIONS

**Steven D. Leavitt:** Conceptualization (lead); Data curation (lead); Formal analysis (lead); Funding acquisition (lead); Investigation (lead); Methodology (lead); Project administration (lead); Resources (lead); Software (lead); Supervision (lead); Validation (lead); Visualization (lead); Writing-original draft (lead); Writing-review & editing (lead). **Jason Hollinger:** Conceptualization (supporting); Data curation (supporting); Formal analysis (supporting); Investigation (supporting); Methodology (supporting); Validation (supporting); Writing-review & editing (supporting). **Sara Summerhays:** Data curation (supporting); Investigation (supporting); Software (supporting); Visualization (supporting). **Isaac Munger:** Data curation (supporting); Methodology (supporting); Visualization (supporting). **Jonah Allen:** Methodology (supporting); Visualization (supporting). **Barb Smith:** Conceptualization (supporting); Funding acquisition (equal); Methodology (supporting); Resources (supporting); Supervision (supporting); Writing-review & editing (supporting).

## DATA AVAILABILITY STATEMENT

DNA sequences: GenBank accessions Nos. MZ243469–MZ244202. Final family-level multiple sequence alignments and topologies; photographs of sampled specimens; and supplementary files: Dryad https://doi.org/10.5061/dryad.mpg4f4r08.

## ORCID

*Steven D. Leavitt* https://orcid.org/0000-0002-5034-9724

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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# APPENDIX 3:

# PROVIDING SCALE TO A KNOWN TAXONOMIC UNKNOWN—AT LEAST A 70-FOLD INCREASE IN SPECIES DIVERSITY IN A COSMOPOLITAN NOMINAL TAXON OF LICHEN-FORMING FUNG

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# Providing Scale to a Known Taxonomic Unknown—At Least a 70-Fold Increase in Species Diversity in a Cosmopolitan Nominal Taxon of Lichen-Forming Fungi

**Yanyun Zhang [1,2,†], Jeffrey Clancy [3,†], Jacob Jensen [3], Richard Troy McMullin [4], Lisong Wang [1,*] and Steven D. Leavitt [5,*]**

[1] Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Heilongtan, Kunming 650201, China; 2021209@ahnu.edu.cn
[2] College of Life Science, Anhui Normal University, Wuhu 241000, China
[3] Department of Biology, Brigham Young University, 4102 Life Science Building, Provo, UT 84602, USA; jclancy1330@gmail.com (J.C.); jnjensen88@gmail.com (J.J.)
[4] Canadian Museum of Nature, Research and Collections, Ottawa, ON K1P 6P4, Canada; tmcmullin@nature.ca
[5] Department of Biology, M. L. Bean Life Science Museum, Brigham Young University, 4102 Life Science Building, Provo, UT 84602, USA
* Correspondence: wanglisong@mail.kib.ac.cn (L.W.); steve_leavitt@byu.edu (S.D.L.)
† These authors contributed equally to this work.

**Abstract:** Robust species delimitations provide a foundation for investigating speciation, phylogeography, and conservation. Here we attempted to elucidate species boundaries in the cosmopolitan lichen-forming fungal taxon *Lecanora polytropa*. This nominal taxon is morphologically variable, with distinct populations occurring on all seven continents. To delimit candidate species, we compiled ITS sequence data from populations worldwide. For a subset of the samples, we also generated alignments for 1209 single-copy nuclear genes and an alignment spanning most of the mitochondrial genome to assess concordance among the ITS, nuclear, and mitochondrial inferences. Species partitions were empirically delimited from the ITS alignment using ASAP and bPTP. We also inferred a phylogeny for the *L. polytropa* clade using a four-marker dataset. ASAP species delimitations revealed up to 103 species in the *L. polytropa* clade, with 75 corresponding to the nominal taxon *L. polytropa*. Inferences from phylogenomic alignments generally supported that these represent evolutionarily independent lineages or species. Less than 10% of the candidate species were comprised of specimens from multiple continents. High levels of candidate species were recovered at local scales but generally with limited overlap across regions. *Lecanora polytropa* likely ranks as one of the largest species complexes of lichen-forming fungi known to date.

**Keywords:** alpine/arctic/Antarctic; ASAP; cosmopolitan; cryptic species; genome skimming; species delimitation; symbiotic phenotype

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## 1. Introduction

Quantifying the full scope of species diversity is perhaps one of the largest undertakings of modern taxonomy [1]. Form and function have historically played fundamental roles in inferring species boundaries and relationships among species [2]. More recently, the incorporation of genetic information has highlighted limitations in past attempts to characterize the earth's biological diversity based exclusively on phenotypes [3]. While the science of naming, describing, and classifying organisms has fallen largely to taxonomists, the implications of taxonomy extend to questions of the origin of novelty, speciation, symbiosis, conservation, and ecology [4–6]. Complementary, modern taxonomists use an ever-expanding toolbox from other disciplines for systematic and taxonomic research, integrating traditional approaches with genetic and ecological data, computational modeling, and empirical species delimitation [7–10].

As with other organismal groups, this integrative taxonomic approach has transformed our understanding of fungal diversity—challenging current taxonomy at multiple levels and highlighting rampant unrecognized diversity [11,12]. Though underestimates and challenges in establishing accurate inventories of fungal diversity may be expected due to the mostly cryptic lifestyle of fungi, reconsidering diversity in well-known, conspicuous fungi has also led to the realization that species diversity in these groups may also be mischaracterized [13–16]. Traditionally, and often due to lack of available genetic data, fungal species boundaries have been delimited based largely on morphological characteristics as they can be incredibly practical, especially in the field. However, robust phenotype-based species delimitations in fungi are often confounded by the existence of morphologically similar species in which intra- and inter-specific variations overlap regarding some characters that have been traditionally used to separate taxa [17].

Factors relating to fungi in obligate symbioses add another layer of complexity in delimiting species-level lineages. In lichen-forming fungi, stable interactions among multiple symbionts—the fungal nutritional specialists that acquire fixed carbon from photoautotrophs and are supported by other microbes [18–21]—result in a persistent thallus which can be considered a symbiotic phenotype that results only from the interaction of unrelated organisms [22]. While the symbiotic phenotype of lichens can provide important insight into species boundaries of lichen-forming fungi—the mycobiont, interactions of the mycobiont with differing suites of microorganisms may result in diverse symbiotic phenotypes of the same fungal species [23,24]. In other cases, carefully considering the lichen-associated symbionts can provide crucial insight for resolving otherwise apparently cryptic fungal species [25]. While the complexities of lichen symbioses and their implications for interpreting lichen-forming fungal diversity have recently been more fully recognized, commensurate advances in practical approaches to empirically delimiting lichen-forming fungi have also occurred [26].

Ongoing advances in sequencing technologies have been key in revealing the presence of previously unknown species diversity in fungi [12], opening the gates for research into lichen symbioses that would have previously been impossible [27–30]. Research focusing on long-standing, well-known problematic groups has been shown to lead to crucial resolution. For example, detailed investigations of a widespread montane, tropical macrolichen representing a single nominal fungal species highlighted that the species diversity of the name-giving mycobiont had been underestimated by orders of magnitude [16,31]. The question remains, is this vast underestimate of species-level diversity in lichen-forming fungi an anomaly or a more widespread phenomenon across lichen fungi?

*Lecanora polytropa* (Hoffm.) Rabenh. (Figure 1)—the name-giving mycobiont for the associated symbiotic phenotype—is distributed across largely disjunct, intercontinental populations and occurs on siliceous rocks (especially granite) in montane, alpine, and arctic/Antarctic habitats (Figure 2). As the name suggests, *L. polytropa*, and related species, exhibit a wide range of morphological diversity (Figure 1). Morphological diversity across the name-giving nominal taxon has been recognized over the course of two centuries of research, with dozens of described forms or varieties. At the same time, limited studies have explored the relationship of *L. polytropa* with other *Lecanora* species [32–36], and the full extent of diversity within the '*L. polytropa* group' itself remains largely uncharted. Delimiting species boundaries is made more difficult by the potential role that the environment plays in shaping morphology. Specimens of *L. polytropa* that grow near copper mines, for instance, often have their color change from the normal yellow-green to turquoise blue [37]. While careful phenotypic assessments have led to the description of some species within the '*L. polytropa* group' [32,33], the notorious variability of this species group, coupled with the occurrence of intermediate morphotypes within the '*L. polytropa* group' [38] highlights the pressing need for a thorough survey of this group.

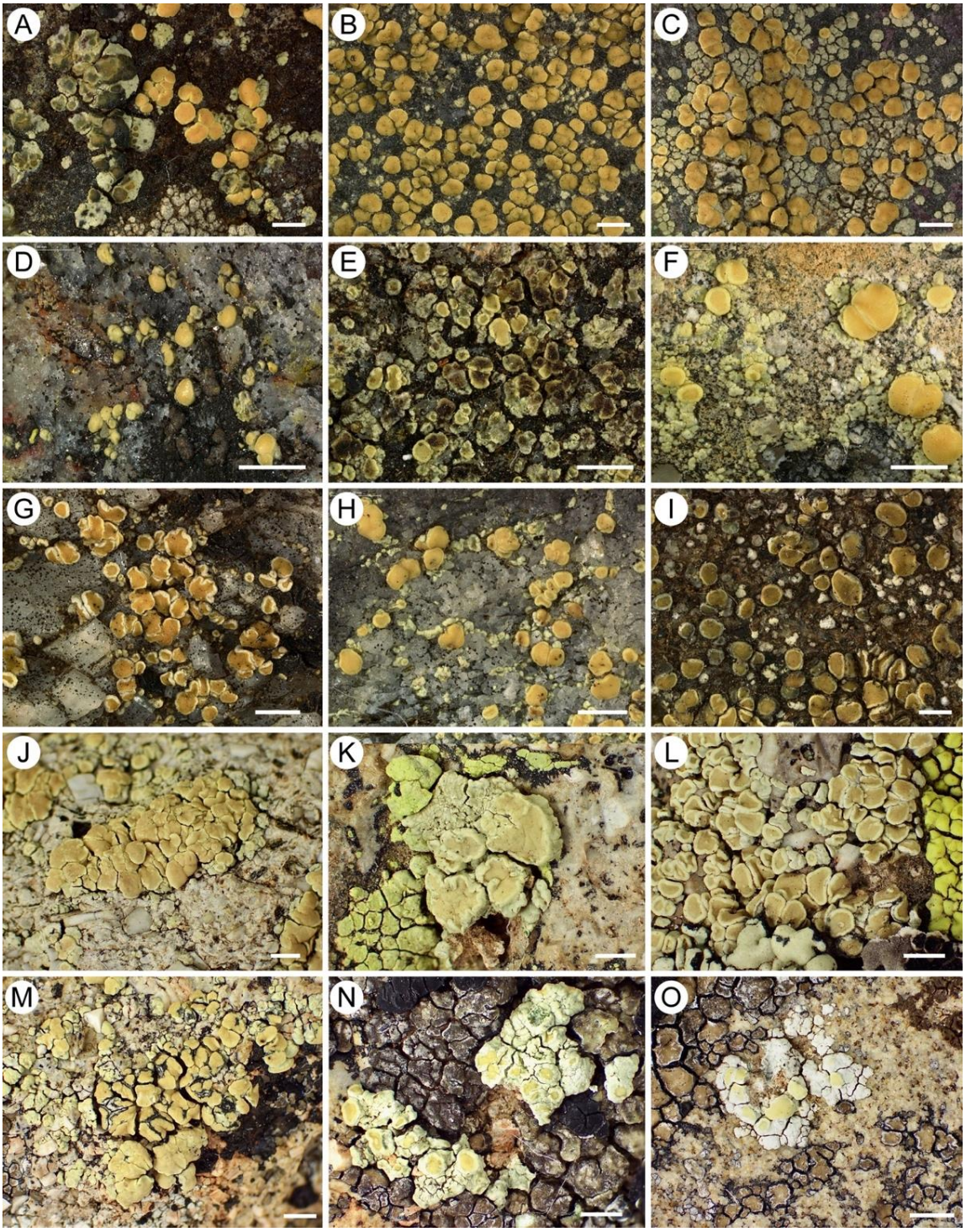


**Figure 1.** Morphological diversity within the *Lecanora polytropa* group. (**A**) *L. intricata* and *L. polytropa* (McMullin 13834 CANL); (**B**) *L. polytropa* (McMullin 17629 CANL); (**C**) *L. polytropa* (McMullin 17695 CANL); (**D**) *L. polytropa* (McMullin 17811 CANL); (**E**) *L. polytropa* (McMullin 13266 CANL); (**F**) *L. polytropa* (McMullin 22536 CANL); (**G**) *L. polytropa* (McMullin 8820CANL); (**H**) *L. polytropa* (McMullin 22539 CANL); (**I**) *L. polytropa* (McMullin 21121 CANL); (**J**) *L. polytropa* (Leavitt SL18256 BRY-C); (**K**) *L. polytropa* (Leavitt SL18280 BRY-C); (**L**) *L. polytropa* (Leavitt SL18356 BRY-C); (**M**) *L. polytropa* (Leavitt SL18454 BRY-C); (**N**) *L. polytropa* (Leavitt SL18455 BRY-C); and (**O**) *L. polytropa* (Leavitt SL18653 BRY-C). Scale bar = 1 mm.

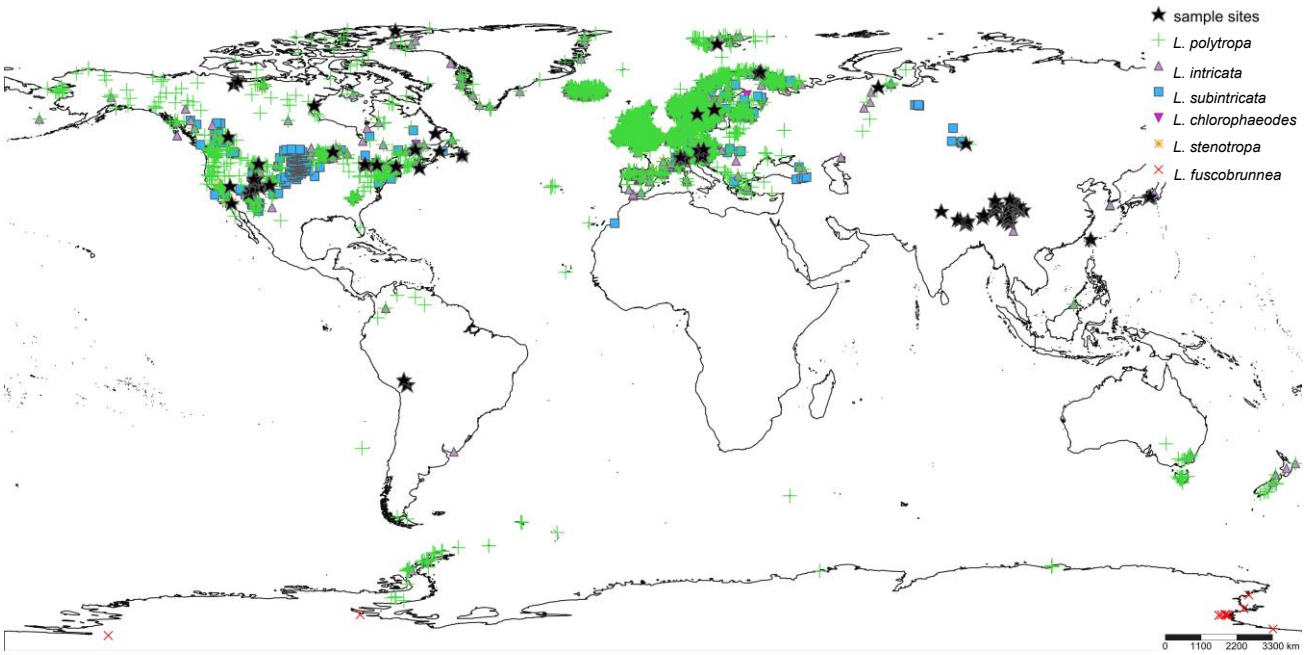


**Figure 2.** Geographic distribution of species within the *Lecanora polytropa* group based on records available from GBIF (https://www.gbif.org; accessed on 3 February 2022). Sampling sites are indicated with a 'star'. The locations of specimens representing *L. fuscobrunnea* (Antarctic endemic, downloaded from GenBank) are not shown. The map was procuded using SimpleMappr (https://www.simplemappr.net; accessed on 3 February 2022).

To better understand the scale of unrecognized species-level diversity in this common, cosmopolitan lichen-forming fungal species, here we circumscribe candidate species within this complex using DNA sequence data. We sampled over 300 specimens collected across multiple, intercontinental populations to meet this aim. From these specimens, we generated DNA sequence data from the standard DNA barcode marker—the internal transcribed spacer region [39]. A subset of specimens was then selected to represent the genetic diversity observed from the ITS data to generate multi-locus and genome-scale datasets. Based on these data, we provide compelling evidence that species diversity within the '*L. polytropa* group' is vastly underestimated, with the nominal taxon *L. polytropa* representing at least 70 candidate species based on current, limited sampling and additional unrecognized species diversity in other species within the '*L. polytropa* group'. In so doing, this study lays a transformative framework for future studies to characterize species diversity more fully within the '*L. polytropa*' species complex.

## 2. Materials and Methods

### 2.1. Taxon Sampling

Our sampling targeted specimens in the '*L. polytropa* group' [35,40]. Recently, the '*L. polytropa* group' was found to be a major lineage within the provisionally named 'MPRPS clade' *sensu* [35] within Lecanoraceae (comprising *Myriolecis*, *Protoparmeliopsis*, *Rhizoplaca*, the '*L. polytropa* group', *Bryonora*, and the "*Lecanora*" *saligna* group). For this study, sampling efforts focused on *L. polytropa sensu lato* populations in Asia, Europe, and North America, supplemented with ITS sequences from GenBank, including *Lecanora chlorophaeodes* Nyl. ($n$ = 3), *L. dispersoareolata* (Schaer.) Lamy (1), *L. fuscobrunnea* Dodge & Baker (31), *L. intricata* (Ach.) Ach. (4), *L. polytropa* (22), *L. solaris* Yakovchenko & Davydov (7), *L.* cf. *subcinctula* (Nyl.) Th. Fr. (2), *L. subintricata* (Nyl.) Th. Fr. (19), *Rhizoplaca aspidophora* (Vain.) Redón (1), and unidentified sequences also recovered within the 'polytropa group' clade (5) (Figure 2). The genus *Carbonea* was shown to be closely related to the 'polytropa group' [40], and all 21 ITS sequences currently available on GenBank were included here. Ultimately, a

total of 340 specimens were included (Supplementary File S1). We note that in several cases, multiple lichen thalli were selected from the same vouchered collection when the voucher included multiple, distinct *L. polytropa sensu lato* thalli. To assess the range of diversity at a limited geographic scale in this nominal taxon, '*L. polytropa* group' specimens were relatively densely sampled from the La Sal Mountains—a sky island on the Colorado Plateau, Utah, USA. Other geographic regions were not sampled as densely, aiming to characterize broader-scale patterns of distributions of putative species-level lineages. We could not obtain fresh material from alpine/subalpine habitats in Africa, Antarctica, Australia, Central America, or New Zealand, and only limited sequences from South America were available [35].

To explore the potential for diagnostic phenotypic traits separating specimens for distinct candidate species-level lineages circumscribed in this study, we characterized (i) general growth forms, (ii) spore sizes, and (iii) secondary metabolites. Morphological characters—e.g., thallus characters, surface color/texture, apothecial disk color, apothecia margin, etc.—were assessed using an Olympus SZH dissecting microscope. Observations and measurements of ascospores were made in water with an Olympus BH-2 microscope, with multiple ascospores measured from at least two apothecia on each specimen. Chemical constituents were identified using thin-layer chromatography (TLC), following standard methods with solvent systems 'C' and 'G' [41,42].

### 2.2. DNA Extraction and Sequencing

Total genomic DNA was extracted from specimens collected for this study using the ZR fungal/bacterial DNA miniprep kit (Zymo Research, Irvine, CA, USA), the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA), or the DNAsecure Plant Kit (Tiangen Biotech, Beijing, China). For all specimens, we attempted to generate sequence data from the internal transcribed spacer region (ITS)—the standard DNA barcoding marker for fungi [39]. For selected specimens representing the genetic diversity observed from the ITS dataset, for example, attempting to represent as many candidate species-level lineages as possible, including multiple representatives for candidate species, if available (see Results), we targeted four additional loci traditionally used in phylogenetic analyses of Lecanoraceae [40]—a portion of the nuclear large-subunit (nuLSU), a fragment of the gene encoding the mitochondrial small subunit (mtSSU), and fragments from two nuclear protein-coding loci, the RNA polymerase II subunit 1 (*RPB1*) and RNA polymerase II subunit 2 (*RPB2*). Temperature profiles for polymerase chain reaction (PCR) amplification for all loci follow previous studies [40]. PCR amplifications were performed using Ready-To-Go PCR Beads (GE Healthcare, Pittsburgh, PA, USA); or alternatively, in 25 µL reactions containing 12.5 µL 2 × Taq PCR Mix (Tiangen Biotech, Beijing, China), 0.5 µL of each primer, 10.5 µL ddH2 O and 1 µL of DNA. PCR products were visualized on 1% agarose gel and cleaned using ExoSAP-IT (USB, Cleveland, OH, USA), following the manufacturer's recommendations. We sequenced complementary strands with the same primers used for PCR amplification, and sequencing reactions were performed using BigDye 3.1 (Applied Biosystems, Foster City, CA, USA). Products were run on an ABI 3730 automated sequencer (Applied Biosystems) at the DNA Sequencing Center at Brigham Young University, Provo, UT, USA.

Single marker and multi-locus approaches may be insufficient to robustly delimit species boundaries, particularly among closely related species [43,44]. Genome-scale data provide unprecedented insight into species boundaries and testing concordance among independent loci [45–47]. Therefore, in addition to Sanger sequencing of traditional phylogenetic markers, metagenomic reads were newly generated from 32 specimens from the '*L. polytropa* group' using short-read shotgun sequencing [48]. Using metagenomic reads from the selected specimens, independent DNA datasets were assembled to investigate concordance among the species partitions inferred from the ITS markers with clades inferred from genome-scale nuclear and mitochondrial datasets [45]. Specimens for Illumina sequencing were selected based on genetic diversity initially observed from sampling in

western North America; as the project expanded, we could not include specimens representing additional diversity observed in subsequent broad geographic sampling for Illumina sequencing. For the specimens selected for metagenomic high-throughput sequencing, total genomic DNA was extracted from a small portion of lichen thalli (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) and 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, Sydney, Australia), 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, Ipswich, MA, USA) 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, UT, USA, using 250 cycle paired-end (PE) reads.

### 2.3. Short-Read Processing and Data Assembly

Raw reads were trimmed using Trimmomatic v0.39 [49] to remove adapter and primer sequences and low-quality reads. Bases at the start and end of reads were trimmed when they had a quality below 3 and 10, respectively, and when the quality of 5-bp sliding windows was <20. All trimmed reads <36 bp were filtered out. We performed a de novo genome assembly using PE reads from *L. polytropa* specimen "Leavitt 16-650" using SPAdes [50]. To identify single-copy nuclear genes for phylogenomic reconstructions from the assembled mycobiont contigs, we used Benchmarking Universal Single-Copy Orthologs to extract up to 1438 gene regions (BUSCO; [51]. Assembled contigs were analyzed using the BUSCO pipeline implemented in the Cyverse.org Discovery Environment [52,53]. The Fungi Odb10 dataset was used to identify BUSCO genes from the assembled *L. polytropa* contigs. Exploratory BLAST searches and assessments of relative sequencing coverage were used to infer that the extracted BUSCO genes likely originated from the *L. polytropa* genome and not other co-occurring fungi. Partial and multi-copy BUSCOs were excluded, and the remaining filtered, single-copy BUSCO genes were used as targets for bait sequence capture using HybPiper [54] to extract these genes regions from each '*L. polytropa* group' metagenomic sample (e.g., [48]). MAFFT [55] was used to generate alignments for individual BUSCO genes using the default parameters, and the alignment algorithm for each locus was chosen automatically by MAFFT. For each alignment, any sample which had an average completion (assembly length/target length) of <0.20 was removed. Genes with average coverage <75% across all BUSCO genes were removed. Phylogenetic gene trees were reconstructed using maximum likelihood (ML) as implemented by IQ-TREE [56]. The substitution model used for each tree was selected using ModelFinder [57]. To assemble genome-scale data from the mycobiont mitochondrial genome, we identified mitochondrial contigs from the SPAdes assembly using BLAST comparisons [58]. The three longest mitochondrial contigs—representing a total of 87.9 Kb—were used as targets in RealPhy v1.12 [59]. For the RealPhy assembly, we used the following parameters to generate the mitochondrial genome alignment: -readLength 100; –perBaseCov 5; –gapThreshold 0.2.

### 2.4. Candidate Species Delimitation Using the Standard Fungal DNA Barcode

Initial candidate species partitions for the *L. polotropa* group were inferred using Assemble Species by Automatic Partitioning (ASAP) [60] based on the multiple sequence alignment of the standard fungal DNA barcode—ITS [39]. ASAP circumscribes species partitions using an implementation of a hierarchal clustering algorithm based on pairwise genetic distances from single-locus sequence alignments [60]. The pairwise genetic distances are used to build a list of partitions ranked by a score, computed using the probability of groups to define panmictic species. ASAP provides an objective approach to circumscribe relevant species hypotheses as a first step in the process of species delimitation.

ITS sequences generated for this study were combined with those GenBank and aligned using the program MAFFT v7 [55,61]. We implemented the G-INS-i alignment algorithm and '1PAM/K = 2' scoring matrix with an offset value of 0.1, the 'unalignlevel' = 0.2, and the remaining parameters were set to default values. The multiple sequence alignment was analyzed using the ASAP Web Server (https://bioinfo.mnhn.fr/abi/public/asap/, accessed on 27 January 2022), with the 'asap-score' considered to select the optimal number of species partitions [60]. In addition to ASAP species delimitations, we also implemented a single-locus tree-based species delimitation method—the Bayesian implementation of the Poisson tree process model (bPTP) [62]. A maximum-likelihood (ML) tree was inferred from the ITS alignment using IQ-TREE [56], which was subsequently analyzed using bPTP with 1,000,000 generations and a burn-in of 10%.

### 2.5. Phylogenetic Analyses and Tests of Genomic Concordance

To assess the monophyly of the '*L. polytropa* group' within Lecanoraceae, ITS, and mtSSU sequences from the present study were combined with the relatively comprehensive "2-locus dataset", including 251 OTUs representing 150 species, originally reported in [40]. Sequences were aligned using MAFFT v7 [55,61], implementing the same parameters described above for ITS. To minimize ambiguities in multiple sequence alignments (MSA), subsequent MSA and phylogenetic reconstructions were restricted to members of the '*L. polytropa* group' and specimens representing the two putative sister lineages inferred from the family-wide, "2-locus dataset"—*Carbonea* species and those representing *L. subintricata* (see Results). For the '*L. polytropa* group' and putative sister groups, the ITS, nuLSU, mtSSU, *RPB1*, and *RPB2* sequences, were aligned using MAFFT v7 as described above. For both the nuLSU and mtSSU alignments, ambiguously aligned regions were excluded using the Gblocks webserver [63], implementing the options for a less stringent selection. A ML tree for the '*L. polytropa* group' was inferred from the concatenated five-marker alignment using IQ-TREE [56]. The concatenated alignment was partitioned by loci, with substitution models selected using ModelFinder [57] and nodal support assessed using 2000 ultra-fast bootstrap replicates [64].

To assess concordance between the candidate species inferred from the standard fungal barcode (ITS) and genome-scale data, we compared ASAP partitions with (i) clades inferred from concatenated single-copy nuclear markers spanning 2.28 Mb, (ii) clades inferred from a mitochondrial alignment spanning 65.5 Kb, and (iii) a phylogenomic approach to species delimitation. Our genome-scale sampling only represented a subset of the candidate species inferred from the ITS data, and comparisons were limited to the 12 ASAP partitions that also had representative samples with short-read data (32 specimens). We reconstructed a phylogeny from a supermatrix comprised of the 1209 single-copy BUSCO markers. Concatenation approaches provide accurate inferences under a range of conditions [65]. We used IQ-TREE v1.6.9 to generate a ML tree from the concatenated BUSCO supermatrix, with nodal support assessed using 1000 ultra-fast bootstrap replicates [64]. We also used SODA [66], an ultra-fast and relatively accurate method for species delimitation, to assess candidate species boundaries inferred from the BUSCO data. SODA uses frequencies of quartet topologies to determine if each branch in a guide tree inferred from gene trees (1209 BUSCO topologies) is likely to have a positive length. It uses the results to infer a new species tree that defines species boundaries. We ran SODA, implemented in ASTRAL [66], with a *p*-value cut-off of 0.001. The mitochondrial topology was reconstructed from the RealPhy alignment using IQ-TREE [56], with the substitution model selected using ModelFinder [57], and nodal support assessed using 1000 ultra-fast bootstrap replicates [64].

## 3. Results

### 3.1. Sequence Data

Newly generated ITS, nuLSU, *RPB1*, *RPB2*, and mtSSU sequences are deposited in GenBank under accession numbers ON179980–ON180462 and ON217582–ON217793. Illumina short reads from the 32 '*L. polytropa* group' specimens are available in the NCBI

Short Read Archive (PRJNA823672). Of the 1438 BUSCO genes searched, 1209 complete, single-copy BUSCO genes were recovered (93.5% of all BUSCO groups). The concatenated alignments of the 1209 nuclear BUSCO markers spanned a total of 2.28 Mb. The REALPHY genome skimming approach for generating mitochondrial data resulted in an alignment of 65456 bp. The concatenated five-marker dataset comprised 3891 aligned nucleotide position characters—ITS ($n$ = 380; 603 bp MSA), nuLSU ($n$ = 122; 843 bp MSA [ambiguous sites removed]), *RPB1* ($n$ = 117; 816 bp MSA), *RPB2* ($n$ = 105; 828 bp MSA), and mtSSU ($n$ = 112; 801 bp MSA [ambiguous sites removed]).

### 3.2. Candidate Species Inferred Using the Standard DNA Barcode (ITS)

The best-scoring ASAP species partitions delimited between 62–103 candidate species within the '*L. polytropa* group'—the 103- and 102-species models had the best asap-scores (Supplementary File S2 and S3). Specimens identified as *L. polytropa* represented up to 75 distinct species partitions in the ASAP analyses of the ITS alignment. Multiple candidate species were also recovered within the nominal taxa *L. concolor* (up to 2 species partitions), *L. dispersoareolata* (2), *L. intricata* (6), *L. solaris* (2), *L. sommervelli* (2), and *L. subintricata* (5). Six species partitions comprised *Carbonea* sequences—the currently unsettled sister-clade to the '*L. polytropa* group'. Based on current sampling, most candidate species partitions are found in geographically limited areas, with only seven species partitions including samples from multiple continents (Supplementary File S1). The tree-based bPTP species delimitation model resulted in 73 candidate species within the '*L. polytropa* group', which were concordant with most of the ASAP species partitions, with ten of the 73 bPTP candidate species combining multiple ASAP partitions from the best-supported models (Supplementary File S4).

### 3.3. Multi-Locus Phylogenetic Inference and Phylogenetic Concordance among Data Sets

In the combined ITS/mtSSU topology, the '*L. polytropa* group' was recovered as monophyletic with strong bootstrap (BS) support within the 'MPRPS clade' *sensu* [35]. In the combined ITS/mtSSU topology, *Carbonea* specimens were recovered as monophyletic within the '*L. polytropa* group'; and *L. subintricata* specimens were recovered as sisters to the entire '*L. polytropa* group' with weak support (58% BS; Supplementary Files S5 and S6). In the 5-marker '*L. polytropa* group' topology, including both *Carbonea* and *L. subintricata* specimens, a midpoint rooted topology showed *Carbonea* specimens as sisters to the '*L. polytropa* group', with *L. subintricata* specimens nested within. Given the uncertainty of the sister clade to the '*L. polytropa* group', we opted to show the midpoint root topology (Figure 3), rather than rooted with *L. subintricata* specimens as inferred from the "2-locus dataset".

In the 5-marker '*L. polytropa* group' topology, the vast majority of candidate species partitions inferred from the ITS MSA using ASAP were recovered as reciprocally monophyletic (Figure 3; Supplementary Files S7 and S8). However, the candidate species partitions inferred from the ITS MSA were not recovered as reciprocally monophyletic in any of the topologies inferred from four traditional markers individually (nuLSU, *RPB1*, *RPB2*, and mtSSU), although some species partitions were monophyletic in some single-gene topologies. Specimens representing previously described species that occur within the *L. polytropa* clade were generally recovered as monophyletic—*L. concolor*, *L. dispersoareolata*, *L. fuscobrunnea*, *L. solaris*, *L. sommervelli*, and *L. subintricata*; although multiple candidate species were inferred from ITS sequence data in most of these nominal taxa (Figure 3; Supplementary File S7). In contrast, sequences representing *L. chlorophaeodes* and *L. intricata* were not recovered as monophyletic.

The limited nuclear phylogenomic sampling comprising specimens from the western USA unambiguously supported the ASAP candidate species partitions represented by short-read data (Figure 4A). The mitochondrial phylogenomic data also generally supported the ASAP species partitions with one instance of putative mitochondrial introgression (Figure 4B). The SODA species delimitation analyses based on 1209 BUSCO gene topologies consistently further subdivided the candidate species partitions delimited from

ITS sequence data using ASAP, delimiting 20 species from the specimens represented by genome-scale data using SODA, in contrast to the 12 ASAP species partitions (Figure 4A).

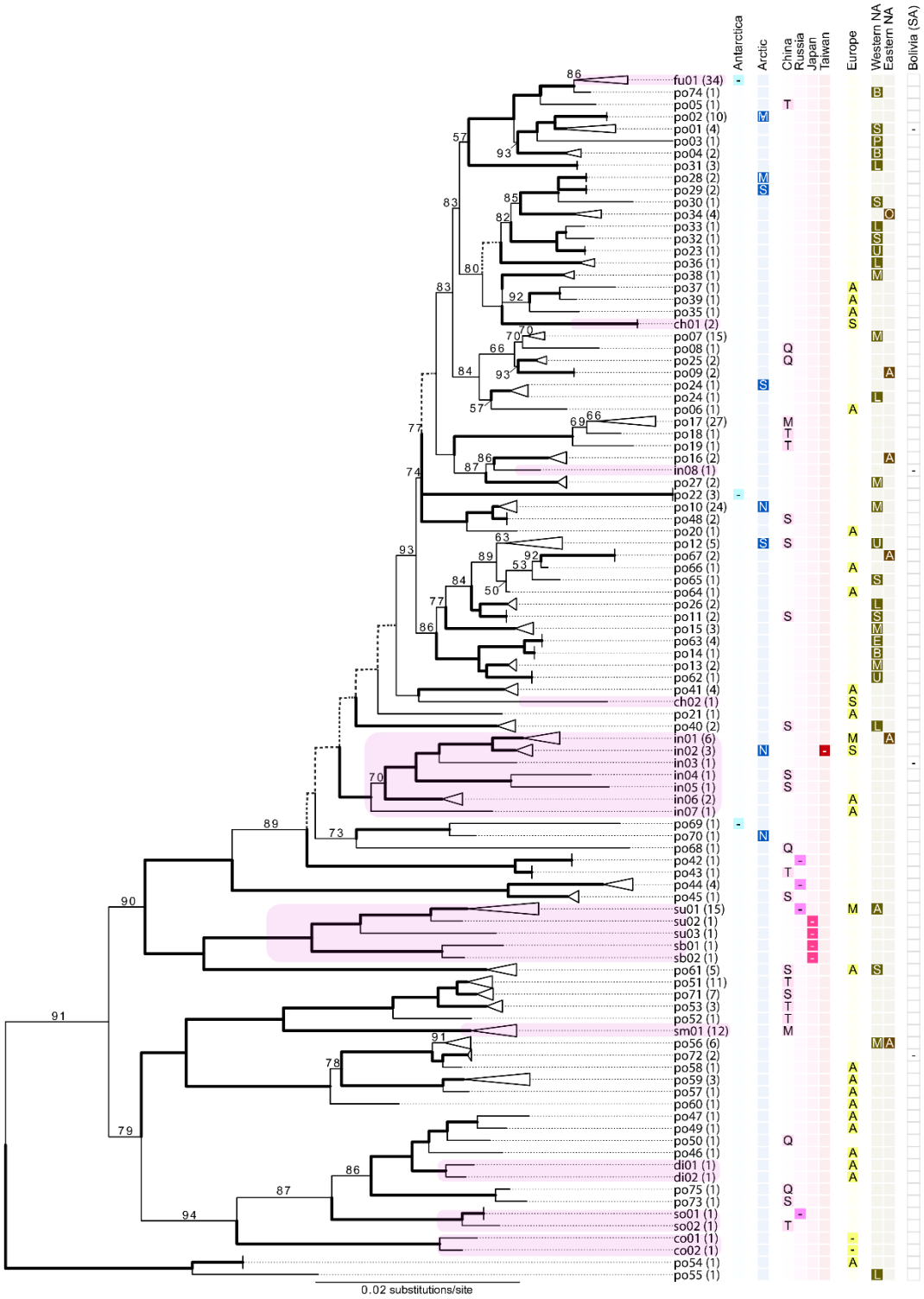


**Figure 3.** (previous page). Midpoint-rooted, five-marker (ITS, nuLSU, *RPB*1, *RPB*2, mtSSU) maximum likelihood topology of the *L. polytropa* group. Clades are labeled as the candidate species inferred from ITS data using ASAP, with the number of specimens representing each candidate species in parentheses. Thickened black branches indicate bootstrap (BS) values between 95–100%, dashed lines indicate BS values below 50%, and otherwise, bootstrap values are indicated at nodes. Clades highlighted in pink represent other taxa recovered in the *L. polytropa* clade—'ch', *L. chlorophaeodes*; 'co',

*L. concolor*; 'di', *L. dispersoareolata*; 'fu', *L. fuscobrunnea*; 'in', *L. intricata*; 'sb', *L.* cf. *subcintula*'; 'sm', *L. somervelii*; 'so', *L. solaris*; 'su', *L. subintricata*—tips labels coincide with names in Supplementary File S1 in the 'Candidate ASAP species partition code' column. The geographic distribution of each candidate species is shown following the tip label: Antarctica (no distinction for different regions); Arctic ('N' = Nunavik; 'S' = Svalbard; 'M' = multiple locations); China ('Q'= Qinghai; 'S'= Sichuan; 'T' = Tibet; 'M' = multiple locations); Russia (no distinction for different regions); Japan (no distinction for different regions); Taiwan (no distinction for different regions); 'Western NA' is western North America ('B' = Beartooth Plateau, MT, USA; 'E' = Escalante Region, UT, USA; 'L' = La Sal Mountains, UT, USA; 'P' = Transverse Range, CA, USA; 'S'= Saguache Range, CO, USA; 'U' = Uinta Mountains, UT, USA; 'M' = multiple locations); 'Eastern NA' is eastern North America ('M' = multiple locations); Bolivia, South America (no distinction for different regions). *Carbonea* specimens were recovered as sisters to the remaining samples and not shown—the complete tree is available as Supplementary File S3.

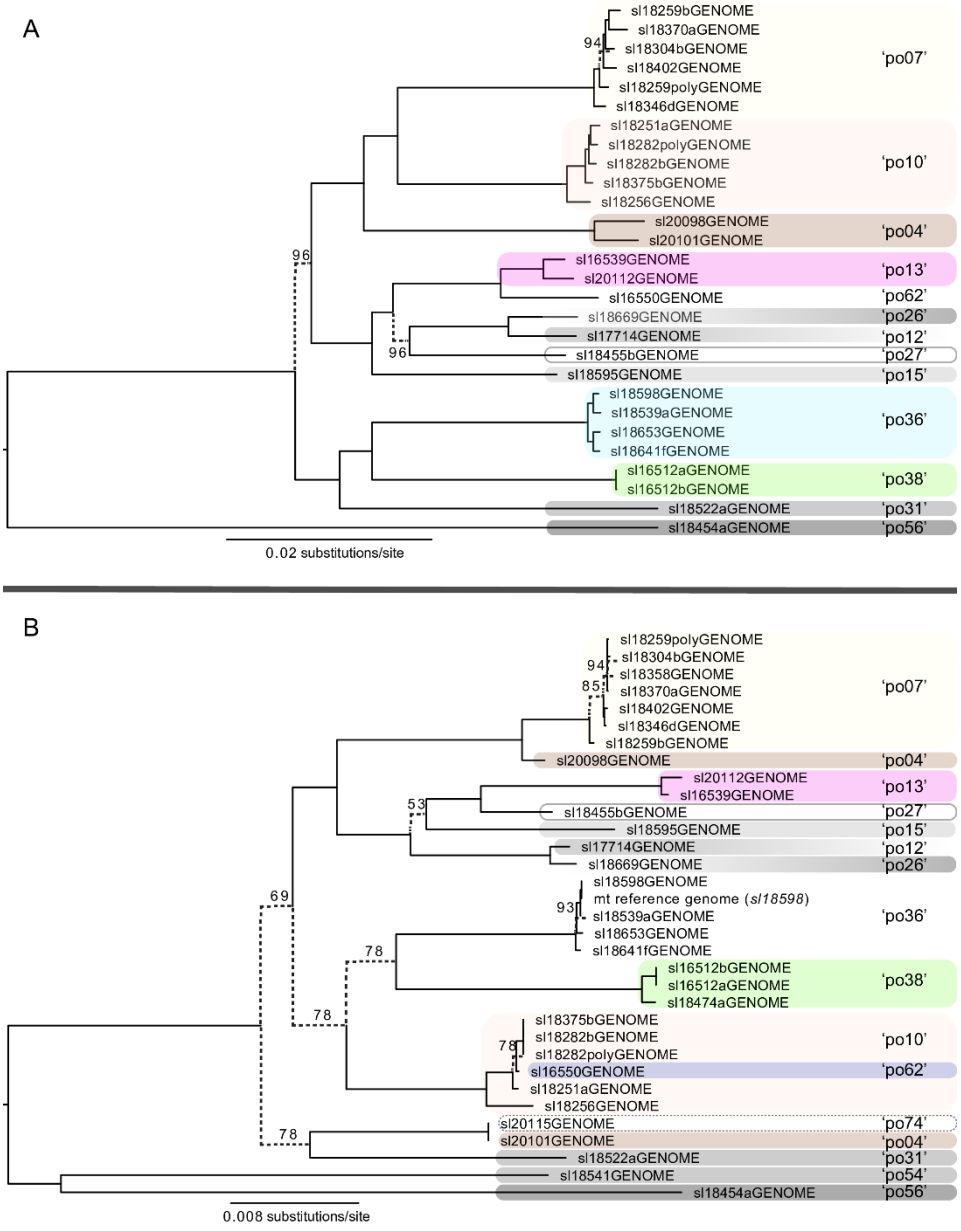


**Figure 4.** Maximum likelihood (ML) topologies inferred from nuclear (**A**) and mitochondrial (**B**) phylogenomic datasets. (**A**). A ML topology from the concatenated alignments of 1209 BUSCO

single. copy nuclear markers spanning 2.28 MBps (Supplementary File S9). Colored clades and clade names are linked to the candidate species inferred from the standard DNA barcoding marker for fungi (ITS), and candidate species inferred from the 1209 BUSCO gene trees using SODA are shown with dashed boxes. Only bootstrap values below 100% are shown. (**B**). A ML topology from the 66.5 KBp mitochondrial alignment generated using RealPhy (Supplementary File S10). Only bootstrap values below 100% are shown

### *3.4. Assessing Morphological Concordance with Species Partitions*

ASAP partitions represented by multiple specimens often comprised polymorphic morphologies. No phenotypic characters were observed that consistently diagnosed any of the ASAP partitions comprised of three or more specimens, although some characters or combinations of characters (e.g., spore size, growth form, secondary metabolite variation, and morphology) loosely corresponded with some distinct species partitions. In a limited number of cases, secondary metabolite concentrations qualitatively varied among specimens in different ASAP partitions. However, given the uneven representation of specimens per candidate species, with many candidate species represented by a very limited number of species, no quantitative comparisons were made. *Lecanora stenotropa* Nyl. is morphologically similar to *L. polytropa* but with a more brownish-green thallus and smaller, narrowly ellipsoid spores. However, based on anatomical observations, no *L. stenotropa* specimens from western North America were sampled, and the relationship of this taxon to other species-level clades in the *L. polytropa* group remains unknown.

## 4. Discussion

Here, molecular sequence data reveal that the well-known, problematic nominal taxon *Lecanora polytropa* likely ranks as one of the largest species complexes of lichen-forming fungi known to date. Based on species delimitations using alignments of ITS sequences, as many as 103 species partitions were circumscribed with the *L. polytropa* clade using ASAP, including ca. 75 candidate species identified as *L. polytropa* and multiple candidate species in other formally described species in the clade. For comparison, perhaps the most species-rich group is the nominal taxon *Dictyonema glabratum*, likely comprising at least 400 distinct species-level lineages [16]. Most other nominal taxa known to mask multiple species-level lineages comprise perhaps up to ten distinct species-level lineages [29,67–72]. Overall, these findings corroborated the perspective that some currently circumscribed conspicuous, well-known lichens may harbor spectacular levels of unrecognized species-level diversity [16]. While phenotypic variation within *L. poytropa s. lat.* is well documented and has historically been interpreted to circumscribe dozens of forms or varieties, a widely accepted workable taxonomy has been elusive for this group over the past two centuries. Below we discuss important implications relative to the likely extent of the species-level diversity in the *L. polytropa* clade.

We provide the most comprehensive sampling to date for members within the *L. polytropa* clade, with over 300 sampled specimens collected from populations across the globe (Figure 2). However, despite the more than 70-fold increase in putative species in the nominal taxon *L. polytropa*, the present sampling effort is likely insufficient to fully capture species-level diversity in this group. For this study, our densest sampling effort targeted a limited number of mountain ranges in western China and the southwestern USA, complemented by opportunistic sampling in other regions. At local scales, for example, individual mountain ranges, high levels of candidate species were recovered (Supplementary File S1). For example, in the La Sal Mountains, in southeastern Utah, USA, up to 14 candidate species were observed. However, candidate species observed on other nearby mountains, e.g., Uinta Mountains (UT, USA), Beartooth Plateau (MT/WY, USA), Saguache Range (CO, USA), had only partly overlapping suites of candidate species (Figure 3). Hence, we speculate that a substantial portion of species diversity in the *L. polytropa* clade remains undiscovered even in the regions most densely sampled for this study. The speculated underestimate of

species diversity in the group is further confounded by the fact that vast regions remain poorly sampled, for example, Europe, Northern Asia, and isolated populations such as those in Australasia (Figure 2). Predictive models for estimating species richness, such as that implemented by Lucking et al. [16], can help in providing quantitative estimates of diversity and directing future sampling.

Given the apparent species richness in the group and the unsettled species boundaries, effective strategies must be developed to robustly delimit species boundaries [26,46]. Based on our results, it appears that the ITS coupled with sequence-based species delimitation approaches, such as ASAP [60], is appropriate as a first pass investigation into species boundaries in the *L. polytropa* clade. Following an initial screening of ITS diversity, targeted genome-scale sequencing will be essential to robustly test evolutionary independence among candidate species-level lineages [46,73]. While our multi-locus phylogenetic reconstruction generally resulted in distinct, well-supported clades coinciding with candidate species inferred from ITS sequence data (Figure 3), the multi-locus dataset was insufficient to resolve many backbone-level relationships nor fully corroborate the independence of candidate species. The genome skimming method implemented here provided a wide range of phylogenomic data that also supported rampant unrecognized diversity in the *L. polytropa* clade. By investigating concordance among different DNA datasets [45], e.g., the ITS (standard fungal barcode) and inferences from the mitochondrial and nuclear genomes, we observed a general pattern that suggests long-term, evolutionary independence among the candidate species within the *L. polytropa* clade. Specifically, data from whole-genome sequencing of a subset of the *L. polytropa* group samples support the inference of rampant, unrecognized species-level diversity (Figures 3 and 4), largely congruent with ASAP delimitations. These results suggest that the candidate species in the *L. polytropa* group are not an artifact of limitations due to single-locus species delimitation methods or intragenomic variation within the multi-copy ribosomal tandem repeat [74]. Rather, there are most likely legitimate reproductive barriers resulting in the observed phylogenetic structure and inferred species partitions.

Although genome skimming approaches, such as those implemented here, can provide crucial genome-scale data [48,75,76], more cost- and time-efficient methods, such as target enrichment sequencing [77,78] or restriction-site associated sequencing (RADseq) [26,47,79] might be more appropriate for this clade given the high number of samples that will likely need to be included. Ultimately, genome-scale data can also be used to reconstruct a robust phylogeny for this group to explore additional evolutionary, phylogeographic, and taxonomic questions. Arguably, genetic data alone is not sufficient for robust delimitations of species boundaries and is best when evaluated in conjunction with other information [80]. While these candidate species provide a powerful framework for taxonomic hypothesis testing, we ultimately support a hypothesis-based, integrative approach to species delimitation [26]. In this study, biased specimen sampling, small sample sizes for most candidate species, and pending detailed anatomical assessments all limit the robustness of the species boundaries inferred here. We hope that these species hypotheses serve as a starting point for integrated approaches required for robust species boundaries and good taxonomy [80,81].

The spectacular species-level diversity and unexpected biogeographic patterns highlight the complex speciation and phylogeographic history of the *L. polytropa* clade (Figure 3). Based on current, limited sampling, we found a mix of a few cosmopolitan lineages and many local/regional endemics. Other lichen-forming fungi have shown similar patterns of complex intercontinental species distributions [82–85]. Presently, little is known of evolutionary processes that may give rise to reproductive isolation and ultimately speciation among populations in the *L. polytropa* clade. Our results indicate frequent, long-distance dispersal throughout the diversification history of this clade (Figure 3). While most candidate species inferred here were not found across broad, intercontinental distributions, ca. 10% comprised specimens from multiple continents. Future work should attempt to elucidate species distributions and different ecological niches among candidate species and

factors influencing ecological specialization, dispersal limitations, etc. For example, some morphologically similar species, such as *L. microloba*, appear to have specialized ecological niches and distributions [36], while our data suggest that others have wide ecological amplitude, resulting in more broadly distributed species. Our hope is that this study provides the impetus for using members of the *L. polytropa* clade as a model to explore phylogeography and speciation in symbiotic fungi.

The results of this study have complex taxonomic implications for *L. polytropa* and closely related species. Given the long, unsettled taxonomic history of *L. polytropa* and associated forms and varieties, a careful revision of the complex taxonomy and available type material will be required to identify which candidate species coincide with *L. polytropa* and other formally described species before naming new species can proceed [26]. This effort may be confounded by the lack of consistent diagnostic features separating evolutionarily distinct lineages, potentially due, in part, to symbiotic interactions [23,86]. Initial phenotypic investigations here failed to reveal consistent taxonomically diagnostic traits, e.g., spore size (Supplementary File S5), corroborating a subset of the distinct candidate species. In some cases, intraspecific phenotype variation/plasticity relating to the thallus morphology, secondary metabolite variation, and spore size was observed. *Lecanora fuscobrunnea*, an Antarctic endemic with distinct apothecia morphology, was found to be closely related to several *L. polytropa* specimens from western North America (Figure 3) and were inferred to be conspecific in several ASAP species partition models (Supplementary File S2). However, given the sparse sample size for most candidate species circumscribed in this study and the limited number of specimens examined, this conclusion should be held only tentatively. In contrast, our results show/corroborate that several morphologically distinct taxa also belong to the *L. polytropa* clade, including *L. chlorophaeodes*, *L. dispersoareolata*, *L. fuscobrunnea*, *L. intricata*, *L. solaris*, *L.* cf. *subcinctula*, and *L. subintricata*. While multiple candidate species also occur in a number of these other nominal taxa, the morphologically distinct groups are generally separated from specimens representing *L. polytropa* (Figure 3). Importantly, we propose that future species descriptions include genetic information in the formal description, as the highly variable morphology is often non-diagnostic, making classification based simply on morphology largely unreliable, as evidenced by two centuries of unresolved species boundaries in the *L. polytropa* clade. Despite the indisputable merits of molecular sequence data in taxonomy, these data are only scarcely used for the formal description of taxa. Novel approaches for DNA-based diagnoses, including diagnostic nucleotide combinations in DNA sequence alignments [87], can be used to provide formal diagnoses for these challenging groups.

Our study lays the groundwork for elucidating important evolutionary insight and formally recognizing undescribed species diversity and taxonomic diversity for this important cosmopolitan clade of symbiotic fungi.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jof8050490/s1. Supplementary File S1: List of specimens from the *L. polytropa* group included in this study. Data include voucher number and herbarium for specimens collected for this study and GenBank accession numbers for sequences from GenBank; DNA number used for labels in multiple sequences alignments; taxonomic assignments for all specimens, including both candidate ASAP species partition codes (shown in Figure 3) and morphology-based specimen identifications; geographic information for specimens (note: geographic origin for sequences from GenBank is incomplete; and in a number of cases, latitude and longitude were inferred [shown in red text]); and GenBank accession numbers for the internal transcribed spacer region (ITS), the nuclear ribosomal large subunit (nuLSU), *RPB*1, *RPB*2, mitochondrial small subunit (mtSSU), and short reads for selected specimens. Supplementary File S2: ASAP scores and rankings for the '*L. polytropa* group' inferred from a multiple sequence alignment of the internal transcribed spacer region (ITS, standard fungal barcode marker) and comprising 380 sequences. Ten best partitions and coinciding asap-scores and ranking (panel on left); and graphical representation of all asap-scores (center and right panels), highlighting the ten best partitions shown in the panel. Supplementary File S3: Comparison of sample assignments to candidate species across the ten best asap partitions. Supplementary File S4:

Results of the Bayesian implementation of the Poisson tree process (bPTP) species delimitation model. A maximum-likelihood (ML) tree was inferred from the ITS alignment using IQ-TREE, which was subsequently analyzed using bPTP with 1,000,000 generations and a burn-in of 10%—see trace file below. Supplementary File S5: Two-marker (ITS + mtSSU) maximum likelihood topology of Lecanoraceae (summarized in Figure 2). Specimen sampling includes L. polytropa samples compiled for this study combined with a broad, Lecanoraceae-wide sampling reported in [40]. Bootstrap support is indicated at each node and the 'L. polytropa group' is shown in red branches, with L. subintricata specimens and Carbonea specimens with blue branches. Supplementary File S6: Concatenated two-marker (ITS + mtSSU) alignment representing Lecanoraceae to assess monophyly of the *L. polytropa* group. The two-marked dataset comprised 630 specimens and spanned 1337 aligned nucleotide position characters: ITS, 781 bp; mtSSU, 556 bp. Supplementary File S7: Midpoint-rooted, five-marker (ITS, nuLSU, *RPB*1, *RPB*2, mtSSU) maximum likelihood topology of the 'L. polytropa group', including Carbonea species and L. subintricata. Bootstrap values greater than 50% are shown for each node. This topology is summarized in Figure 3, and locality info for each specimen can be found in Supplementary File S1. Supplementary File S8: Concatenated five-marker (ITS, nuLSU, *RPB*1, *RPB*2, mtSSU) alignment representing the *L. polytropa* group and putative sister clades. The five-marker dataset comprised 382 specimens and spanned 3891 aligned nucleotide position characters—ITS ($n$ = 380; 603 bp MSA), nuLSU ($n$ = 122; 843 bp MSA [ambiguous sites removed]), *RPB*1 ($n$ = 117; 816 bp MSA), *RPB*2 ($n$ = 105; 828 bp MSA), and mtSSU ($n$ = 112; 801 bp MSA [ambiguous sites removed]). The ITS portion of the concatenated alignment (comprising alignment positions 1–603) was used for the ASAP and BPTP species delimitations. Supplementary File S9: Concatenated alignment of 1209 nuclear BUSCO markers, spanning a total of 2.28 Mb. Of the 1438 BUSCO genes searched, 1209 complete, single-copy BUSCO genes were recovered (93.5% of all BUSCO groups). Supplementary File S10: The REALPHY genome skimming approach for generating mitochondrial data resulted in an alignment of 65,456 bp.

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**Data Availability Statement:** Newly generated ITS, nuLSU, RPB1, RPB2, and mtSSU sequences are deposited in GenBank under accession numbers ON179980–ON180462 and ON217582–ON217793. Illumina short reads from the 32 '*L. polytropa* group' specimens are available in the NCBI Short Read Archive (PRJNA823672). All alignments are available provided as Supplementary Files.

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