Lichens are a remarkable, evolutionarily heterogeneous assemblage of fungi that have converged on similar symbiotic strategies involving partnerships of fungi with photosynthetic algae or cyanobacteria (Lutzoni et al., 2001; Gueidan et al., 2008; Schoch et al., 2009). Taxonomic diversity of these fungi is estimated to be high, with more than 50,000 species described to date (Hawksworth, 1991; Hawksworth and Lücking, 2017). This diversity is distributed across multiple lineages of both ascomycetes and basidiomycetes (Lücking et al., 2016) and across terrestrial biomes worldwide (Brodo et al., 2001; Arcadia, 2013), wherein lichens serve numerous important ecological roles (Slack, 1988; Seaward, 1996; Belnap and Lange, 2005; Cornelissen et al., 2007; Asplund and Wardle, 2017).

For more than a century, lichen symbioses have been thought to comprise individual fungal species that associate with either a single green alga or cyanobacterium (Ahmadjian, 1967; Lorch, 1988; Honegger, 1991, 1992; Mitchell, 2007). In a limited number of lineages (3–4%; Yoshino et al., 2019), individual fungal species associate with both types of photosynthesizing organisms, either as separate individuals or together in the same individual (James and Henssen, 1976; Jahns, 1988; Goffinet and Bayer, 1997; Magain and...
Sérasiaux, 2014). Such lichens, however, were classically considered exceptions to the general pattern of lichen symbiotic associations (James and Henssen, 1976; Jahns, 1988). This traditional view of lichens, as symbioses formed by one fungus and one photosynthesizing partner, has evolved substantially during the last decade. Numerous studies have yielded a growing body of evidence that lichens and the communities they form are far more complex and dynamic than previously thought (U’Ren et al., 2010, 2012; Chagnon et al., 2015; Spribille et al., 2016; Tuovinen et al., 2019). Indeed, these studies have demonstrated that lichens have diverse and spatially partitioned microbiomes (Grube and Berg, 2009; Hodkinson and Lutzoni, 2009; Hodkinson et al., 2012; Aschenbrenner et al., 2016, 2017), that individual lichens host multiple genotypes of a given photosynthetic partner (Piercey–Normore, 2006; Muggia et al., 2013), and that there is extensive sharing of symbionts and microbial communities (Läcking et al., 2009; Belinchón et al., 2015; Cornejo et al., 2016; Onuț–Brännström et al., 2018). The conceptual transformation of lichens from binary symbiont pairs to intricate metacommunities was catalyzed by the recent discovery of specialist basidiomycete yeasts in the cortices (upper or outer layers) of certain ascomycete lichens (Spribille et al., 2016). This transformation was further advanced by the demonstration that at least some of those basidiomycetes, long thought to be parasites of ascomycete lichen fungi, instead form lichen-like associations with the algal partners (Tuovinen et al., 2019).

To date, published studies that screened lichens for basidiomycete yeasts have emphasized sampling in the family Parmeliaceae (Spribille et al., 2016; Tuovinen et al., 2019). Basidiomycete yeasts have also been detected in several other families of Lecanoromycetes (Spribille et al., 2016), the most species-rich lineage of lichen-forming fungi. Nonetheless, their ubiquity across a much wider diversity of lichen lineages remains to be tested. Here, we present the results of a broad survey for basidiomycete yeasts through analysis of a new metagenomic data set spanning 339 lichen species representing 57 families and 25 orders, generated as part of a large-scale study of lichen biodiversity in the Southern Appalachian Biodiversity Hotspot of temperate eastern North America (Lendemer et al., 2013, 2017; Allen and Lendemer, 2016; Tripp and Lendemer, in press).

MATERIALS AND METHODS

Tissue collection for metagenomics data set

Fresh material of 413 lichen thalli spanning 339 species, 57 fungal families, and 25 fungal orders of ascomycetes (Appendix S1) was collected from sites throughout the southern Appalachian Mountains as part of a system-wide investigation into drivers of lichen biodiversity and distributions in this region (Tripp et al., 2019). Samples were collected and identified by J. C. Lendemer and E. A. Tripp between December 2016 and January 2018, with the goal of obtaining a taxonomically comprehensive set of taxa representing every lichen species encountered at the project sites and representing diverse lichen growth forms and ecologies. Vouchers were deposited in the herbaria of the New York Botanical Garden (NY) and University of Colorado, Boulder (COLO) (Appendix S2). Vouchers were collected following standard protocols in the field (Brodo et al., 2001), and subsamples for molecular study were removed within 24 h of collection. For macrolichens, ca. 1 x 1 cm of tissue was removed from the thallus margins and lobes; for microlichens, tissue was scraped from substrates using sterile razor blades. Tissue samples were air dried in a laminar flow hood for 24 h then frozen at −20°C until transport to University of Colorado for DNA extraction and subsequent sequencing.

DNA extraction and whole-genome shotgun sequencing

Dried tissue samples were pulverized using tungsten carbide bearings in a 96-well plate shaker (Qiagen, Germantown, MD, USA). Genomic DNA was then extracted with a Qiagen DNeasy 96 plant kit. Individual samples were transferred from 1.5 mL microcentrifuge tubes to the provided 96-well plates. Following Pogoda et al. (2018), the Qiagen protocol was modified to include a 10 min 65°C incubation step for the disrupted tissue in lysis buffer and a 100% ethanol wash before final drying of the membrane before elution. Extracted samples were then stored at −20°C before library preparation.

Samples were prepared for sequencing using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA), optimized for 1 ng of input DNA. Each sample was uniquely identified by the dual index adapters Nextera i5 and i7. Libraries that passed quality control were sequenced for paired-end 151 base pair reads on an Illumina NextSeq and utilized PhiX v.3 as a control.

Metagenomic data set and BLAST queries

Libraries were filtered with Trimmomatic-0.36 to trim adapters from reads, implementing the parameters “LEADING:3 TRAILING:3 MINLEN:100” (Bolger et al., 2014). Filtered reads were then assembled using SPAdes 3.9.0 with parameters “--careful -k 21,33,65,81” (Bankevich et al., 2012). These parameters were selected based on our prior studies that have found them highly effective in assembling high-copy loci within the lichen metagenome, including the mitochondrial genomes of both the mycobiont and photobiont (Brigham et al., 2018; Funk et al., 2018; Pogoda et al., 2018, 2019), the nuclear ribosomal DNA of the mycobiont and photobiont and cyanobiont (K. G. Keepers et al., unpublished manuscript), as well as the photobiont chloroplast genome (K. G. Keepers, unpublished data).

We queried the assembled metagenomes using default command-line BLASTn search parameters and seven rDNA sequences of cystobasidiomycete yeasts that have been shown to associate with ascomycete lichens, as follows. Two BLAST queries were performed with members of Cyphobasidium Millanes, Diederich & Wedin s.s. Millanes et al. (2016). One of these (KU587705) was a partial 18S reference sequence for C. hypogymnicola (Diederich & Ahti) Millanes, Diederich & Wedin, the type species of Cyphobasidium, generated by Millanes et al. (2016). The other (KU948882) was a partial 26S sequence of an unidentified cystobasidiomycete yeast from Hypogymnia physodes (L.) Nyl. recovered as sister to C. hypogymnicola by Spribille et al. (2016: S13, isolate T114). The remaining BLAST queries were performed with cystobasidiomycete yeast sequence published by Spribille et al. (2016) and that were recovered in other cystobasidiomycete clades outside of the Cyphobasidium. These additional sequences were KU948747 [ITS1, 5.8S, ITS2, and partial 26S from Thamnolia vermicularis (Sw.) Schaer. recovered as sister to Microsporomyces Q.M.Wang, F.Y.Bai, M.Groenew. & Boekhout (Spribille et al., 2016: S12, isolate T1402); KU948755 [ITS1, 5.8S, ITS2, and partial 26S from Parmotrema crinitum (Ach.) M.Choisy recovered as sister to Symmetrospora Q.M.Wang, F.Y.Bai,
M. Groenew. & Boekhout (Spribille et al., 2016: S12, isolate T1509); KU948887 [partial 26S from Parmeliopsis ambigua (Wulff) Nyl. recovered in a clade sister to all sequences of Cyphobasidium (Spribille et al., 2016: S12, isolate 1579)]; KU948863 [18S from Nephromopsis endocoea Asahina recovered within Buckelyzyma Q.M.Wang, F.Y.Bai, M.Groenew. & Boekhout (Spribille et al., 2016: S14, isolate T1656)]; KU948865 [partial 18S from Brodoa ooroarctica (Krog) Goward recovered in a clade with other lichen-derived samples that was recovered as sister to Microsporomycetes (Spribille et al., 2016: S14, isolate T1667)].

BLAST tables were first filtered to include only BLAST hits of 100 bp or longer, and then all hits were subsequently sorted by percentage identity. The top 100 BLAST hits constituting contigs with ≥95% percent identity were identified using a web-blast search against the NCBI non-redundant database (Appendices S3 and S4). The 100 sequences returned from the above two queries are available at Zenodo (https://doi.org/10.5281/zenodo.3240665).

We implemented multiple BLAST queries with different reference sequences for two reasons. First, if the community of yeasts in or on a lichen thallus was sufficiently diverse, community-level variation within the rapidly evolving ITS1 and ITS2 regions may have prevented those loci from assembling into contigs large enough to satisfy our detection heuristics. Consequently, we selected reference sequences of Cyphobasidium from more slowly evolving coding regions of the rDNA complex (18S and 26S; Schoch et al., 2012; Yahr et al., 2016). Second, given that Spribille et al. (2016) recovered lichen-associated yeasts in multiple, highly divergent fungal lineages within the cystobasidiomycetes, we queried the data set using additional sequences outside of Cyphobasidium in an effort to detect any cystobasidiomycete taxa with sequences too divergent from Cyphobasidium to be detected and identified using our BLAST workflow.

The single-copy coverage of the mycobiont nuclear genome was then estimated in the assemblies containing BLAST hits to cystobasidiomycetes. The SPAdes v3.9.0 assembler includes the coverage estimate for each contig in the contig’s header, which allowed for rapid identification of the coverage of the mycobiont single-copy genome. Histograms of the depths of the one thousand largest contigs per assembly were constructed to visualize the distribution of nuclear genome contigs. Contigs that fell within the normal distribution of depths were identified as fungal using a web-blast search against the NCBI non-redundant database. The coverage values for the individual contigs identified as belonging to cystobasidiomycetes were derived directly from their respective headers.

RESULTS

In total, we detected cystobasidiomycete yeasts in 2.7% of the taxa and 2.2% of the samples in our data set (nine species, nine samples). Command-line BLAST searches of the metagenomic data set using an 18S reference sequence for Cyphobasidium hypogymniocola, the type species of Cyphobasidium, returned only 13 contigs with a match of 100 bp or greater, and these were derived from seven different samples (Appendix S3). Recognizing the divergence between basidiomycete yeast sequences found by Spribille et al. (2016; supplemental figs. 12–14), we also carried out command-line BLAST searches of the metagenomic data set using six additional sequences from lichen-associated yeasts recovered in clades within cystobasidiomycetes that were phylogenetically distant from KU587705, including additional representative loci. These searches cumulatively returned only a three additional cystobasidiomycete sequences (Appendix S4).

DISCUSSION

The cystobasidiomycete sequences detected in our metagenomic data set derived from tissue samples of Bryoria nadvornikiana (Gyeln.) Brodo & D.Hawksw., Heteroderma leucomelas (L.) Poelt, Lecidea roseotincta Coppins & Tonsberg, Opegrapha vulgata (Acth.) Ach., Parmotrema hypotropum (Nyl.) Hale, P. subsumptum (Nyl.) Hale, Usnea cornuta Ach., U. strigosa (Ach.) A.Eaton, and U. subgracilis Vain. Of these nine species, seven are foliose and have a leafy morphology (H. leucomelas, P. hypotropum, P. subsumptum) or fruticose and have a shrubby morphology (B. nadvornikiana, U. cornuta, U. strigosa, U. subgracilis). All but one of those seven belong to the family Parmeliaceae as have the majority of hosts in which cystobasidiomycete fungi previously detected (Spribille et al., 2016). The seventh species, Heteroderma leucomelas, belongs to the Physciaceae, which is another family where cystobasidiomycete sequences have previously been detected (Spribille et al., 2016). Lecidea roseotincta is a crust-forming lichen that belongs to the family Lecanoraceae (Schmull et al., 2011). Opegrapha vulgata is a crustose member of the Arthoniomyces, a class of fungi from which cystobasidiomycetes have not previously been reported.

Notably, we failed to detect cystobasidiomycete fungi in 97% (330 of 339 species) of the taxa sampled in this study. It is possible that the failure to detect yeasts in 330 of the 339 species may be attributable to sequencing at an insufficient depth. However, the same amount of raw sequence data (2 Gb) was used as input into each de novo assembly, therefore eliminating the possibility of a detection bias due to variable DNA input into the assemblies. Further, we did detect cystobasidiomycetes in nine samples, and in several samples, the coverage of the cystobasidiomycete was comparable to, or higher than, that for the single-copy nuclear genome of the primary lichen mycobiont (Fig. 1 and Table 1; average cystobasidiomycete to mycobiont ratio of coverages 0.51 ± 0.36, ratio range 0.16–1.55).

It is also possible that detections were false positives due to sensitivity of WGS sequencing to microscopic contaminants of lichens that grow on the same substrates and in close association with the sampled species (K. G. Keepers et al., unpublished manuscript). Such contamination cannot be ruled out for any data derived from direct sampling of lichen thalli. Nonetheless, it is unlikely in our data set given that we detected cystobasidiomycetes in only 2.2% of samples and all but two of these detections were in lichens from lineages where the yeasts have previously been detected independently by others.

Given our results, the extreme skew in our analyses toward non-detection of yeasts suggests low occurrence, low abundance, and/or uneven distribution of cystobasidiomycetes in natural communities of lichens. The latter is supported by fluorescent in situ hybridization (FISH) staining of cystobasidiomycetes in lichen thalli that showed yeasts to be restricted to the lichen cortex (Spribille et al., 2016) and hence spatially restricted to a small area in proportion to the overall thallus. However, the majority of lichens sampled produce cortical tissues (exceptions are ecoricate taxa such as Lepraria Ach.), and our tissue sampling protocol captured the lichen cortex in all cases when present. Nonetheless, low occurrence, low abundance, and/or spatially restricted occurrence could explain why,

\[ \text{**Equation**} \]

\[ Y = aX + b \]

The results of the regression analysis are shown in the following table:

<table>
<thead>
<tr>
<th>X</th>
<th>Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>

The coefficient of determination (R^2) is 1.0, indicating a perfect fit of the data to the linear model.
in the absence of PCR and amplicon sequencing of targeted molecular markers, we did not detect basidiomycete yeasts in a larger number of Parmeliaceae lineages where they have previously been demonstrated to occur. Taken together, our results call into question whether Cyphobasidium and other related yeasts previously detected in lichen thalli are obligately associated with all individuals of every lichen species in which they have been detected. In fact, members of the cystobasidiomycetes, to which Cyphobasidium belongs, are widely characterized by their fungal parasite lifestyles (Diederich, 1996; Bauer et al., 2006; Millanes et al., 2011, 2016). Hence, these yeasts may function as parasites that infect specific host lichens when conditions are conducive to infection, as has been recently asserted (Hawksworth et al., 2016; Oberwinkler, 2017). Experimental studies and additional genomic work targeting detection of these yeasts in more samples and through additional sequencing techniques would help elucidate their role in the lichen microcosm.

CONCLUSIONS

The diverse metacommunities of lichen thalli have become the focus of considerable study, especially from the perspective of biotic constraints on the development and distribution of symbioses in nature. The discovery of lichen-associated basidiomycete yeasts has been particularly noteworthy, catalyzing a call to reconceptualize lichens as dynamic metacommunities. Lichen-associated basidiomycete yeasts have been implicated as previously overlooked members of the lichen symbiome, at both phylogenetic and spatial scales. However, here we have shown that analysis of metagenomic data from phylogenetically broad sampling of hundreds of lichen species within a lichen biodiversity hotspot detected very few occurrences of Cyphobasidium or other cystobasidiomycete yeasts. These results suggest that such yeasts may not be as ubiquitous as previously thought.

ACKNOWLEDGEMENTS

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TABLE 1. NCBI GenBank accessions for cystobasidiomycete yeast sequences and their host lichens detected during this study, cross-referenced with study isolate numbers (Appendix S2), yeast sequence depth, and mycobiont rDNA sequence coverage.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Cystobasidiomycete GenBank accession</th>
<th>Mycobiont rDNA GenBank accession</th>
<th>Host lichen taxon</th>
<th>Cystobasidiomycete rDNA depth</th>
<th>Lichen mycobiont single-copy rDNA coverage range</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEN_313</td>
<td>MN037399</td>
<td>MK092093</td>
<td>Heterodermia leucomelos</td>
<td>1.43</td>
<td>4.5</td>
<td>0.32</td>
</tr>
<tr>
<td>FEN_271</td>
<td>MN037400</td>
<td>MN038162</td>
<td>Usnea strigosa</td>
<td>3.54</td>
<td>6</td>
<td>0.59</td>
</tr>
<tr>
<td>FEN_107</td>
<td>MN037401</td>
<td>MK106005</td>
<td>Bryoria nadvornikiana</td>
<td>1.04</td>
<td>5.5</td>
<td>0.19</td>
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<tr>
<td>FEN_107</td>
<td>MN037402</td>
<td>MK106005</td>
<td>Bryoria nadvornikiana</td>
<td>1.47</td>
<td>5.5</td>
<td>0.27</td>
</tr>
<tr>
<td>FEN_368</td>
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<td>MG720066</td>
<td>Usnea subgigascis</td>
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<td>7.5</td>
<td>0.17</td>
</tr>
<tr>
<td>FEN_313</td>
<td>MN037404</td>
<td>MN092093</td>
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<td>1.24</td>
<td>4.5</td>
<td>0.28</td>
</tr>
<tr>
<td>FEN_85</td>
<td>MN037405</td>
<td>MN038163</td>
<td>Lecidea roseatincta</td>
<td>1.36</td>
<td>4</td>
<td>0.34</td>
</tr>
<tr>
<td>FEN_117</td>
<td>MN037406</td>
<td>KY1315997</td>
<td>Opegrapha vulgata</td>
<td>1.93</td>
<td>2.5</td>
<td>0.77</td>
</tr>
<tr>
<td>FEN_262</td>
<td>MN037407</td>
<td>MN038164</td>
<td>Parmotrema hypotropum</td>
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<td>3</td>
<td>0.39</td>
</tr>
<tr>
<td>FEN_117</td>
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<td>KY1315997</td>
<td>Opegrapha vulgata</td>
<td>2.12</td>
<td>2.5</td>
<td>0.85</td>
</tr>
<tr>
<td>FEN_117</td>
<td>MN037409</td>
<td>KY1315997</td>
<td>Opegrapha vulgata</td>
<td>2.90</td>
<td>2.5</td>
<td>1.16</td>
</tr>
<tr>
<td>FEN_259</td>
<td>MN032489</td>
<td>MN038165</td>
<td>Parmotrema subsumptum</td>
<td>2.03</td>
<td>4.5</td>
<td>0.45</td>
</tr>
<tr>
<td>FEN_63</td>
<td>MN032112</td>
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<td>Usnea coruta</td>
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<td>4.5</td>
<td>0.28</td>
</tr>
<tr>
<td>FEN_271</td>
<td>MN032113</td>
<td>MN038162</td>
<td>Usnea strigosa</td>
<td>1.55</td>
<td>6</td>
<td>0.26</td>
</tr>
</tbody>
</table>
Botanical Garden] and National Science Foundation IGERT award 1144807 for the IQBiology program at University of Colorado's BioFrontiers Institute.

AUTHOR CONTRIBUTIONS

J.L. and K.K. wrote the manuscript and collaborated on data presentation. J.L. and E.T. led field and genomic sampling components of the study, with collaboration from C.M. C.P. led wet lab components with guidance from N.K. K.K. led data analysis with guidance from NK and input from J.L., E.T., N.K. and C.M. obtained support and developed the southern Appalachian lichen project. All authors reviewed and revised drafts of the manuscript.

DATA ACCESSIBILITY

Taxon sampling and voucher metadata are provided in Appendices S1 and S2, respectively. Voucher data are available via the C.V. Starr Virtual Herbarium at NY and the Consortium of North American Lichen Herbaria (CNALH). Sequence data of BLAST results aimed at detecting cytochrome oxidase subunit II yeast are available via Zenodo (https://doi.org/10.5281/zenodo.3240665). Cystobasidiomycete yeast sequences detected in this study and rDNA of the host lichens have been deposited in NCBI (Table 1).

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. Taxon sampling for the metagenomic data set used in this study.

APPENDIX S2. Molecular voucher metadata for metagenomic samples included in this study.

APPENDIX S3. Top 100 hits from BLAST search of the metagenomic data set using the reference sequence (KU587705.1).

APPENDIX S4. Top 100 hits from BLAST search of the metagenomic data set using six different reference sequences.

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