Shotgun sequencing decades-old lichen specimens to resolve phylogenomic placement of type material

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Abstract. Natural history collections, including name-bearing type specimens, are an important source of genetic information. These data can be critical for appropriate taxonomic revisions in cases where the phylogenetic position of name-bearing type specimens needs to be identified, including morphologically cryptic lichen-forming fungal species. Here, we use high-throughput metagenomic shotgun sequencing to generate genome-scale data from decades-old (i.e., more than 30 years old) isotype specimens representing three vagrant taxa in the lichen-forming fungal genus *Rhizoplaca*, including one species and two subspecies. We also use data from high-throughput metagenomic shotgun sequencing to infer the phylogenetic position of an enigmatic collection, originally identified as *R. haydenii*, that failed to yield genetic data via Sanger sequencing. We were able to construct a 1.64 Mb alignment from over 1200 single-copy nuclear gene regions for the *Rhizoplaca* specimens. Phylogenomic reconstructions recovered an isotype representing *Rhizoplaca haydenii* subsp. *arbuscula* within a clade comprising other specimens identified as *Rhizoplaca haydenii* subsp. *arbuscula*, while an isotype of *R. idahoensis* was recovered within a clade with substantial phylogenetic substructure comprising *Rhizoplaca haydenii* subsp. *haydenii* and other specimens. Based on these data and morphological differences, *Rhizoplaca haydenii* subsp. *arbuscula* is elevated to specific rank as *Rhizoplaca arbuscula*. For the enigmatic collection, we were able to assemble the nearly complete nrDNA cistron and over 50 Mb of the mitochondrial genome. Using these data, we identified this specimen as a morphologically deviant form representing *Xanthoparmelia* aff. *subcumberlandia*. This study highlights the power of high-throughput metagenomic shotgun sequencing in generating larger and more comprehensive genetic data from taxonomically important herbarium specimens.

Key words: fungaria, herbaria, Illumina, metagenomics, museum, natural history collections, *Rhizoplaca*, vagrant

Introduction

Natural history collections are repositories of an impressive quantity and variety of biological information (Lane 1996; Schilthuizen et al. 2015; Wen et al. 2015). Ongoing developments with DNA sequencing technologies are enabling researchers to tap into genetic data that were previously inaccessible (Cooper 1994; Lan & Lindqvist 2019). Incorporating genetic data from natural history collections representing rare/threatened species and species that are otherwise hard to access into genetic-based analyses can provide critical insights (Heckberg et al. 2016; Hundsdorfer et al. 2017; McGuire et al. 2018). For example, genetic data from museum specimens has been used to track temporal and spatial changes in species distributions (Lozier & Cameron 2009), investigate population genetics (Spurgin et al. 2014; Schmid et al. 2018), and evaluate conservation implications (Anco et al. 2018; Brandt et al. 2018). Additionally, accessing molecular sequence data from natural history collections may be an invaluable resource for resolving taxonomic questions (Cappellini et al. 2014), including generating genetic data from type specimens (Hawksworth 2013; Silva et al. 2017; McGuire et al. 2018). Furthermore, DNA from historical museum specimens can provide novel insights into ambiguously identified collections (Chambers & Hebert 2016).
Sequencing DNA from historical specimens raises a number of challenges not encountered with fresh material. In addition to natural processes that lead to DNA degradation post-mortem (Lindahl 1993), some museum procedures for pest management or storage may also adversely affect museum collections as a source of usable DNA (Cooper 1994). In many cases, museum specimens contain short, fragmented DNA molecules, limiting the utility of PCR amplification (Burrell et al. 2015; Holmes et al. 2016). Specimens collected and preserved without sequencing in mind may also contain DNA from organisms stored nearby, collected alongside the specimen, or organisms that have grown on or within museum specimens (Pääbo 1988). Furthermore, destructive sampling, including DNA extraction, may potentially damage irreplaceable specimens (Hawksworth 2013). Degraded or contaminated DNA is often not suitable for traditional PCR amplification and Sanger sequencing, limiting the ability to incorporate museum specimens into DNA barcode identification surveys or phylogenetic or population genetic analyses. However, high-throughput sequencing technologies are now routinely used to generate genome-scale data from degraded DNA commonly found in museum specimens (Staats et al. 2013; Besnard et al. 2015; McCormack et al. 2016). This type of genome-scale data based on museum specimens is playing an increasingly important role in species delimitation and phylogenomic studies (Rittmeyer & Austin 2015; Wood et al. 2018).

Natural history collections have also played a key role in phylogenetic research efforts involving lichen-forming fungi, with DNA routinely extracted from herbarium specimens (Sohrabi et al. 2010; Gueidan et al. 2015; Kistenich et al. 2019), including a 151-year-old museum specimen (Redchenko et al. 2012). However, in other cases, generating sequence data from some lichens can be problematic with the passing of even short amounts of time, on the scale of months to years, without careful, intentional preparation and storage to maximize the probability of extracting usable DNA. Even with specimens collected specifically for genetic research, additional care must be taken to preserve usable DNA, and successful extraction of high-quality DNA may be limited after only a few months. With the prevalence of cryptic species inferred from molecular systematic studies of lichen-forming fungi (Crespo & Pérez-Ortega 2009), ascertaining the relationships of name-bearing organisms stored nearby, collected alongside the specimen, or organisms that have grown on or within museum specimens (Pääbo 1988). Furthermore, destructive sampling, including DNA extraction, may potentially damage irreplaceable specimens (Hawksworth 2013). Degraded or contaminated DNA is often not suitable for traditional PCR amplification and Sanger sequencing, limiting the ability to incorporate museum specimens into DNA barcode identification surveys or phylogenetic or population genetic analyses. However, high-throughput sequencing technologies are now routinely used to generate genome-scale data from degraded DNA commonly found in museum specimens (Staats et al. 2013; Besnard et al. 2015; McCormack et al. 2016). This type of genome-scale data based on museum specimens is playing an increasingly important role in species delimitation and phylogenomic studies (Rittmeyer & Austin 2015; Wood et al. 2018).

Materials and methods

Specimen sampling

To explore the potential use of Illumina metagenomic shotgun sequencing to efficiently ascertain usable molecular sequence data from decades-old museum collections, including type collections, we targeted a total of five specimens ranging from 30 to 34 years old (Table 1). Specifically, we focused on vagrant specimens from the Rhizoplaca melanophthalma group, including three isotypes – R. haydenii subsp. arbuscula, R. idahoensis, and R. melanophthalma subsp. cerebriformis; a second historical collection representing R. idahoensis; and a morphologically unusual specimen originally identified as R. haydenii and collected in a krumholtz-dominated subalpine habitat outside of the known distribution of R. haydenii (Fig. 1C). Isotype collections representing R. haydenii subsp. arbuscula included specimens with somewhat deviating morphologies – specimens with more robust thalli (Fig. 1A) relative to other thalli with narrower, finely branching lobes (Fig. 1B). Narrower, finely branching lobed specimens morphologically similar to thalli from an R. haydenii subsp. arbuscula iso-type collection were represented in previously generated metagenomic samples, including individuals collected near the type locality (Keuler et al. 2019). Therefore, we selected a more robust thallus from the isotype collection for metagenomic shotgun sequencing. We were unable to secure type material representing R. haydenii subsp. haydenii for DNA sequencing. Therefore, we collected R. haydenii subsp. haydenii specimens from two populations near the type locality in eastern Wyoming (Table S1) – ‘Laramie Plains’ (H-NYL 28325; [basionym published...
for this study. Using material from the new collections, four individual specimens representing the range of morphological variation were selected for sequencing. New specimens sequenced for this study were then combined with sequences from specimens reported by Keuler et al. (2019; Table S1). All specimens were visually assessed using an Olympus SZH zoom stereo dissecting microscope, and secondary metabolites were identified using thin layer chromatography (TLC). Lichen compounds were extracted in acetone using a small, clean piece of thallus material (subsequently used for DNA isolation); the acetone extract was then separated using solvents C and G following the methods of Orange et al. (2001).

DNA extraction and sequencing

Total genomic DNA was extracted using the E.Z.N.A. Plant DNA DS Mini Kit (Omega Bio-tek) following the manufacturers’ recommendations. Previous efforts to generate sequence data from five to twenty-year-old Rhizoplaca specimens have been successful in other studies. However, for this study, multiple attempts to generate

Figure 1. Vagrant Rhizoplaca specimens, including isotypes, and a morphologically aberrant vagrant Xanthoparmelia specimen. A – Rhizoplaca haydenii subsp. arbuscula (Rosentreter 3861; isotype – sequenced for this study); B – Rhizoplaca haydenii subsp. arbuscula (Rosentreter 3861; isotype – not sequenced); C – Xanthoparmelia aff. subcumberlandia (Newberry 936 – sequenced for this study), originally identified as R. haydenii; D – Rhizoplaca idahoensis (Rosentreter 3719; isotype – sequenced for this study); E – Rhizoplaca haydenii subsp. haydenii (Leavitt 18-1006 – sequenced for this study); F – Rhizoplaca melanophthalma subsp. cerebriformis (Rosentreter 3876; isotype – sequenced for this study). These specimens are housed at the Herbarium of Non-Vascular Cryptogams (BRY-C). Scales: 1 cm in all panels.
genetic data via Sanger sequencing for the *R. haydenii*-like specimens collected in 1989 from subalpine habitat in the Uinta Mountains of northeastern Utah were unsuccessful. DNA extractions from these specimens revealed low quantities of highly fragmented DNA. From this total genomic DNA, high-throughput sequencing libraries were prepared using the standard Illumina whole-genome sequencing (WGS) library preparation process with Adaptive Focused Acoustics for shearing (Covaris) followed by low quantities of highly fragmented DNA. From this library, e.g., the internal transcribed spacer region (ITS), markers (see Results). BLAST searches using markers within the nrDNA, e.g., the internal transcribed spacer region (ITS), nrLSU, and IGS were added to the TreeBase alignment for western North American

| Taxon                                      | Voucher                  | Locality                  | Collection date | # of filtered Illumina reads | ITS mean coverage | RBP1 mean coverage |
|--------------------------------------------|--------------------------|---------------------------|-----------------|-----------------------------|-------------------|-------------------|
| *R. haydenii* subsp. arbuscula             | R. Rosentreter 3861      | USA, Idaho, Lemhi Co.     | 08 June 1986    | 31,069,276 (2×150)         | 402.1             | 29.1              |
| (usnic acid chemotype)                     | (isotype)                |                           |                 |                             |                   |                   |
| *R. idahoensis* (usnic acid chemotype)    | R. Rosentreter 3719      | USA, Idaho, Lemhi Co.     | 27 August 1985  | 28,832,106 (2×150)         | 134.1             | 40.4              |
| (isotype)                                  |                          |                           |                 |                             |                   |                   |
| *R. idahoensis* (usnic acid chemotype)    | A. DeBolt 750            | USA, Idaho, Lemhi Co.     | 26 June 1987    | 26,370,812 (2×150)         | 171.9             | 31.4              |
| (isotype)                                  |                          |                           |                 |                             |                   |                   |
| *R. melanoniphalma* subsp. cerebriformis   | R. Rosentreter 3876      | USA, Idaho, Lemhi Co.     | 08 June 1986    | 28,558,924 (2×150)         | 662.5             | 39.7              |
| (usnic and psoromic acids chemotype)       | (isotype)                |                           |                 |                             |                   |                   |
| *Xanthoparmelia* aff. subcumberlandia      | C. Newberry 936          | USA, Utah, Duchesne Co.   | 26 July 1989    | 8,234,622 (2×150)          | 24.1              | <0.5              |
| (usnic, norstictic, and stictic acids chemotype) |                       |                           |                 |                             |                   |                   |
Figure 2. Phylogeny of the Rhizoplaca melanophthalma species group inferred from a 1.64 Mb BUSCO alignment. Isotype specimens are indicated in bold text. Bootstrap support values are indicated at nodes, with thickened branches highlighting bootstrap support values = 100%. Distinct forms in the ‘arbuscula’ and ‘haydenii/idahoensis’ clades are indicated.

Phylogenetic analysis

Evolutionary relationships were inferred using a supermatrix approach, which has been shown to accurately infer relationships across a wide range of scenarios (Tonini et al. 2015). We inferred relationships of the four vagrant Rhizoplaca samples, including three isotypes, with other members of the R. melanophthalma species complex using the REALPHY phylogenomic dataset analyzed under a maximum likelihood (ML) criterion as implemented in IQ-TREE v1.6.7 (Nguyen et al. 2015), with 1,000 ultra-fast bootstrap replicates (Hoang et al. 2018). The concatenated nine-locus Xanthoparmelia dataset was analyzed in IQ-TREE v1.6.7, as described above, with the exception that each locus was treated as a separate partition, with no additional partitioning within loci (e.g., codon position, etc.). In all cases, nodal support was mapped onto the best-scoring ML topologies.

Results

TLC indicated that the specimens representing R. haydenii subsp. arbuscula, R. haydenii subsp. haydenii and R. idahoensis (a total of two specimens) contained usnic acid, while R. melanophthalma subsp. cerebriformis contained usnic and psoromic acids (Table 1). The morphologically unusual specimen originally identified as R. haydenii (Newberry 936 [BRY-C]) contained usnic, norstictic, and stictic acids.

Illumina metagenomic shotgun sequencing generated between 8.2 and 31.1 million PE reads for each specimen
and was closely related to three specimens representing clade, *R. porteri* subsp. *robust*, forms. The final isotype specimen, representing *R. porteri* (Fig. 2) included in this study (all from Lemhi Valley, specimens with narrower, finely branching lobes (see provisionally named clades 'D' and 'E' (Fig. 3). The previous reconstructions, supporting two distinct clades within the genus *Xanthoparmelia*, while the other three species belonged to the *R. melanophthalma* complex. Coverage of the single copy RPB1 marker ranged from 29× to 40× for the *Rhizoplaca* species; and < 0.5× for the *Xanthoparmelia* species reconstructed in REALPHY comprised a total of 1,650,966 aligned nucleotide position characters (File S1). Phylogenomic inference using this alignment in IQ-TREE recovered a generally well-supported (e.g., bootstrap values > 95%) topology consistent with previous reconstructions (Fig. 2).

Both *R. idahoensis* specimens sequenced for this study, including an isotype (Rosentreter 3719 [BRY-C]), were recovered within the *R. haydenii/R. idahoensis* clade as a distinct, well-supported clade within specimens representing the polyphyletic *R. haydenii*. Another lineage containing two specimens morphologically similar to *R. idahoensis* (Leavitt 094f & Leavitt 103f [BRY-C]) was also recovered within the *R. haydenii/R. idahoensis* clade but was clearly distinct from the type specimen (Fig. 2). The four *R. haydenii* subsp. *haydenii* specimens collected from locations near the type locality – Laramie, WY – were nearly genetically identical and recovered within the *R. haydenii/R. idahoensis* clade; and all *R. haydenii* subsp. *haydenii* specimens from Wyoming were recovered within a single monophyletic clade, with phylogenetic substructure corresponding to distinct geographic regions. The *R. haydenii* subsp. *arbuscula* isotype (Rosentreter 3861 [BRY-C]), was recovered within a second major clade, distinct from the *R. haydenii/R. idahoensis* clade, the *R. arbuscula* group (Fig. 2), comprised of other *R. haydenii* subsp. *arbuscula* specimens collected from the region. All of the *R. haydenii* subsp. *haydenii* specimens with narrower, finely branching lobes (see Fig. 1B) included in this study (all from Lemhi Valley, ID, USA) were phylogenetically distinct from the more robust forms. The final isotype specimen, representing *R. melanophthalma* subsp. *cerifer* (Rosentreter 3876 [BRY-C]) was recovered within the *R. porteri* clade, and was closely related to three species representing *R. porteri* (Fig. 2).

The *Xanthoparmelia* phylogeny (Fig. 3) inferred from a nine-locus dataset (File S2), including the unusual *Xanthoparmelia* specimen from the Uinta Mountains of northeastern Utah (Fig. 1C), was congruent with previous reconstructions, supporting two distinct clades provisionally named clades ‘D’ and ‘E’ (Fig. 3). The clade containing the unusual *Xanthoparmelia* specimen was recovered within a weakly supported clade comprised of other stictic acid-containing *Xanthoparmelia* species, which has previously been supported as a distinct genetic cluster, cluster ‘E1-a’ (Leavitt et al. 2013c). Many members of this clade correspond morphologically with *X. subcumberlandia* Elix & T. H. Nash. Because we were unable to generate data from single-copy nuclear genomic regions, we provide the nearly complete nDNA operon and fragments of the mitochondrial genome assembly as supplementary files (File S3).

### Taxonomy

**Rhizoplaca arbuscula** Rosentreter, St. Clair & Leavitt, comb. et stat. nov. (Fig. 1A, B).

**Description.** Free on soil; thallus globose with abundant marginal whitish nodules; lobes narrowly divided, flat to round in cross section; medulla lacking both placodiolic and psoromic acids.

**Etymology.** Named after the tree-like resemblance of the narrowly divided lobes.

### Discussion

Here we report the first, to our knowledge, successful use of high-throughput sequencing specifically targeting lichen type specimens to resolve the evolutionary relationships of the mycobiont. Based on the genome-scale data generated in this study and morphological differences, we propose to elevate *R. haydenii* subsp. *arbuscula* to species, formally named here as *R. arbuscula*. We also show that data from high-throughput metagenomic sequencing can be used to identify ambiguously identified historical lichen collections. Below we discuss the implications for vagrant members of the *Rhizoplaca melanophthalma* species complex (Leavitt et al. 2011a), as well as lichen research in general.

The *Rhizoplaca melanophthalma* species complex (Leavitt et al. 2011a) has received considerable attention, including the description of a number of new species supported largely by genetic data (Leavitt et al. 2013a; Leavitt et al. 2016; Grewe et al. 2017). Most recently, sampling of vagrant forms in this complex has revealed striking phylogeographic structure in the *R. haydenii* clade including *R. haydenii* subsp. *arbuscula*, *R. haydenii* subsp. *haydenii*, and *R. idahoensis* (Keuler et al. 2019). It is now apparent that extensive, distribution-wide sampling
of members of this clade will be essential for inferring the evolutionary history of this group in order to accurately make appropriate taxonomic revisions and better understand phylogeographic patterns and processes of speciation.

Within the broad ‘R. haydenii’ clade, two well-supported clades were recovered as clearly distinct – the ‘R. arbuscula’ and ‘R. haydenii/R. idahoensis’ lineages (Fig. 2). Based on morphological differences between specimens in the ‘R. arbuscula’ clade and specimen recovered in the ‘R. haydenii/R. idahoensis’ clade and corroborating support from phylogenomic data, we propose that R. haydenii subsp. arbuscula be formally described as a distinct species – R. arbuscula. At a finer scale, within the ‘R. arbuscula’ clade, two distinct forms were observed, one with a shrubbier thallus and the others with more narrowly divided and elongated lobes, both of which are represented in isotype collections (Fig. 1A, B). However, both forms are closely related and distinct from R. haydenii subsp. haydenii (Fig. 2). The range of variation in R. arbuscula specimens is distinct from the variation in R. haydenii subsp. haydenii specimens, which have much broader lobes and are generally found with bluish/dark green pigments.

In this study, the relationships of isotypes representing R. haydenii subsp. arbuscula (elevated here to R. arbuscula) and R. idahoensis were recovered with strong support within the Rhizoplaca melanopthalma species complex (Fig. 2). Specimens representing R. haydenii subsp. haydenii were recovered in multiple distinct clades. Rhizoplaca haydenii subsp. haydenii was recovered as polyphyletic, with R. idahoensis, R. cf. idahoensis and an interesting saxicolous specimen from western Montana (Leavitt 715f [BRY-C]), nested within the broader ‘R. haydenii/R. idahoensis’ clade (Fig. 2). The phylogeographic structure in the ‘R. haydenii/R. idahoensis’ clade is striking, and additional studies will be required to more accurately determine population structure,
When working with historical specimens where PCR and Sanger sequencing may fail to yield data, high-throughput sequencing provides an effective alternative for generating crucial molecular data (Green et al. 2006; Rowe et al. 2011). High-coverage metagenomic data from type specimens is also more versatile, in terms of scientific utility, as the data can be integrated into a variety of genetic datasets, ranging from single- and multi-locus datasets (Leavitt et al. 2018) to reduced-representation phylogenomic datasets (Grewe et al. 2017) to other phylogenomic and genomic applications, including investigations into other associated symbiotic partners (Paul et al. 2018).

While guidelines for collecting botanical samples intended for genomic work have been developed (Funk et al. 2017), to our knowledge established best practices for collecting lichen samples for genomic work have not yet been developed. Extraction of DNA from members of the Rhizoplaca melanophthalma group depends to a large degree on specimen processing and curation. Specimens collected dry, processed right after collection and housed in herbaria with low humidity may yield high-quality DNA years after they were collected (personal observation). However, in other cases we have been unable to extract DNA from relatively recent collections (1 to 5 years) of Rhizoplaca specimens. A variety of factors likely influence DNA quantity and quality in lichen collections. Relative to herbaria predominantly housing vascular plants, lichen collections tend to not have as much DNA degradation since most lichen herbaria are not treated with chemicals (Hall 1988). Lichens from arid regions are often dry when collected or are more easily dried without the use of artificial drying methods; and based on our anecdotal observations, the integrity of the DNA tends to be preserved at a higher level even in older collections. In contrast, extracting high-quality DNA from lichens occurring in humid areas is generally more problematic. In many cases, lichen specimens from humid regions must be dried using artificial heating methods which often degrade the DNA. Furthermore, our experience suggests that lichen collections stored in plastic containers/bags, even for short periods of time, are less likely to yield high-quality DNA. Similarly, moistening and pressing the specimens after collection, especially if a heated drier is used, may damage DNA from lichens intended for genomic work.

Successfully generated DNA from name-bearing types will be particularly valuable for resolving taxonomic issues in complexes comprised of morphologically cryptic lineages (Hawksworth 2013). With the increased recognition of morphologically cryptic species of lichen-forming fungi (Crespo & Pérez-Ortega 2009), attempts to recover genetic data from previously designated types where ample material exists will minimize the risk of erroneously designating epitypes (Hawksworth 2013). However, we note that in many cases non-destructive microscopic studies are sufficient to unequivocally place type specimens in relationship to recently collected specimens. Similarly, shotgun sequencing approaches for generating genome-scale data may not be necessary in all cases. Standard PCR amplification and Sanger sequencing may
be sufficient to accurately assign name-bearing types; and high-throughput sequencing of PCR amplicons has also been shown to effectively generate sequence data from historical lichen collections (Kistenich et al. 2019). As sequencing technologies continue to change and the cost of generating genome-scale data decreases, researchers must carefully evaluate the most appropriate data for effectively addressing specific questions. In conclusion, our study provides additional evidence of the value of historical lichen collections as a source of genetic material (Bruns et al. 1990; Brock et al. 2009), highlighting the role of high-throughput sequencing technologies in generating larger and more comprehensive genetic data from taxonomically important herbarium specimens.

**Acknowledgements**

We dedicate this publication to our friend and colleague, Dr. James Lawrey, on the occasion of his 70th birthday. Dr. Lawrey’s academic career and exemplary professional interactions have been inspiring. We acknowledge fruitful collaboration with the Snake River Plain herbarium at Boise State University. This research was supported by the M. L. Bean Life Science Museum, Brigham Young University, Provo, Utah, USA. We thank Ed Wilcox, DNA Sequencing Center, Brigham Young University, Provo, Utah, USA, for help with sequencing.

**Supplementary electronic material**

- **Table S1.** Summary of specimens included in the study, including taxon, clade, DNA code, locality, GPS coordinates, number of filtered reads, and voucher ID. [Download file]
- **File S1.** The BUSCO alignment of the *R. melanophthalma* species complex reconstructed in REALPHY comprised a total of 1,650,966 aligned nucleotide position characters. [Download file]
- **File S2.** Concatenated alignment of ribosomal (nrLSU, IGS, ITS, and a group I intron) and protein-coding (*β*-tubulin, GAPDH, MCM7, *RPB1*, and *RPB2*) sequence data from *Xanthoparmelia* clades ‘D’ and ‘E’. [Download file]
- **File S3.** *Xanthoparmelia* nrDNA cistron; and three mitochondrial contigs from the *de novo* assembly of short reads. [Download file]
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