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Is targeted community DNA metabarcoding suitable for biodiversity inventories of lichen-forming fungi?

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ABSTRACT

Comprehensive lichen inventories at biomonitoring reference sites provide valuable ecological information but are notoriously difficult to obtain. Due to the limited number of specialists and challenges with generating objective species inventories, investigating alternative or complementary strategies for compiling lichen inventories is paramount to improve biomonitoring strategies. The aim of this study was to determine if targeted community DNA metabarcoding provides an efficient, objective and reliable approach for characterizing lichen-forming fungal diversity in biomonitoring research. To evaluate if reliable biodiversity data could be obtained efficiently and objectively, metacommunity samples were collected by trained field technicians and a professional lichenologist at a previously established biomonitoring reference site in east-central Nevada, USA. Using amplicon-based high-throughput sequencing of a portion of the fungal DNA barcoding marker, the internal transcribed spacer region, molecular operational taxonomic units (OTUs) were inferred and used as a proxy for species diversity. OTUs from individual sampling efforts and two DNA extraction methods were compared to data from the original, voucher-based inventory at the same site. Treating OTUs as a proxy for species diversity, a 1.6-fold increase in diversity of lichen-forming fungi was observed when comparing the targeted community DNA metabarcoding to the initial, voucher-based inventories – 72 OTUs vs. 43 species. Strikingly, substantial overlap in captured OTU diversity was observed between minimally trained technicians and a professional lichenologist metacommunity samples. Based on these results, targeted community DNA metabarcoding is a promising strategy for creating objective lichen inventories. However, metabarcoding cannot discriminate the presence of established thalli from that of spores or propagules (in absence of established thalli) present in the sample/area. Therefore, targeted community DNA metabarcoding for assessing lichen biodiversity offers a complementary, rather than a substitutive sampling strategy.

1. Introduction

Biodiversity inventories play important roles across a wide range of biological subdisciplines, including conservation, ecology, evolution, etc. (Balmford and Gaston, 1999). However, traditional biodiversity assessments are costly in terms of time, money and taxonomic expertise (Yu et al., 2012). Amplicon-based community DNA barcoding (targeted metabarcoding) can provide critical advantages as an alternative, or complementary, inventory strategy by boosting data acquisition through automating the identification of multiple species from a single bulk sample (Taberlet et al., 2012). In monitoring research, targeted metabarcoding of sensitive biological communities can provide important insights into the spatial-temporal continuum of biodiversity at the level of species and even populations (Baselga et al., 2013). However, the efficacy of targeted community DNA metabarcoding has been assessed for only a limited number of groups used in biomonitoring research, such as macroinvertebrates (Emilson et al., 2017), benthic eukaryotic communities (Chariton et al., 2015), marine vertebrates (Andruszkiewicz et al., 2017), and others (Baird and Hajibabaei, 2012; Keck et al., 2018). In all cases, reliable reference DNA libraries are required to successfully identify samples to species (Dincă et al., 2011; Kelly et al., 2011; Knebelsberger et al., 2014).

Rather than abiotic metrics, sensitive bio-indicators are used to directly assess disturbances and ecological health in biological communities. Groups that have been used as bio-indicators include: terrestrial and aquatic invertebrates (Hodkinson and Jackson, 2005), nematodes (Bongers and Ferris, 1999), bryophytes (Frego, 2007; Pesch and Schroeder, 2006), vascular plants (Coulston et al., 2003), and lichens

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(Leavitt and St. Clair, 2015; Nimis et al., 2002). Lichens are particularly useful as bio-indicators because many species live for decades, or even hundreds of years, showing cumulative responses to changes in atmospheric pollution levels, land management practices, and climate change (Conti and Cecchetti, 2001). Additionally, some species are known to be sensitive to important pollutants, and their presence or absence is indicative of current ecological conditions (Giordani and Brunialti, 2015). Measurable responses of individual lichen thalli – e.g., differential accumulation of atmospheric pollutants – and lichen communities – e.g., changes in community composition and population density – provide a mechanism for quantitatively assessing ecosystem health (McCune, 2000), as well as a record of the kinds and relative quantities of potential air pollutants (Hawksworth, 1971; Loppi and Pirintsos, 2003; St. Clair et al., 2002).

Important to lichen-based biomonitoring programs is the ability to effectively produce reliable data (Vondrák et al., 2016), and a number of standardized sampling methodologies have been developed (Conti and Cecchetti, 2001; Giordani and Brunialti, 2015; McCune and Lesica, 1992). Taxonomic surveys and ecological sampling are two well-developed sampling strategies. While the aim of each is fundamentally the same – namely, to generate lists and distributional data of lichens present in a community – there is an information trade-off between the two methods (McCune and Lesica, 1992). Ecological sampling is able to provide robust quantitative data of species richness but captures lower levels of species diversity than a taxonomic inventory (Newmaster et al., 2005). Taxonomic surveys generally generate a more complete qualitative summary of diversity and often document species relative abundance/frequency (Ellis and Coppins, 2017; McMullin et al., 2010). However, patterns of richness and species composition obtained from these distinct sampling strategies have been shown to be correlated, potentially resulting in congruent inferences in terms of identifying and managing priority sites (Ellis and Coppins, 2017).

Most traditional sampling methods require substantial amounts of time and labor to correctly identify the species collected (Giordani et al., 2009). Of specific concern are the effects that different levels of training may have on the ability to generate consistent, objective results, particularly given the limitations even with surveys performed by experts (Giordani et al., 2009; Vondrák et al., 2016). Specimen-based lichen inventories are time consuming, often requiring taxonomic specialists to provide accurate identifications (Hunter and Webb, 2002); and accurate specimen identification of lichen specimens is not a trivial task. Misidentifications are relatively common in field surveys of lichen-forming fungi due to the fact the many identifications require microscopic, chemical, and/or DNA sequence data. Other specimens may lack the phenotypic characters due to developmental stage, environmental influences, etc. Furthermore, given the paucity of morphological and/or diagnostic traits in lichens, nominal species mask cryptic diversity in many lineages of lichen-forming fungi (Crespo and Lumbsch, 2010).

Challenges in generating consistent, objective results for ecological monitoring research are exacerbated in regions with highly heterogeneous landscapes/habitats where lichens may be dispersed sparsely or unevenly across the landscape (Giordani et al., 2009). Traditional, ecological sampling strategies cannot be consistently implemented across reference sites that don't meet the specific sampling protocol. For example, the Forest Inventory and Analysis National Program (FIA) protocol (Will-Wolf, 2010) provides standardized sampling protocol for macrolichens from woody substrates. However, in many habitats in the Intermountain Region of western USA macrolichens are much less common and cannot be consistently found across references sites (Leavitt and St. Clair, 2015). Furthermore, dominant and potentially sensitive components of lichen communities, e.g., rock- and soil-dwelling lichens, are not included in FIA sampling strategies (Belnap and Eldridge, 2001), leaving these important members of lichen communities largely unassessed.

DNA metabarcoding studies may have different, or partially overlapping, aims when compared to traditional inventories. In addition to generating site-specific inventories, metabarcoding may be used to detect propagules from potential community-members that have not established, thus providing important information about environmental filtering. Using metabarcoding approaches it may be difficult to distinguish reads from propagules vs. established lichens without complementary field surveys, although perhaps comparisons of relative read abundance may offer valuable insights. Therefore, great care must be taken in design and interpretation of DNA barcoding studies (Taberlet et al., 2012).

Targeted metabarcoding allows taxonomic identification from mixed samples (Zimmermann et al., 2015) but has never been utilized for ecological monitoring research using lichens. In contrast to individual specimen- and/or voucher-based approaches that rely on the identification of physical lichen specimens, diversity is assessed through DNA metabarcoding of mixed lichen community samples. While traditional voucher-based inventories are time intensive and require taxonomic expertise for accurate specimen identifications, DNA-based inventories shift the burden to efficient sampling strategies, cost-effective sequencing methods, and appropriate bioinformatic approaches (Baird and Hajibabaei, 2012). The purpose of this study was to evaluate the utility of using a targeted community DNA metabarcoding approach for facilitating biodiversity assessments of lichen-forming fungi. Specifically, we asked whether high-throughput sequencing of targeted ITS2 amplicons from lichen metacommunity samples could provide a more efficient and objective approach for characterizing lichen-forming fungal diversity when compared to sampling efforts toward a more traditional, voucher-based inventory. To assess these aims, we (i) compare a DNA-based inventory approach with a traditional voucher-based inventory to characterize lichen-forming fungal diversity, (ii) compare DNA-based inventories from trained field technicians with a professional lichenologist to assess consistency among sampling effort, and (iii) consider two DNA extractions methods to elucidated potential bias based on laboratory methods.

2. Materials & methods

2.1. Study area and study design

To evaluate the suitability of targeted community DNA metabarcoding for lichen inventories, we selected a previously established biomonitoring reference site in east-central Nevada, USA. The reference site was located in a semi-arid, pinyon-juniper woodland near the mouth of Broom Canyon in the Humboldt-Toiyabe National Forest, Ely Ranger District, Nye County, Nevada, U.S.A. Broom Canyon is located at ca. 2030 m.a.s.l. on the west-facing slope of the White Pine Mountain Range, east of Railroad Valley and west of the Currant Mountain Wilderness Area. The site is approximately one hectare, bounded on the south by Broom Creek, an unimproved jeep track on the North, and centered near the following coordinates: 38.889°N and -115.499° W. For comparison to traditional taxonomic inventories, a checklist of lichens at the site, including vouchered collections, was available from the previous lichen inventory completed during the summer of 2011. The initial inventory reported 43 species of lichen-forming fungi in 24 genera (Supplementary Table S1).

2.2. Field methods

Samples for targeted metabarcoding were collected in October 2016, and the field team consisted of two trained technicians and a professional lichenologist (SDL). Prior to the fieldwork, the technicians were trained (for ca. 3 h) on how to recognize lichens, the importance of sampling diverse microhabitats, the range of potential substrates (e.g., rock, soil, vascular plants, lignum, etc.), and specific field protocols. Workers were instructed to collect small fragments of lichen thalli representing perceived diversity across all potential substrates. At the reference site, sampling time was limited to two hours. Lichen

Table 1
Summary of metacommunity samples assessed in this study.

Code	Description of origin of lichen community sample	Amount collected (g)	DNA extraction method	Number of Illumina reads
$T_{A\text{-}2016}$	'Technician A' – 2016 community sampling for lichen community metabarcoding.	ca. 1.2 g	Quick-DNA Fungal/Bacterial Kit (Zymo)	56,336
$T_{B\text{-}2016}$	'Technician B' – 2016 community sampling for lichen community metabarcoding.	ca. 0.85 g	Quick-DNA Fungal/Bacterial Kit (Zymo)	53,752
$P_{2016\text{-}Zymo}$	Professional lichenologist – 2016 community sampling for lichen community metabarcoding.*	ca. 0.75 g*	Quick-DNA Fungal/Bacterial Kit (Zymo)	36,790
$P_{2016\text{-}PowerMax}$	Professional lichenologist – 2016 community sampling for lichen community metabarcoding.*	ca. 7.9 g*	PowerMax Soil DNA Isolation Kit (Qiagen)	52,596
V_{2011}	2011 vouchered collection from the initial morphology-based lichen inventory.	ca. 1.2 g	PowerMax Soil DNA Isolation Kit (Qiagen)	44,882

* DNA was extracted from ca. 0.75 g of the P_{2016} using the Quick-DNA Fungal/Bacterial Kit (Zymo); and the remaining material – ca. 7 g – was used for DNA extraction using the PowerMax Soil DNA Isolation Kit (Qiagen).

fragments were collected with sterilized tweezers and placed directly into Nasco Whirl-Pak 18 oz. collecting bags. For comparison to the original lichen inventory (Supplementary Table S1), small fragments of lichens were removed from the 2011 vouchered collections at the Herbarium of Non-Vascular Cryptogams (BRY-C) at Brigham Young University, Provo, Utah, USA – herbarium collections Nos. 53533–53583. Many of the 2011 vouchers included additional, or accessory, lichens, and effort was made to sample any additional material from other lichens observed along with the targeted lichen. As with the new field samples, lichen fragments were also collected from the 2011 samples using sterilized tweezers and placed directly into a Nasco Whirl-Pak sample bag.

2.3. Laboratory methods

DNA was extracted from four separate community samples collected at the Broom Canyon site – two samples from the technicians, one from the professional lichenologist, and the fourth from the 2011 field collections (Table 1). Each composite sample was then homogenized using a sterilized mortar and pestle. Lichen community DNA was extracted from a 200 mg subset of each of the homogenized 2016 community samples using the ZR Fungal/Bacterial DNA Miniprep Kit (Zymo Research) following the manufacturer's recommendations. From each sample, four independent DNA extraction replicates were performed and subsequently pooled. To assess potential differences in DNA extraction methods and the differences in the amount of sample material, e.g. a subset of bulk samples vs. more comprehensive inclusion of sample material, DNA was also extracted from the professional lichenologist's 2016 community sample using the PowerMax Soil DNA Isolation Kit (Qiagen) ($P_{2016\text{-}PowerMax}$, Table 1). This kit accommodates DNA extraction from much larger sample sizes and was used to extract DNA from ca. 7 g of the homogenized '$P_{2016\text{-}PowerMax}$' community sample following the manufacturer's recommendations. DNA was also extracted from the 2011 community sample – 'V_{2011}' – using the PowerMax Soil DNA Isolation Kit (Qiagen). This comparison of potential differences in DNA extraction methods is not intended to be a comprehensive exploration of potential laboratory methods that may potentially introduce bias but to provide exploratory insight into a single laboratory factor to consider when designing metabarcoding studies from bulk samples.

The internal transcribed spacer region (ITS, 5.8S and ITS2; hereafter 'ITS') has been proposed as the standard barcoding marker for specimen identification in fungi (Schoch et al., 2012). In fungi, the ITS region has been shown to outperform other commonly use genetic markers in discriminating species within a DNA barcoding context (Schoch et al., 2012), although a comprehensive, well-curated reference DNA library is required to successfully identify samples to species (Kelly et al., 2011; Orock et al., 2012). Furthermore, in some cases a single locus, such as the ITS, may not be able to discriminate among closely related taxa, thereby resulting in spurious identifications (Leavitt et al., 2016; Will et al., 2005). DNA barcoding may also detect the presence of DNA from specimens that are not established at a site, e.g., propagules, environmental DNA, etc.; and quantitative approaches to distinguished established lichen-forming fungi from propagules remain unexplored. In spite of these potential shortcomings, DNA barcoding using the ITS marker remains a powerful tool for characterizing fungal diversity (Abarenkov et al., 2018). Fragments in either the ITS1 or ITS2 size range can be readily sequenced on the Illumina MiSeq platform. For our study, we amplified the ITS2 region based on previous recommendations (Bálint et al., 2014; Bazzicalupo et al., 2013; Schmidt et al., 2013). The ITS2 region from our community samples was amplified (Table 1) and prepared for sequencing at RTL Genomics (Lubbock, TX) using a two-step process. The forward primer was constructed (5′-3′) using the Illumina i5 sequencing primer (TCGTCGGCAGCGTCAGATGTGTATAA GAGACAG) and the ITS3 (GCATCGATGAAGAACGCAGC) primer (White et al., 1990). The reverse primer was constructed (5′-3′) using

the Illumina i7 sequencing primer (GTCTCGTGGGCTCGGAGATGTGT ATAAGAGACAG) and the ITS4 (TCCTCCGCTTATTGATATGC) primer (White et al., 1990). Polymerase chain reaction amplifications (PCR) were performed in 25 µl reaction volumes with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, California), using 1 µl of each 5 µM primer, and 1 µl of template DNA. Reactions were performed with ABI Veriti thermocyclers (Applied Biosytems, Carlsbad, California) using the following thermal profile: 95 °C for 5 min., then 25 cycles of 94 °C for 30 sec., 54 °C for 40 sec., 72 °C for 1 min., followed by one cycle of 72 °C for 10 min. and 4 °C hold. Products from the first stage amplification were added to a second PCR based on qualitatively determine concentrations. Primers for the second PCR were designed based on Illumina Nextera PCR primers as follows: Forward – AATGATACGGC GACCACCGAGATCTACAC[i5index]TCGTCGGCAGCGTC and Reverse – CAAGCAGAAGACGGCATACGAGAT[i7index]GTCTCGTGGGCTCGG. The second stage amplification was run the same as the first stage except for 10 cycles. Amplification products were then visualized on eGels (Life Technologies, Grand Island, New York). Products were then pooled equimolar and each pool was size selected in two rounds using SPRIselect (BeckmanCoulter, Indianapolis, Indiana) in a 0.7 ratio for both rounds. Size selected pools were then quantified using the Quibit 2.0 fluorometer (Life Technologies) and loaded into an Illumina MiSeq (Illumina, Inc. San Diego, California) 2 × 300 flow cell at 10 pM and sequenced at RTLGenomics.

2.4. Metagenomic analyses

The sequenced reads were processed through denoising and chimera detection. The forward and reverse reads were merged together using the PEAR Illumina paired-end read merger (Zhang et al., 2014). Reads were filtered using an internally developed quality trimming algorithm (RTL Genomics) where the quality score of each read is taken as a running average across the sequence and trimmed back at the last base where the total average read quality score is greater than 25. Prefix dereplication was performed using the USEARCH algorithm (Edgar, 2010) to form clusters (4% dissimilarity among sequences of the same cluster) so that each sequence of equal or shorter length to the centroid sequence must be a 100% match to the centroid sequence for the length of the sequence. All sequences < 100 bp in length were excluded. Molecular operational taxonomic unit (OTU) selection was performed using the UPARSE OTU selection algorithm (Edgar, 2013) to classify clusters into OTUs. Chimera checking was performed on the output OTUs using the UCHIME chimera detection algorithms executed in *de novo* mode (Edgar et al., 2011). Each clustered centroid was then mapped to its corresponding OTUs and either marked as chimeric and excluded or marked as non-chimeric, and all chimeric sequences were excluded. Each sequence was corrected base by base in order to remove noise. Taxonomic identifications of the centroid sequence from each cluster was performed using the USEARCH algorithm and based on the UNITE fungal database, version 7.2 (https://unite.ut.ee). Taxonomic assignments were generated under the assumption that the best match is the correct one, termed 'FullTaxa' here. In a more conservative approach, each taxonomic level is assigned only if the confidence value is greater than or equal to 0.51, termed 'TrimmedTaxa'. However, the UNITE database is still incomplete and many of the lichen-forming fungal species sampled for this study may not be represented. Therefore, the taxonomic assignments are to be considered only approximate identifications and may not reflect the true species.

3. Results

The total number of reads per sample are reported in Table 1. Diversity and general patterns were similar when taxonomic assignments were generated under the assumption that the best match is the correct one ('FullTaxa') and when each taxonomic level was assigned only if the confidence value is greater than or equal to 0.51, ('TrimmedTaxa') (see supplementary files 1 & 2); and here we discuss results from the 'FullTaxa' assignments. Nine to 21% of all reads were classified as 'no hit' (Supplementary Files 1 & 2), meaning that the taxonomic classification of the OTU – even at the Kingdom/Domain level – could not be made. The vast majority of reads (70–85%) were inferred to be fungi; and from these, a total of 106 fungal OTUs were inferred across the five samples (Supplementary Table S2). No OTUs were assigned to Basidiomycota. Four additional OTUs were inferred and assigned membership in Plantae, three representing the lichen-associated algal genus *Trebouxia* (Chlorophyta) and one was identified as a *Salvia* species (Streptophyta) (Supplementary Table S2).

Fungal OTUs were assigned membership to six Ascomycete classes – Arthoniomycetes (1 OTU), Dothideomycetes (17), Eurotiomycetes (21), Lecanoromycetes (62), Leotiomycetes (1), Sordariomycetes (1), and two additional 'unclassified' fungal OTUs. Of the 106 fungal OTUs inferred here, 78 were identified as lichen-forming fungi found in two classes – Dothideomycetes and Lecanoromycetes – representing eight orders: Acarosporales (13 OTUs), Caliciales (8), Candelariales (3), Lecanorales (9), Lecideales (3), Pertusariales (11), Teloschistales (15), and Verrucariales (16) (Supplementary Table S2).

A total of 72 lichen-forming fungal OTUs were inferred from the 2016 community samples ($T_{A\text{-}2016} + T_{B\text{-}2016} + P_{2016\text{-}Zymo} + P_{2016\text{-}PowerMax}$), plus an additional six OTUs from the 2011 vouchered collection (V_{2011}) not found in the 2016 community samples (Table 2). The six OTUs inferred from the 2011 vouchered collection (V_{2011}) were assigned to six different genera, *Athallia*, *Caloplaca*, *Lecanora*, *Physcia*, *Protoparmeliopsis*, and *Verrucaria*, with representatives of *Athallia*, *Physcia*, and *Protoparmeliopsis* found only in the V_{2011} samples. Significant overlap among OTUs was observed when the 2016 community samples ($T_{A\text{-}2016} + T_{B\text{-}2016} + P_{2016\text{-}Zymo} + P_{2016\text{-}PowerMax}$) were compared with the OTUs from the 2011 vouchers collection (V_{2011}). However, each sampling pool also included unique OTUs not present in the others (Fig. 1A). Treating molecular-based OTUs as a proxy for species diversity, there was an approximate 1.6-fold increase in diversity when the targeted community DNA metabarcoding approach was compared to the traditional, voucher-based inventories – 72 OTUs (Table 2) vs. 43 species (Supplementary Table S1). At a generic level, OTUs inferred from the DNA metabarcoding samples belonged to 35 genera based on the 2016 community samples and 28 from bulk metacommunity samples from the 2011 vouchered specimens. Twenty-four genera were initially identified from the voucher-based identifications.

Comparisons of the number of OTUs sampled from each of the four independent community samples from 2016 revealed that, of the 72 inferred OTUs, 51 were present in both the professional's pooled samples ($P_{2016\text{-}Zymo} + P_{2016\text{-}PowerMax}$) and the pooled technicians' samples ($T_{A\text{-}2016} + T_{B\text{-}2016}$) (Fig. 1B), an overlap of over 70%. Eighteen of the 72 reported OTUs were only present in the professional's pooled sample, while the pooled technician samples included three independently-occurring OTUs. However, comparisons among independent community samples extracted using the ZR Fungal/Bacterial DNA Miniprep Kit – $T_{A\text{-}2016}$ vs. $T_{B\text{-}2016}$ vs. $P_{2016\text{-}Zymo}$ – revealed more variation among individual samples (Fig. 1C), with each independent sample representing ca. 60–88% of the cumulative OTU diversity.

A comparison of the two DNA extraction methods – $P_{2016\text{-}PowerMax}$ vs. $P_{2016\text{-}Zymo}$ – (Table 1) showed that the kit typically used for extracting DNA from larger composite samples, the PowerMax Soil DNA Isolation Kit, resulted in six additional lichen-forming fungal OTUs, 69 vs. 63 (Fig. 1D). There were no OTUs that were exclusive to the Zymo extraction pool ($P_{2016\text{-}Zymo}$).

4. Discussion

Our results clearly show that a targeted community DNA metabarcoding approach captures more diversity than traditional voucher-based inventories (Table 2). We also document that there was

Table 2
OTU species assignment and number of associated reads. Bolded values indicate species-level OTUs identified in only a single sample.

OTU species assignment	V_{2011}	$T_{A\text{-}2016}$	$T_{B\text{-}2016}$	$P_{2016\text{-}PowerMax}$	$P_{2016\text{-}Zymo}$
	Vouchers	Technicians		Professional	
Acarospora cf. *contigua*	–	54	1	2	1
Acarospora cf. *dissipata*	–	198	979	76	43
Acarospora glaucocarpa	–	–	–	**52**	**10**
Acarospora laqueata	–	5	20	5	–
Acarospora molybdina	181	1105	184	277	194
Acarospora rosulata	9	944	406	557	386
Acarospora sp	16	390	895	72	63
Acarospora strigata	299	878	2522	851	666
Acarospora umbilicata	–	85	29	2	3
Acarospora wahlenbergii	–	1	–	238	163
Aspicilia caesiocinerea	–	–	10	218	82
Aspicilia cf desertorum	509	1247	987	537	402
Aspicilia cinerea	–	–	4	16	25
Aspicilia desertorum	303	268	–	286	189
Aspicilia esculenta	24	111	83	202	94
Aspicilia rivulicola	–	27	196	123	60
Aspicilia vagans	106	169	68	448	243
Athallia saxifragarum	**4**	–	–	–	–
Bagliettoa calciseda	–	–	920	173	73
Buellia russa	9	–	–	19	6
Calogaya decipiens	2	1	35	–	–
Caloplaca aractina	19	22	4	16	–
Caloplaca biatorina	130	458	289	183	70
Caloplaca chlorina	40	–	–	41	12
Caloplaca ferrugineoides	**3**	–	–	–	–
Caloplaca haematites	1	48	–	5	2
Caloplaca scrobiculata	133	4	–	7	2
Candelaria sp	327	31	–	68	20
Candelariella terrigena	112	927	3072	429	247
Catapyrenium lachneum	–	–	2	9	1
Circinaria contorta	31	13	2	20	12
Dermatocarpon taminium	–	279	–	105	51
Endocarpon adscendens	35	15	2	39	13
Endocarpon petrolepideum	1	–	–	838	885
Gyalolechia flavorubescens	236	–	–	81	26
Lecanora dispersa	35	6	–	2	–
Lecanora fuscobrunnea	**14**	–	–	–	–
Lecanora garovaglii	–	–	–	**138**	**76**
Lecidea laboriosa	–	17	7	21	6
Lecidea sp.	–	–	–	**35**	**15**
Lecidea tessellata	–	–	–	**205**	**90**
Lecidella carpathica	–	–	–	**55**	**30**
Lobothallia praeradiosa	109	–	–	88	58
Lobothallia sp.	–	143	185	238	121
Megaspora rimisorediata	518	–	–	89	19
Phaeophyscia ciliata	982	11	5	201	69
Phaeophyscia hirtella	251	279	–	9	1
Phaeophyscia primaria	237	52	2	33	43
Phaeophyscia sp.	666	113	12	65	19
Physcia dubia	**6**	–	–	–	–
Physconia grisea	175	–	95	182	138
Physconia perisidiosa	15	–	6	10	31
Placocarpus sp.	–	–	–	**13**	**7**
Polysporina arenacea	2	–	60	215	73
Polysporina subfuscescens	121	307	723	261	102
Psora decipiens	75	30	158	71	18
Protoparmeliopsis peltata	**75**	–	–	–	–
Pycnora xanthococca	–	63	11	245	147
Rhizoplaca chrysoleuca	–	–	–	**19**	**10**
Rhizoplaca melanophthalma	483	238	–	387	232
Rusavskia elegans	34	790	197	206	89
Sarcogyne arenosa	–	63	–	4	3
Staurothele areolata	57	149	107	486	222
Staurothele frustulenta	17	55	9	71	72
Teloschistes contortuplicatus	98	–	–	45	21
Tuckermannopsis chlorophylla	–	–	–	51	26
Verrucaria devensis	10	–	–	–	–
Verrucaria elaeina	306	257	63	265	104
Verrucaria funckii	–	5	5	1	–
Verrucaria lecideoides	539	971	10	364	186
Verrucaria nigrescens	44	123	12	57	17
Verrucaria sp.	–	–	–	**11**	**3**
Verrucula biatorinaria	–	–	–	**38**	**14**
Verruculopsis poeltiana	2	4	4	–	–
Xanthocarpia marmorata	35	17	23	4	11
Xanthocarpia ochracea	18	56	6	62	20
Xanthomendoza montana	137	129	–	141	34
Xanthomendoza trachyphylla	–	535	245	33	9
Comparison of total # of non-singleton OTUs	49*	46	43	69	63
Comparison of total # of non-singleton OTUs	49*	54 (technicians)		69 (professional)	
Comparison of total # of non-singleton OTUs	49*	72 (all 2016 samples)			

* 43 lichens were listed in the original, morphology-based inventory (see Supplementary Table S1).

substantial overlap in captured species diversity between minimally trained technicians and a professional lichenologist (Fig. 1B). Furthermore, targeted DNA metacommunity barcoding closely reflected the diversity found in vouchered collections (Fig. 1A). Despite the apparent promise of using high-throughput sequencing of lichen-forming fungal communities for biodiversity assessments and its potential application to biomonitoring research, a number of challenges must still be addressed. Below we discuss the relevance of our findings to (i) the objectivity and efficiency of using targeted DNA metabarcoding for assessing diversity in lichen-forming fungi, (ii) limitations related to uncertain taxonomic assignments and the need for a curated, comprehensive DNA reference library, and (iii) several other practical considerations.

4.1. Objectively and efficiently assessing diversity in lichen-forming fungi

Biodiversity inventories are an important component of basic ecological and taxonomic research (Giordani and Brunialti, 2015). However, comprehensive species lists are often difficult to generate for many groups, including lichens (Vondrák et al., 2016). Previous studies have highlighted the trade-off between species capture and quantitative accuracy in ecological inventories of lichens (McCune and Lesica, 1992). Generally, taxonomic surveys are able to provide more robust species lists per site than ecological sampling (Ellis and Coppins, 2017). However, for lichen-forming fungi, patterns of richness and species composition have been shown to be correlated and either can be useful in identifying and managing priority sites (Ellis and Coppins, 2017). Individual sampling efforts and traditional lichen inventories at given sites have been shown to vary, even among professional lichenologists; and none were able to document more than 70% of the total species obtained from their combined sampling efforts (Vondrák et al., 2016).

In general, established field survey methods are demonstrably quite robust (Ellis and Coppins 2017; Giordani and Brunialti, 2015; Giordani et al., 2009; McCune and Lesica, 1992), and the validity and limitations of comparisons have been responsibly assessed. More recent attempts to generate comprehensive site-specific inventories, such as the DNA metabarcoding approach described here, are still in developmental stages. A part from generating a comprehensive, site-specific inventory,

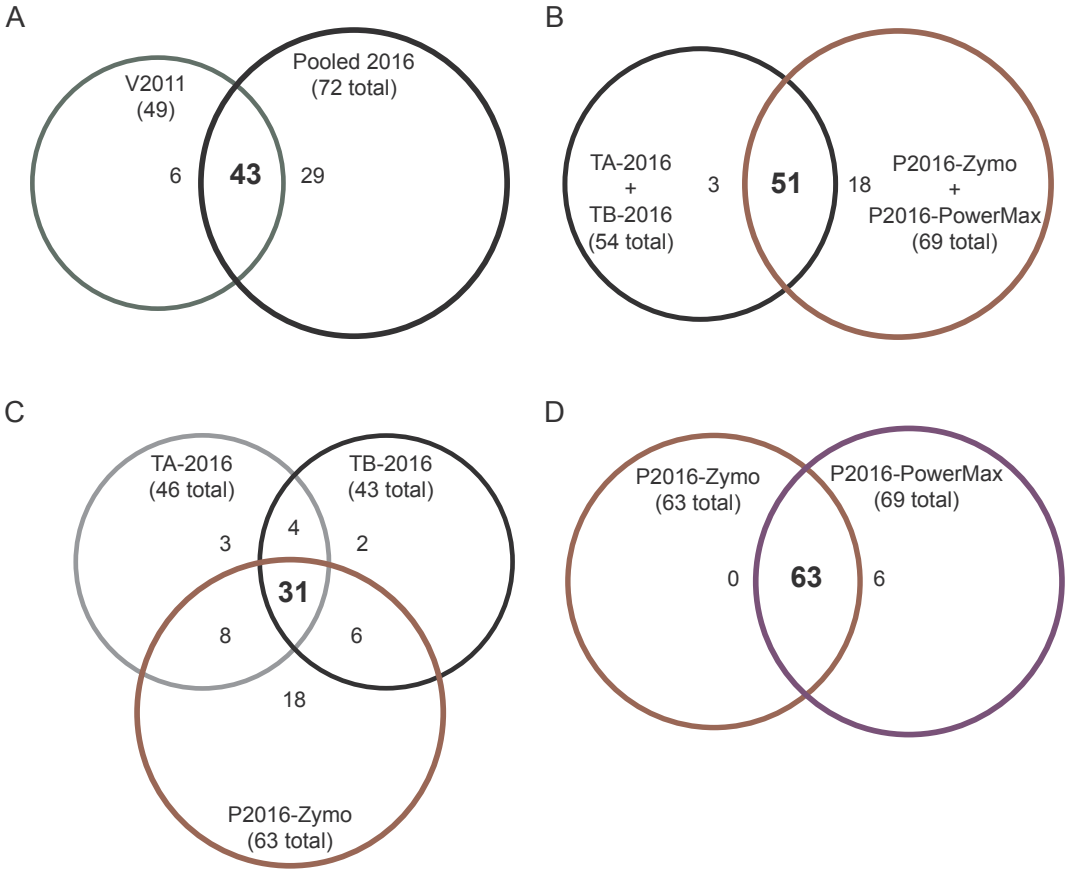

Fig. 1. Venn diagram comparisons of recovered molecular OTUs from different metacommunity sampling efforts. A. Comparison of OTUs from the 2011 vouchered collection (V_{2011}) and the pooled 2016 samples ($T_{A\text{-}2016} + T_{B\text{-}2016} + P_{2016\text{-}Zymo} + P_{2016\text{-}PowerMax}$). B. Comparison of OTUs from the pooled technician samples vs. the pooled professional samples. C. Comparison of OTUs from the individual sampling efforts – all samples were extracted with the Quick-DNA Fungal/Bacterial Kit (Zymo). D. Comparison of OTUs from two different DNA extraction protocols from the professional's metacommunity sample. In each panel, the size of set is proportional to the number of OTUs captured; however, the size of the depicted overlap between or among sets is not proportional.

the detail of the taxonomic survey depends on the research question and scale of interest. If a researcher is concerned with regional trends, capturing every species at every plot may be less important than getting many plots to have better spatial coverage (Root et al., 2015). Similarly, if the aim of the survey is to assess nitrogen pollution, a comprehensive perspective of diversity may be less important than effectively characterizing the range of nitrophiles to score the impact of nitrogen at the site (Jovan and McCune, 2005). As with any biomonitoring research, the aims and objectives of any study must be matched with the appropriate sampling strategy and a one-size-fits-all approach will likely be limited in terms of the inferences that can be made.

While obtaining a comprehensive, site-specific lichen inventory may be challenging on many levels, particularly as the size of the sampling area increases (Vondrák et al., 2016), designing surveys that can effectively and consistently capture a significant portion of the diversity is essential for meaningful ecological and biomonitoring research (Ellis and Coppins, 2017). In order to examine the objectivity and consistency of our targeted community DNA metabarcoding approach (see Materials and Methods above), we compared the number of molecular OTUs present in the technicians' samples with the number present in the professional's sample. Our results indicated an overlap of more than 70% between the technicians' pooled community samples and the professional lichenologist's community sample (Fig. 1B). However, the individual technician's samples represented 60% & 64% of the cumulative OTU diversity, while the professional lichenologist's sample included ca. 89% of the cumulative diversity (Fig. 1C). The overlap between the field technicians with limited experience and the professional lichenologist is impressive, particularly considering the documented variation among inventories generated by professional, experienced lichenologists (Vondrák et al., 2016). A comparison of results using targeted community DNA metabarcoding among professionally trained lichenologists would provide important insights to the overall question. We note that the traditional 2011 lichen inventory (Supplementary Table S1) was based on vouchered collections identified by LLS; and the 2011 inventory had significant overlap with the 2016 community samples (Fig. 1A) which did not include collections made by LLS. The significant biodiversity overlaps between the various approaches documented in this study suggest that targeted community DNA metabarcoding could potentially provide a relatively objective and consistent approach for rapid diversity assessments. In addition, a visual, qualitative assessment and comparison of lichen diversity at Broom Canyon during the 2016 community sampling against the 2011 inventory indicated no significant change in the lichen community at Broom Canyon between 2011 and 2016. This leaves the discrepancy in diversity between the 2011 inventory and the 2016 survey unexplained. This highlights the fact that DNA metabarcoding of lichen-forming fungi may capture additional diversity from (i) morphologically similar species that may not be present in voucher-based collections, (ii) lichen thalli lacking taxonomically diagnostic characters, e.g., immature thalli, (iii) unestablished lichen propagules, (iv) and/or allowing the workers to more effectively sample a wider range of substrates/habitats by removing the constraint of collecting vouchered specimens.

High-throughput sequencing technologies consistently reveal higher levels of diversity than traditional morphology-based taxonomic surveys (Cowart et al., 2015; Zimmermann et al., 2015). Not only did we observe substantial overlap among individual sampling efforts, but with

the targeted metabarcoding approach, we also documented significantly higher levels of species diversity, assuming OTUs were an appropriate proxy for species. In this study, our sampling strategy differed from environmental DNA (eDNA) sampling approaches (Bellemain et al., 2010; Ficetola et al., 2008) by directly targeting lichen thalli and excising small fragments of lichen thalli representing the range of diversity recognized in the field. Our targeted sampling strategy allowed the sampling of a significantly higher number of lichen thalli per hour, making it possible for workers to effectively cover a one-hectare site in less than two hours. Our field sampling protocol also allowed the inclusion of small, immature lichen thalli, thalli lacking diagnostic features, thalli found on difficult-to-collect substrates, etc. that might otherwise not be represented in voucher-based inventories. By removing the time constraint involved with collecting specimens appropriate for traditional vouchered collections, the sampling approach implemented here would permit workers to spend additional time surveying the site and collecting samples. Overall, our targeted community DNA metabarcoding approach demonstrated an approximately 1.6-fold increase in diversity, with 72 OTUs in 37 different genera, as compared with 43 species in 24 genera from the voucher-based inventory (Supplementary Table S1).

The broader impacts of eliminating the constraints of voucher-based collecting to aid in capturing additional diversity using targeted community DNA metabarcoding needs to be studied more thoroughly. However, we do not advocate the elimination of voucher-based collecting. Physical, traditionally curated specimens and museum collections play fundamentally important roles across a range of scientific and societal subdisciplines (Suarez and Tsutsui, 2004), including lichenology (Lendemer and Harris, 2016). Developing effective strategies for integrating collection-based sampling with targeted metabarcoding will be essential to effectively advance biodiversity assessments and biomonitoring programs.

To better evaluate the differences in diversity estimates between DNA metabarcoding approaches and vouchered-based inventories, creating mock communities of common lichen-forming fungi to assess the impact of DNA extraction methods, PCR cycling parameters and primer selection, and sequencing platforms will be essential. Mock communities can be extended by varying the amount of template DNA to explore the ability of molecular-based approaches to detect all members of the community and their relative abundances, similar to the expected proportions of lichen propagules and established lichens.

4.2. Dispersal versus establishment limitations

Our results reflect the presence of additional species of lichen-forming fungi that were not observed in the field. Whether or not these species are actually present as established lichen thalli or simply represent the presence of propagules is unknown. For example, if air pollution has removed or limited the establishment of a species, there may be abundant propagules, potentially detected in the barcoding sample, but no established lichen thalli, thus missing from the vouchered inventory. Lichen propagules have been successfully captured and characterized using high-throughput sequencing approaches, and propagules of asexually reproducing lichens appear to have larger geographical ranges than propagules of sexual species (Tripp et al., 2016). The detection of lichen-forming fungal DNA is not enough to establish the “real presence” of lichens in a community (Comtet et al., 2015).

The detection of spores, or otherwise unestablished lichens (Banchi et al., 2018), may artificially inflate diversity estimates. The utility of metabarcoding approaches may extend beyond floristic inventories, potential aiding in the detection of “seed rain” or potential species pools at sites. Previous studies have found a positive relationship between species abundance and sequencing read abundance (Evans et al., 2016). A broad range of sequence read abundance/OTU was observed in this study, ranging from 1 to 2522 reads/OTU (Table 2). Whether the specimens consistently represented by low read abundance across samples, e.g., *Catapyrenium lachneum* and *Verrucaria funckii* (Table 2), provide evidence of effective dispersal followed by establishment-limitations will require additional study. However, the opportunistic bulk sampling strategy implemented here could potentially bias inferences of species abundance/establishment made based on sequencing read abundance. Future studies coupling community DNA barcoding with voucher-based inventories will likely provide important insights into dispersal capacity and establishment limitations in lichen-forming fungi. Furthermore, studies designed to explicitly test for the presence of spores, e.g., spore traps, sampling substrates not colonized by lichens, etc., will be essential to elucidate dispersal vs. establishment limitations of lichens (Tripp et al., 2016).

4.3. Uncertain taxonomic assignments highlights need for reference library

In this study, we used OTUs as a proxy for species diversity; however, it is well known that a universal inter- intraspecific DNA sequence similarity threshold does not exist (Schmidt et al., 2015; Will et al., 2005). We attempted to link the OTUs inferred in this study with formal taxonomic labels (Table 2). However, given the lack of a comprehensive DNA reference database representing all the potentially occurring species at the site, taxonomic assignments in this study are, at best, approximations and should not be interpreted as a credible lichen checklist for this site (Baird and Hajibabaei, 2012; TABERLET et al., 2012). In fact, it is likely that the majority of species sampled in this study are not currently represented in existing DNA sequence databases, making accurate taxonomic assignments presently impossible in even the best-case scenario, e.g., OTU clustering equates to species-level lineages and a barcode gap exists among many closely related species.

Our study highlights the ongoing need for development of professionally curated DNA reference libraries for lichen-forming fungal species in order to improve the utility of targeted community DNA metabarcoding. A number of valuable resources are presently available, including the Barcode of Life Data System (Ratnasingham and Hebert, 2007) and the UNITE database (Abarenkov et al., 2010; Kõljalg et al., 2005), with the latter being particularly useful with fungi. Molecular-based specimen identification and species delimitation studies have been shown to provide an efficient start for taxonomic workflows (Collins and Cruickshank, 2012; Kekkonen and Hebert, 2014). In spite of the utility of these DNA reference databases, a significant proportion of the sequences in public databases are compromised in terms of taxonomic annotation, technical quality, or insufficient annotations (Collins and Cruickshank, 2012; Nilsson et al., 2016; Nilsson et al., 2006). Furthermore, the conflation of species discovery and specimen identification within a barcoding context has led to the frequently inappropriate use of commonly used analytical methods and misinterpretation of results (Collins and Cruickshank, 2012).

Ultimately, the development of comprehensive, voucher-based, professionally curated DNA reference libraries will help alleviate some of the more substantial issues with targeted community DNA metabarcoding for specimen identification of lichen-forming fungi. While discrimination between the most closely related species remains challenging, a number of studies have highlighted the efficacy of DNA barcoding for specimen identification in clades of lichen-forming fungi where morphology-based specimen identification is notoriously challenging (Divakar et al., 2016; Leavitt et al., 2014; Leavitt et al., 2013). In a floristic inventory context, Kelly et al. (2011) demonstrated the potential for identifying a high percentage of lichen-forming fungi to the correct species and genus using DNA barcoding, particularly in those cases where the lichen flora is well known. However, in regions or with taxonomic groups where lichen diversity is poorly characterized and reference DNA barcode libraries are lacking, DNA barcoding has been shown to be ineffective in accurately characterizing taxonomic diversity (Orock et al., 2012). The ongoing development of reliable DNA reference databases, improved protocols for the consistent

generation of DNA barcodes, and standardized pipelines for consistent OTU delimitation and taxonomic assignment will be essential to improving the prospects for the effective use of targeted DNA metabarcoding in diversity assessments (Pilgrim et al., 2011; Will et al., 2005).

In this study, we found that 9–21% of all reads were classified as 'no hit', meaning that no taxonomic assignment could be made at any level. While this could be a reflection of an incomplete DNA reference library, these reads may also reflect PCR artifacts (Acinas et al., 2005) or sequencing errors (Schirmer et al., 2015). Under current metagenomic approaches, the artifacts are, to a large degree, unavoidable; and care must be taken to not artificially inflate diversity estimates as a result of methodological errors (Wooley and Ye, 2010).

4.4. Practical considerations

Targeted community DNA metabarcoding for assessing lichen biodiversity offers a complementary, rather than a substitutive sampling strategy. Vouchered specimens should remain an integral part of biodiversity monitoring research. Professional lichenologists' surveys at biomonitoring reference sites continue to be relevant to (i) collect vouchered specimens for morphological characterization of lichen diversity, including potential new species, (ii) assess relative abundance of lichens, (iii) evaluate if species recovered as molecular OTUs are, in fact, established at the site, and (iv) provide a qualitative overview of the site, including features that may not be recovered in DNA metacommunity sampling approaches.

In this study, technicians' metacommunity samples (fragments of lichen thalli) were much smaller than the sample collected by the professional (Table 1), suggesting that sampling more thallus fragments likely allows for detection of higher levels of diversity. It may be useful to establish guidelines for collecting a specified amount (mass) of material in a metacommunity sample to increase the probability of capturing higher levels of species diversity. Some DNA extraction protocols, like PowerMax Soil DNA Isolation Kit (Qiagen), can be used to extract DNA from up to 10 g of sample material. When compared with protocols designed for smaller sample sizes, extractions taken from larger samples recovered more diversity (Fig. 1D). However, recommending metacommunity sample sizes is likely to be dependent on the range of diversity and distribution of lichen growth forms. The fieldwork carried out in connection with this study represented the first experience in the field for the technicians involved in the study. The overlap observed among technician and professional samples was present despite the technicians' lack of experience and minimal training. With more experience and training, we may expect field technicians to collect samples with higher levels of overlap, and to produce more comprehensive species inventories.

Short barcodes (> 500 bp), such as the ITS2 region used in this study, only provide limited information for species discrimination, taxonomic assignment and phylogenetic inferences. Recent developments with high-throughput sequencing platforms, such as Pacific Biosciences (PacBio) instruments, can effectively sequences full-length ITS barcodes and longer rDNA amplicons for DNA metabarcoding in fungal communities (Kennedy et al., 2018; Tedersoo et al., 2018). We anticipate that these new technologies will play major roles in advancing effective targeted DNA community barcoding studies in biomonitoring and ecological research. Incorporating this information into community phylogenetic and conservation studies should take high priority in biodiversity monitoring research programs (Miller et al., 2017; Tucker et al., 2017).

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Appendix A. Supplementary data

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References

Abarenkov, K., Henrik Nilsson, R., Larsson, K.-H., Alexander, I.J., Eberhardt, U., Erland, S., Høiland, K., Kjøller, R., Larsson, E., Pennanen, T., Sen, R., Taylor, A.F.S., Tedersoo, L., Ursing, B.M., Vrålstad, T., Liimatainen, K., Peintner, U., Kõljalg, U., 2010. The UNITE database for molecular identification of fungi – recent updates and future perspectives. New Phytol. 186, 281–285.

Abarenkov, K., Somervuo, P., Nilsson, R.H., Kirk, P.M., Huotari, T., Abrego, N., Ovaskainen, O., 2018. Protax-fungi: a web-based tool for probabilistic taxonomic placement of fungal internal transcribed spacer sequences. New Phytol. 220, 517–525.

Acinas, S.G., Sarma-Rupavtarm, R., Klepac-Ceraj, V., Polz, M.F., 2005. PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. Appl. Environ. Microbiol. 71, 8966.

Andruszkiewicz, E.A., Starks, H.A., Chavez, F.P., Sassoubre, L.M., Block, B.A., Boehm, A.B., 2017. Biomonitoring of marine vertebrates in Monterey Bay using eDNA metabarcoding. PLoS One 12, e0176343.

Baird, D.J., Hajibabaei, M., 2012. Biomonitoring 2.0: a new paradigm in ecosystem assessment made possible by next-generation DNA sequencing. Mol. Ecol. 21, 2039–2044.

Bálint, M., Schmidt, P.-A., Sharma, R., Thines, M., Schmitt, I., 2014. An Illumina metabarcoding pipeline for fungi. Ecol. Evol. 4, 2642–2653.

Balmford, A., Gaston, K.J., 1999. Why biodiversity surveys are good value. Nature 398, 204.

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

Baselga, A., Fujisawa, T., Crampton-Platt, A., Bergsten, J., Foster, P.G., Monaghan, M.T., Vogler, A.P., 2013. Whole-community DNA barcoding reveals a spatio-temporal continuum of biodiversity at species and genetic levels. Nat. Commun. 4, 1892.

Bazzicalupo, A.L., Bálint, M., Schmitt, I., 2013. Comparison of ITS1 and ITS2 rDNA in 454 sequencing of hyperdiverse fungal communities. Fungal Ecol. 6, 102–109.

Bellemain, E., Carlsen, T., Brochmann, C., Coissac, E., Taberlet, P., Kauserud, H., 2010. ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. BMC Microbiol. 10, 189.

Belnap, J., Eldridge, D., 2001. Disturbance and Recovery of Biological Soil Crusts. Springer Berlin Heidelberg, pp. 363–383.

Bongers, T., Ferris, H., 1999. Nematode community structure as a bioindicator in environmental monitoring. Trends Ecol. Evol. 14, 224–228.

Chariton, A.A., Stephenson, S., Morgan, M.J., Steven, A.D.L., Colloff, M.J., Court, L.N., Hardy, C.M., 2015. Metabarcoding of benthic eukaryote communities predicts the ecological condition of estuaries. Environ. Pollut. 203, 165–174.

Collins, R.A., Cruickshank, R.H., 2012. The seven deadly sins of DNA barcoding. Mol. Ecol. Resour. 13, 969–975.

Comtet, T., Sandionigi, A., Viard, F., Casiraghi, M., 2015. DNA (meta) barcoding of biological invasions: a powerful tool to elucidate invasion processes and help managing aliens. Biol. Invasions 17, 905–922.

Conti, M.E., Cecchetti, G., 2001. Biological monitoring: lichens as bioindicators of air pollution assessment – a review. Environ. Pollut. 114, 471–492.

Coulston, J.W., Smith, G.C., Smith, W.D., 2003. Regional assessment of ozone sensitive tree species using bioindicator plants. Environ. Monitering Assess. 83, 113–127.

Cowart, D.A., Pinheiro, M., Mouchel, O., Maguer, M., Grall, J., Miné, J., Arnaud-Haond, S., 2015. Metabarcoding is powerful yet still blind: a comparative analysis of morphological and molecular surveys of seagrass communities. PLoS One 10, e0117562.

Crespo, A., Lumbsch, H.T., 2010. Cryptic species in lichen-forming fungi. IMA Fungus 1, 167–170.

Dincă, V., Zakharov, E.V., Hebert, P.D.N., Vila, R., 2011. Complete DNA barcode reference library for a country's butterfly fauna reveals high performance for temperate Europe. Proc. R. Soc. B: Biol. Sci. 278, 347.

Divakar, P.K., Leavitt, S.D., Molina, M.C., Del-Prado, R., Lumbsch, H.T., Crespo, A., 2016. A DNA barcoding approach for identification of hidden diversity in Parmeliaceae (Ascomycota): *Parmelia sensu* stricto as a case study. Bot. J. Linn. Soc. 180, 21–29.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461.

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10, 996.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200.

Ellis, C.J., Coppins, B.J., 2017. Taxonomic survey compared to ecological sampling: are the results consistent for woodland epiphytes? The Lichenologist 49, 141–155.

Emilson, C.E., Thompson, D.G., Venier, L.A., Porter, T.M., Swystun, T., Chartrand, D., Capell, S., Hajibabaei, M., 2017. DNA metabarcoding and morphological macroinvertebrate metrics reveal the same changes in boreal watersheds across an

environmental gradient. Sci. Rep. 7, 12777.
Evans, N.T., Olds, B.P., Renshaw, M.A., Turner, C.R., Li, Y., Jerde, C.L., Mahon, A.R., Pfrender, M.E., Lamberti, G.A., Lodge, D.M., 2016. Quantification of mesocosm fish and amphibian species diversity via environmental DNA metabarcoding. Mol. Ecol. Resour. 16, 29–41.
Ficetola, G.F., Miaud, C., Pompanon, F., Taberlet, P., 2008. Species detection using environmental DNA from water samples. Biol. Lett. 4, 423.
Frego, K.A., 2007. Bryophytes as potential indicators of forest integrity. For. Ecol. Manage. 242, 65–75.
Giordani, P., Brunialti, G., 2015. Sampling and Interpreting Lichen Diversity Data for Biomonitoring Purposes. In: In: Upreti, D.K., Divakar, P.K., Shukla, V., Bajpai, R. (Eds.), Recent Advances in Lichenology: Modern Methods and Approaches in Biomonitoring and Bioprospection Volume 1. Springer India, New Delhi, pp. 19–46.
Giordani, P., Brunialti, G., Benesperi, R., Rizzi, G., Frati, L., Modenesi, P., 2009. Rapid biodiversity assessment in lichen diversity surveys: implications forquality assurance. J. Environ. Monit. 11, 730–735.
Hawksworth, D.L., 1971. Lichens as a litmus for air pollution: a historical review. Int. J. Environ. Stud. 1, 281–296.
Hodkinson, I.D., Jackson, J.K., 2005. Terrestrial and aquatic invertebrates as bioindicators for environmental monitoring, with particular reference to mountain ecosystems. Environ. Manage. 35, 649–666.
Hunter Jr., M.L., Webb, S.L., 2002. Enlisting Taxonomists to Survey Poorly Known Taxa for Biodiversity Conservation: a Lichen Case Study. Conserv. Biol. 16, 660–665.
Jovan, S., McCune, B., 2005. Air-quality bioindication in the Greater Central Valley of California, with epiphytic macrolichen communities. Ecol. Appl. 15, 1712–1726.
Keck, F., Vasselon, V., Rimet, F., Bouchez, A., Kahlert, M., 2018. Boosting DNA metabarcoding for biomonitoring with phylogenetic estimation of operational taxonomic units' ecological profiles. Mol. Ecol. Resour. 18, 1299–1309.
Kekkonen, M., Hebert, P.D.N., 2014. DNA barcode-based delineation of putative species: efficient start for taxonomic workflows. Mol. Ecol. Resour. 14, 706–715.
Kelly, L.J., Hollingsworth, P.M., Coppins, B.J., Ellis, C.J., Harrold, P., Tosh, J., Yahr, R., 2011. DNA barcoding of lichenized fungi demonstrates high identification success in a floristic context. New Phytol. 191, 288–300.
Kennedy, P.G., Cline, L.C., Song, Z., 2018. Probing promise versus performance in longer read fungal metabarcoding. New Phytol. 217, 973–976.
Knebelsberger, T., Landi, M., Neumann, H., Kloppmann, M., Sell, A.F., Campbell, P.D., Laakmann, S., Raupach, M.J., Carvalho, G.R., Costa, F.O., 2014. A reliable DNA barcode reference library for the identification of the North European shelf fish fauna. Mol. Ecol. Resour. 14, 1060–1071.
Kõljalg, U., Larsson, K.-H., Abarenkov, K., Nilsson, R.H., Alexander, I.J., Eberhardt, U., Erland, S., Høiland, K., Kjøller, R., Larsson, E., Pennanen, T., Sen, R., Taylor, A.F.S., Tedersoo, L., Vrålstad, T., 2005. UNITE: a database providing web-based methods for the molecular identification of ectomycorrhizal fungi. New Phytol. 166, 1063–1068.
Leavitt, S.D., Divakar, P.K., Crespo, A., Lumbsch, H.T., 2016. A Matter of Time — Understanding the Limits of the Power of Molecular Data for Delimiting Species Boundaries. Herzogia 29, 479–492.
Leavitt, S.D., Esslinger, T.L., Hansen, E.S., Divakar, P.K., Crespo, A., Loomis, B.F., Lumbsch, H.T., 2014. DNA barcoding of brown Parmeliae (Parmeliaceae) species: a molecular approach for accurate specimen identification, emphasizing species in Greenland. Org. Divers. Evol. 14, 11–20.
Leavitt, S.D., Fernández-Mendoza, F., Pérez-Ortega, S., Sohrabi, M., Divakar, P.K., Lumbsch, H.T., St. Clair, L.L., 2013. DNA barcode identification of lichen-forming fungal species in the Rhizoplaca melanophthalma species-complex (Lecanorales, Lecanoraceae), including five new species. MycoKeys 7, 1–22.
Leavitt, S.D., St. Clair, L.L., 2015. Bio-monitoring in Western North America: What Can Lichens Tell Us About Ecological Disturbances? In: Upreti, D.K., Divakar, P.K., Shukla, V., Bajpai, R. (Eds.), Recent Advances in Lichenology. Springer India, pp. 119–138.
Lendemer, J.C., Harris, R.C., 2016. The New York Botanical Garden Lichen Herbarium: a unique resource for fungal biodiversity research and education. Brittonia 68, 334–340.
Loppi, S., Pirintsos, S.A., 2003. Epiphytic lichens as sentinels for heavy metal pollution at forest ecosystems (central Italy). Environ. Pollut. 121, 327–332.
McCune, B., 2000. Lichen communities as indicators of forest health. The Bryologist 103, 353–356.
McCune, B., Lesica, P., 1992. The trade-off between species capture and quantitative accuaracy in ecological inventory of lichens and bryophytes in forest in Montana. The Bryologist 95, 296–304.
McMullin, R.T., Duinker, P.N., Richardson, D.H.S., Cameron, R.P., Hamilton, D.C., Newmaster, S.G., 2010. Relationships between the structural complexity and lichen community in coniferous forests of southwestern Nova Scotia. For. Ecol. Manage. 260, 744–749.
Miller, E.T., Farine, D.R., Trisos, C.H., 2017. Phylogenetic community structure metrics and null models: a review with new methods and software. Ecography 40, 461–477.
Newmaster, S.G., Belland, R.J., Arsenault, A., Vitt, D.H., Stephens, T.R., 2005. The ones we left behind: comparing plot sampling and floristic habitat sampling for estimating bryophyte diversity. Divers. Distrib. 11, 57–72.
Nilsson, R.H., Abarenkov, K., Kõljalg, U., 2016. Molecular Techniques in Mycological Studies and Sequence Data Curating: Quality Control and Challenges. In: Li, D.-.W. (Ed.), Biology of Microfungi. Springer International Publishing, Cham, pp. 47–64.
Nilsson, R.H., Ryberg, M., Kristiansson, E., Abarenkov, K., Larsson, K.H., Koljalg, U., 2006. Taxonomic reliability of DNA sequences in public sequence databases: A fungal perspective. PLoS ONE 1, e59.
Nimis, P.L., Scheidegger, C., Wolseley, P., 2002. Monitoring with lichens — monitoring lichens. Kluwer Academics, Dordrecht.
Orock, E.A., Leavitt, S.D., Fonge, B.A., St. Clair, L.L., Lumbsch, H.T., 2012. DNA-based identification of lichen-forming fungi: Can publicly available Sequence databases aid in lichen diversity inventories of Mount Cameroon (West Africa)? The Lichenologist 44, 833–839.
Pesch, R., Schroeder, W., 2006. Mosses as bioindicators for metal accumulation: Statistical aggregation of measurement data to exposure indices. Ecol. Ind. 6, 137–152.
Pilgrim, E.M., Jackson, S.A., Swenson, S., Turcsanyi, I., Friedman, E., Weigt, L., Bagley, M.J., 2011. Incorporation of DNA barcoding into a large-scale biomonitoring program: opportunities and pitfalls. J. North Am. Benthological Soc. 30, 217–231.
Ratnasingham, S., Hebert, P.D., 2007. BOLD: The Barcode of Life Data System (http://www. barcodinglife. org). Mol. Ecol. Notes 7, 355-364.
Root, H.T., Geiser, L.H., Jovan, S., Neitlich, P., 2015. Epiphytic macrolichen indication of air quality and climate in interior forested mountains of the Pacific Northwest, USA. Ecol. Ind. 53, 95–105.
Schirmer, M., Ijaz, U.Z., D'Amore, R., Hall, N., Sloan, W.T., Quince, C., 2015. Insight into biases and sequencing errors for amplicon sequencing with the Illumina MiSeq platform. Nucleic Acids Res. 43, e37.
Schmidt, P.-A., Bálint, M., Greshake, B., Bandow, C., Römbke, J., Schmitt, I., 2013. Illumina metabarcoding of a soil fungal community. Soil Biol. Biochem. 65, 128–132.
Schmidt, T.S.B., Matias Rodrigues, J.F., von Mering, C., 2015. Limits to robustness and reproducibility in the demarcation of operational taxonomic units. Environ. Microbiol. 17, 1689–1706.
Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., Consortium, F.B., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc. Natl. Acad. Sci. 6241–6246, 6241–6246.
St. Clair, S.B., St. Clair, L.L., Weber, D.J., Mangelson, N.F., Eggett, D.L., 2002. Element Accumulation Patterns in Foliose and Fruticose Lichens from Rock and Bark Substrates in Arizona. The Bryologist 105, 415–421.
Suarez, A.V., Tsutsui, N.D., 2004. The value of museum collections for research and society. Bioscience 54, 66–74.
Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., Willerslev, E., 2012. Towards next-generation biodiversity assessment using DNA metabarcoding. Mol. Ecol. 21, 2045–2050.
Tedersoo, L., Tooming-Klunderud, A., Anslan, S., 2018. PacBio metabarcoding of Fungi and other eukaryotes: errors, biases and perspectives. New Phytol. 217, 1370–1385.
Tripp, E.A., Lendemer, J.C., Barberán, A., Dunn, R.R., Fierer, N., 2016. Biodiversity gradients in obligate symbiotic organisms: exploring the diversity and traits of lichen propagules across the United States. J. Biogeogr. 43, 1667–1678.
Tucker, C.M., Cadotte, M.W., Carvalho, S.B., Davies, T.J., Ferrier, S., Fritz, S.A., Grenyer, R., Helmus, M.R., Jin, L.S., Mooers, A.O., Pavoine, S., Purschke, O., Redding, D.W., Rosauer, D.F., Winter, M., Mazel, F., 2017. A guide to phylogenetic metrics for conservation, community ecology and macroecology. Biol. Rev. 92, 698–715.
Vondrák, J., Malíček, J., Palice, Z., Coppins, B., Kukwa, M., Czarnota, P., Sanderson, N., Acton, A., 2016. Methods for obtaining more complete species lists in surveys of lichen biodiversity. Nordic J. Bot. 34, 619–626.
White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, N., Gelfand, D., Sninsky, J., White, T.J. (Eds.), PCR protocols. Academic Press, San Diego, pp. 315–322.
Will, K.W., Mishler, B.D., Wheeler, Q.D., 2005. The Perils of DNA Barcoding and the Need for Integrative Taxonomy. Syst. Biol. 54, 844–851.
Will-Wolf, S., 2010. In: Analyzing lichen indicator data in the Forest Inventory and Analysis Program U.S. Department of Agriculture, Forest Service. Pacific Northwest Research Station, Portland, OR, pp. 61.
Wooley, J.C., Ye, Y., 2010. Metagenomics: facts and artifacts, and computational challenges. J. Comput. Sci. Technol. 25, 71–81.
Yu, D.W., Ji, Y., Emerson, B.C., Wang, X., Ye, C., Yang, C., Ding, Z., 2012. Biodiversity soup: metabarcoding of arthropods for rapid biodiversity assessment and biomonitoring. Methods Ecol. Evol. 3, 613–623.
Zhang, J., Kobert, K., Flouri, T., Stamatakis, A., 2014. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30, 614–620.
Zimmermann, J., Glöckner, G., Jahn, R., Enke, N., Gemeinholzer, B., 2015. Metabarcoding vs. morphological identification to assess diatom diversity in environmental studies. Mol. Ecol. Resour. 15, 526–542.