PHILOSOPHICAL TRANSACTIONS B

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Cite this article: Daru BH, Bowman EA, Pfister DH, Arnold AE. 2018 A novel proof of concept for capturing the diversity of endophytic fungi preserved in herbarium specimens. *Phil. Trans. R. Soc. B* **374**: 20170395.
http://dx.doi.org/10.1098/rstb.2017.0395

Accepted: 29 September 2018

One contribution of 16 to a theme issue 'Biological collections for understanding biodiversity in the Anthropocene'.

Subject Areas:
ecology

Keywords:
foliar endophytes, fungi, global change, herbaria, plant specimens, plant microbiome

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Electronic supplementary material is available online at https://dx.doi.org/10.6084/m9.figshare.c.4258310.

THE ROYAL SOCIETY PUBLISHING

A novel proof of concept for capturing the diversity of endophytic fungi preserved in herbarium specimens

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Herbarium specimens represent important records of morphological and genetic diversity of plants that inform questions relevant to global change, including species distributions, phenology and functional traits. It is increasingly appreciated that plant microbiomes can influence these aspects of plant biology, but little is known regarding the historic distribution of microbes associated with plants collected in the pre-molecular age. If microbiomes can be observed reliably in herbarium specimens, researchers will gain a new lens with which to examine microbial ecology, evolution, species interactions. Here, we describe a method for accessing historical plant microbiomes from preserved herbarium specimens, providing a proof of concept using two plant taxa from the imperiled boreal biome (*Andromeda polifolia* and *Ledum palustre* subsp. *groenlandicum*, Ericaceae). We focus on fungal endophytes, which occur within symptomless plant tissues such as leaves. Through a three-part approach (i.e. culturing, cloning and next-generation amplicon sequencing via the Illumina MiSeq platform, with extensive controls), we examined endophyte communities in dried, pressed leaves that had been processed as regular herbarium specimens and stored at room temperature in a herbarium for four years. We retrieved only one endophyte in culture, but cloning and especially the MiSeq analysis revealed a rich community of foliar endophytes. The phylogenetic distribution and diversity of endophyte assemblages, especially among the Ascomycota, resemble endophyte communities from fresh plants collected in the boreal biome. We could distinguish communities of endophytes in each plant species and differentiate likely endophytes from fungi that could be surface contaminants. Taxa found by cloning were observed in the larger MiSeq dataset, but species richness was greater when subsets of the same tissues were evaluated with the MiSeq approach. Our findings provide a proof of concept for capturing endophyte DNA from herbarium specimens, supporting the importance of herbarium records as roadmaps for understanding the dynamics of plant-associated microbial biodiversity in the Anthropocene.

This article is part of the theme issue 'Biological collections for understanding biodiversity in the Anthropocene'.

1. Introduction

Herbarium specimens represent important records of the morphological, ecological and genetic diversity of plants [1–5]. Often these specimens are deposited with metadata that can inform questions relevant to global change, including historical records of distributions, phenology and functional traits [6–18]. It is increasingly appreciated that plant microbiomes can influence these aspects of plant biology, shaping the functional traits of plants and thus their responses to abiotic and

biotic stress, their physiology, the timing of their demographic events and their distributions at diverse scales [19–21].

The rich diversity of microbes in living plants, and information about their evolution and biogeography, recently have become more accessible—particularly through the application of diverse sequencing technologies and culture-independent methods [22–24]. Such approaches have highlighted the phylogenetic and functional diversity of plant microbiomes, showcasing aspects of their phenology and how they respond to environmental factors in the context of short-term experiments or environmental shifts ([25–27]; see also [28,29]). However, little is known regarding the historic distribution of microbes associated with plants, restricting our understanding of how such microbial communities may have changed over time in a given plant species or environment, particularly in response to changes in climate and other conditions induced by human activity. We thus lack records relevant to microbial phenology, function and distributions over time.

One solution is to examine the microbiota of plant tissue archived as herbarium specimens. Such microbes include fungal endophytes, a diverse and polyphyletic group of fungi that occur within healthy plant tissues such as roots and leaves [30]. Fungal endophytes form important associations with plants worldwide and have been observed in living tissue of every plant species examined to date via culture-based or culture-independent methods [30]. Often overlooked because they cause no visible symptoms of disease [31], endophytes are increasingly appreciated for their beneficial impacts on plant physiology and their roles in mitigating abiotic and biotic stress [30,32–40]. The vertically transmitted endophytes of cool-season grasses have received especially extensive attention [41], but the horizontally transmitted endophytes associated with photosynthetic tissues of all plants are striking in their phylogenetic- and species richness at scales ranging from individual leaves to landscapes [42–46]. Overall, foliar fungal endophytes represent a tremendous richness of fungal species, particularly among the largest fungal phylum (Ascomycota) [47], and thus they contribute meaningfully to global biodiversity [48]. Their abundance, diversity and composition can provide insight into the environmental conditions in which plants occur [29,34] and help connect the dual dynamics of fungal ecology and plant ecology in a changing world. However, little is known about their historical associations with plants, nor how their distributions and functional importance may have shifted over time.

Vast collections of plant specimens archived in herbaria can open a window into the history of endophyte diversity, and how this diversity has been impacted through the course of global change [49]. However, it is unclear whether, or to what extent, the dried material that comprises plant specimens in most herbaria is amendable to studies of endophyte diversity. Traditionally, fungal endophytes have been isolated on growth media from surface-sterilized plant material, and emergent colonies have been identified using morphology and/or analyses of molecular sequence data [43,50–52]. Subsequent use of culture-independent approaches such as cloning from fresh plant material revealed a high richness of endophytes that may not be isolated in culture [53–55]. Such perspectives have recently been expanded further by next-generation amplicon sequencing [45,46,56,57], capturing the rich set of endophytes that may be recalcitrant to cultivation on standard media or that might not be detected with the lower-throughput method of cloning.

Next-generation sequencing approaches have been used with fresh plant material in diverse settings, but to our knowledge, they have not yet been applied to examine the endophytic microbiota of herbarium specimens. Previous efforts that have used herbarium material to assess microbial diversity have focused on individual microfungi, including pathogens (e.g. [2,58–63]), and have demonstrated that pathogen DNA can be captured from preserved tissues if carefully tailored methods are used. These studies have shown that certain challenges—such as the issue of DNA degradation in herbarium specimens [64,65]—can be overcome when herbarium specimens are examined for fungal associates. For the study of endophytes, additional challenges include low biomass of individual endophytic fungi in leaves, the potential for multiple endophyte species to occur in the same parts of leaves (rather than only a single species), the likelihood of surface contamination by other fungi, and the use of chemical preservation techniques that may limit endophyte growth in culture or the capacity to amplify them via molecular methods [66,67].

We examined the use of culture-based and culture-independent methods for capturing endophyte communities from dried leaf material in a herbarium collection. Specifically, we use culturing, cloning and next-generation sequencing to evaluate the diversity of fungal endophytes in preserved specimens of two plant species, *Andromeda polifolia* and *Ledum palustre* subsp. *groenlandicum* (Ericaceae). Both are distributed in the northern parts of the Northern Hemisphere across a gradient of climate and human population density [68,69]. As part of the rich flora of the increasingly imperiled boreal biome, they provide a basis to unlock the genetic information of plant-associated microbes stored in herbaria.

2. Material and methods

(a) Sampling

We retrieved 10 mature leaves each of *A. polifolia* and *L. palustre* subsp. *groenlandicum* (Ericaceae) from herbarium specimens provided by Jason Karakehian of the Harvard University Herbaria (HUH). The specimens were collected originally from a bog in St John, New Brunswick, Canada, in 2013. They were prepared by pressing and drying, and then were preserved under standard herbarium conditions in cabinets in Cambridge, MA, USA, for a period of four years before use in this study. Permission to sample tissue was obtained before leaves were selected for the present study. We collected a small piece (approx. 2 cm^2) of material from each leaf specimen under standard sampling protocol for HUH, which ensured minimal destruction of specimens. Voucher information, including collection date and collectors are shown in the electronic supplementary material, table S1.

(b) Workflow and experimental design

The conceptual workflow for our study is outlined in figure 1. Each leaf sample was cut into 192 segments of 2 mm^2 each (3840 segments in total for the study), surface-sterilized and then partitioned haphazardly for fungal isolation (culture-based approach) or DNA extraction (culture-independent approaches: cloning and next-generation amplicon sequencing). Surface sterilization followed [70]: sequential immersion and agitation in 95% ethanol for 30 s, 0.525% NaOCl for 120 s and 70% ethanol for 120 s (hereafter, method A). Because specimens were brittle and dry, and we were uncertain whether the standard sterilization method would damage fungal DNA within tissues, we evaluated two other treatments: (method B)

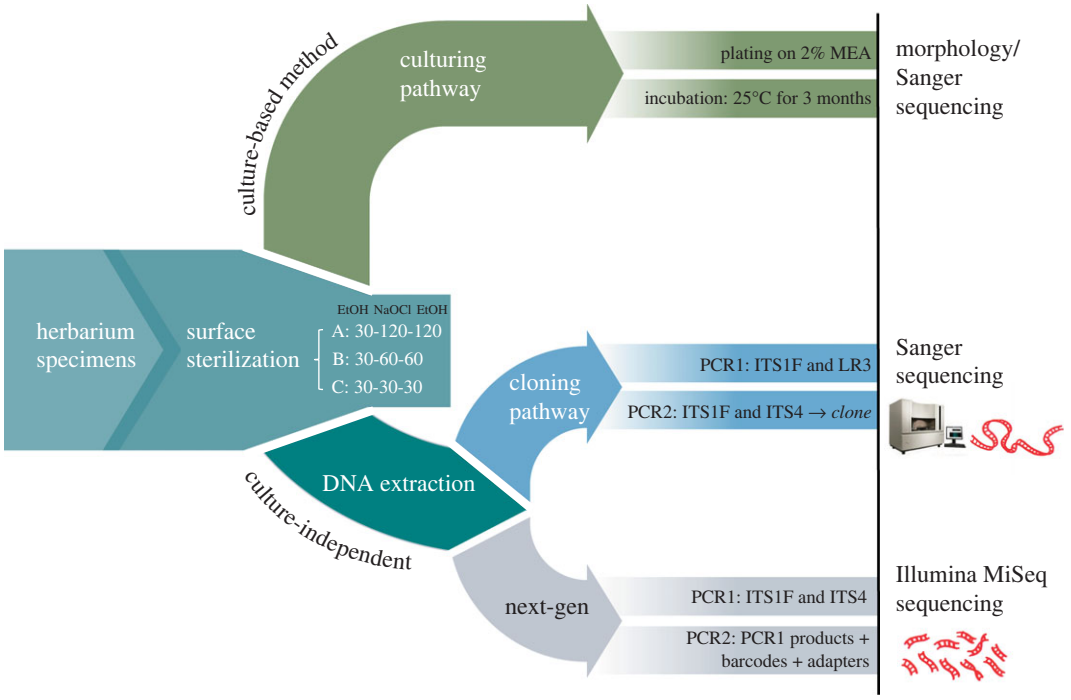

Figure 1. Analytical workflow and experimental design for isolating DNA of fungal endophytes from plant herbarium specimens. The workflow follows two broad pathways: culture-based and culture-independent methods. MEA, malt extract agar, one of many media that can be used to isolate endophytes in culture.

30–60–60 s and (method C) 30–30–30 s, indicating immersion time (in seconds) in 95% ethanol, 0.525% NaOCl and 70% ethanol, respectively (figure 1).

Leaf segments were dried briefly under sterile conditions before initiating the fungal isolation or DNA extraction pathways. To limit contamination by exogenous microbes and DNA [65,70,71], we conducted all work in a dedicated, sterile environment in which all surfaces and tools were treated with bleach, DNA Away (Thermo Scientific, USA) and ultraviolet light (30 min) immediately before use [45,72]. Additional controls are described below.

(i) Culture-based method

We plated 96 surface-sterilized segments per leaf sample onto the surface of 2% malt extract agar (MEA; Amresco, USA) under sterile conditions [43–45]. Plates were sealed with Parafilm and incubated at room temperature (approx. 25°C) with approximately 12 L : 12 D cycles for three months [44]. One endophyte emerged in culture (see below). It was transferred to axenic culture and stored in sterile water as a permanent voucher at the University of Arizona. DNA was extracted from the isolate using a RedExtract-N-Amp plant PCR kit (Sigma-Aldrich, St Louis, MO, USA) following the manufacturer's instructions. The internal transcribed spacer region (ITSrDNA) and adjacent D1–D2 region of the nuclear ribosomal large subunit were amplified by polymerase chain reaction (PCR) as a single fragment using primers ITS1F and LR3 (see [73,74]). Each 20 µl reaction mixture included 8 µl of water, 10 µl of RedTaq (Sigma-Aldrich), 0.8 µl of each primer (10 µM concentration), 1.3 µl of bovine serum albumin (BSA) at a concentration of 15 mg ml^{-1} and 1 µl of DNA extract. Cycling parameters followed Hoffman *et al.* [75]: 94°C for 3 min, 36 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 1 min, and 72°C for 10 min. The positive amplicon was cleaned with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and submitted to the University of Arizona Genetics Core for bidirectional Sanger sequencing using the Big Dye Terminator v.3.1 (Applied Biosystems, Foster City, CA, USA). The sequence was assembled and edited in Sequencher v.4.10.1 (Gene Codes Corporation, Ann Arbor, MI, USA) prior to analyses.

(ii) Culture-independent pathway

For culture-independent analyses, we extracted total genomic DNA from leaf tissue and then used fungal-specific primers to selectively amplify fungal DNA for cloning and Illumina MiSeq sequencing (figure 1). We used the PowerPlant Pro Kit (Qiagen, USA) to extract DNA from each of four sets of 24 leaf segments per specimen, representing the same total leaf area per leaf as that used for culturing [76]. DNA quality was tested using a NanoDrop 2000/2000c Spectrophotometer (Thermo Scientific, USA). The samples were found to have low 260/280 ratios (i.e. the ratio of absorbance at 260 and 280 nm), indicating potential inhibitors for PCR. Extractions were cleaned using the DNeasy PowerClean Pro Cleanup Kit (Qiagen, USA) to remove proteins and phenols prior to PCR.

Cloning pathway. A hemi-nested PCR approach was used to amplify the fungal ITSrDNA region from the total genomic DNA obtained from leaves. In the first PCR, the ITSrDNA and adjacent D1–D2 region of the nuclear ribosomal large subunit were amplified in a total volume of 20 µl reaction mixture containing 2.1 µl of water, 10 µl of RedTaq (Sigma-Aldrich), 0.8 µl of each primer (10 µM concentration of primers ITS1F and LR3), 1.3 µl of BSA (15 mg ml^{-1} concentration) and 5 µl of DNA extract. Cycling parameters followed Hoffman *et al.* [75], as described above. PCR products were visualized on a 1% agarose gel stained with SYBR Green. In the second PCR, amplification was repeated as above with minor alterations: 1 µl of amplicon (PCR products from the first PCR) was used as template for the second amplification step using primers ITS1F and ITS4 [73,74] and water was increased to bring the total reaction volume to 20 µl.

Products from the second PCR were cloned using a StrataClone PCR Cloning Kit (Agilent Technologies, Santa Clara, CA, USA) and screened using the blue–white screening technique according to the manufacturer's protocol. Positive colonies were transferred to fresh 'library' plates and incubated at 37°C for 24 h to increase colony size. Eight positive clones per specimen were selected and then PCR-amplified with primers M13F and M13R, as described above. We checked for contamination from reagents or laboratory handling by running analyses in parallel in which water, extraction reagents and PCR products from previous amplifications each were used in place of the DNA template.

We observed no contamination in these analyses and thus focus only on the sequence data obtained from specimens for further analyses. Sanger sequencing was performed using Big Dye Terminator v.3.1 (Applied Biosystems, Foster City, CA, USA) at the University of Arizona Genomics Core. Sequences were processed as described above.

Illumina MiSeq amplicon sequencing. Amplification was carried out using a two-step PCR approach [72,77]. The first PCR (PCR1) was used to amplify fungal ITSrDNA, and a second PCR was used to add Illumina adapters and unique sample barcodes. In PCR1, we used phase-shifted primers ITS1F and ITS4 with universal sequences CS1 and CS2 attached (Integrated DNA Technologies Inc., USA). Each 20 µl reaction contained 10 µl of Phusion Flash High Fidelity Master Mix (Thermo Scientific, USA), 0.2 µl of 0.5 µM of each primer, 1 µl of BSA at a concentration of 20 mg ml^{-1}, 5 µl of DNA template and 3.6 µl of molecular biology grade water (Fisher Scientific, USA). The reaction mixture was amplified by PCR as follows: 98°C for 10 s, 28 cycles of 98°C for 1 s, 57°C for 5 s, 72°C for 20 s, and a 1 min extension at 72°C [77]. PCR1 was run in triplicate for each extraction and visualized on a 2% agarose gel stained with SYBR Green. PCRs from each leaf then were pooled and diluted with molecular grade water based on band intensity (1 : 3 or 1 : 10) [72,77].

Pooled and diluted products from PCR1 (including extraction blanks and negative controls, see below) were used as the template for PCR2, in which sample-identification barcodes and Illumina adapters were added (IBEST Genomics Resource Core, Moscow, ID, USA). Each 20 µl reaction contained 10 µl of Phusion Flash High Fidelity Master Mix, 0.75 µl of the barcoded primers at 2 µM concentration, 0.24 µl of BSA at a concentration of 20 mg ml^{-1}, 1 µl of pooled and diluted PCR1 product, and 8.01 µl of molecular biology grade water. The PCR programme was run for 7 cycles as follows: 98°C for 10 s, 28 cycles of 98°C for 1 s, 51°C for 5 s, and 72°C for 20 s, and a 1 min extension at 72°C [72,77]. The product was visualized on a 2% agarose gel. DNA concentration was quantified using Qubit (Thermo Scientific, USA) and the Qubit dsDNA HS Assay Kit (Thermo Scientific, USA). We pooled 20 ng of DNA from each sample into a single tube, which was shipped on dry ice to the University of Idaho IBEST Genomics Resources Core for Illumina MiSeq sequencing [72,77].

To limit contamination [45,72,77], PCR mixes were prepared in a sterile, dedicated 'pre-PCR' hood (i.e. an environment that was never exposed to amplified DNA). We used a 'post-PCR' hood for library preparation following PCR1 (i.e. PCR1 pooling and dilutions; addition of diluted PCR1 products to PCR2 master mix; PCR2 amplicon pooling). We decontaminated all surfaces and tools in the pre- and post-PCR hoods, including pipettes, as described above. We used separate reagents, pipettes, aerosol-resistant pipette tips and consumables for pre- and post-PCR work. We pooled controls from PCR1 and used them as a template for PCR2 to ensure that no contamination occurred during pooling or PCR2 set-up. Although we detected no contamination, we combined 5 µl of each PCR negative control and extraction blank in a separate pool and subjected them to the same pre-sequencing treatment as positive amplicon pools. We sequenced these negative controls in parallel with our samples.

To qualify our inferences from the MiSeq analyses, we simultaneously sequenced two mock communities consisting of fungal DNA (electronic supplementary material, table S2): one with even concentrations across all species and one with tiered concentrations. The mock community consisted of total genomic DNA extracted directly from 31 phylogenetically diverse fungal taxa spanning the major fungal phyla (electronic supplementary material, table S2). Communities were created by pooling 10 µl of each isolate at the concentrations shown in the electronic supplementary material, table S2. We amplified 2 µl of each mock community in triplicate for PCR1 via the protocol mentioned above. Products from PCR1 were pooled and diluted (1 : 10) with molecular grade water and 1 µl was used as the DNA template for PCR2 (see above for PCR2 parameters). For sequencing at IBEST, 20 ng of the even and tiered mock communities was pooled together with the rest of the samples. Mock community sequences were assessed for quality (as below) before evaluation to assess the presence of primer- or sequencing bias.

MiSeq reads were demultiplexed at the IBEST Genomics Core using standard protocols. Forward reads had overall higher quality than the reverse reads, so we used only forward reads in our analyses [78]. Forward reads were assessed for quality via two methods [72]: (i) FastQC [79] followed by aggregation of reports using Multi-QC [80] to assess the quality of the MiSeq data; and (ii) the -fastq_eestats2 command in USEARCH v.10 [81] using different expected error filters (-ee_cutoffs 0.25,0.5,1.0) and length thresholds (-length_cut-offs 150,30,10) to assess the number of reads that would pass these parameters. Based on these assessments, we selected a length and maxEE cut-off that would result in a high-quality reads per sample while simultaneously maintaining maximum read length. Using the command -fastq_filter in USEARCH [81], we filtered and trimmed fastq files at a maximum expected error of 1.0 and length of 200 bp.

After quality assessment, sequences were dereplicated with the command -fastx_uniques (parameters -sizout). We used the commands -unoise3 (parameters -zotus -minsize 1) and -cluster_smallmem (parameters -id = 0.95 -relabel Otu -centroids) in USEARCH v.10, UCHIME, and UNOISE2 [81–83], to check for chimaeras and cluster sequences into operational taxonomic units (OTUs; sequences corresponding to taxonomic clades or monophyletic groups) at 95% sequence similarity, consistent with previous work on endophytic Ascomycota [44,45,84]. Sample-by-species matrices were created using the USEARCH command -otutab [81]. Singleton OTUs were removed prior to data analysis.

(iii) Data analyses

We assembled the full dataset from culturing, cloning and MiSeq (nonsingletons only) into OTUs at 95% sequence similarity. Increasing the threshold of sequence similarity of OTUs to greater stringency at 97% or 99% did not change our primary conclusions (data not shown). As MiSeq reads are shorter than those produced from Sanger sequencing, we used ITSx [85] to extract the ITS1 region from sequences obtained from culturing and cloning prior to clustering into OTUs. Representative sequence data from each OTU were compared against GenBank via BLAST (http://ncbi.nih.gov) and evaluated for phylogenetic placement in T-BAS [86] (electronic supplementary material, table S3). Any OTU matching a plant rather than a fungus was removed from the analysis. We removed all OTUs that were represented by fewer than 10 reads in the MiSeq dataset and did not appear in the culture-based or cloning datasets. We also removed all OTUs that were observed in controls at a frequency greater than or equal to 10% of the number of reads observed from herbarium samples (electronic supplementary material, table S3). The final dataset as a whole comprised 114 OTUs, including one sequenced isolate obtained in culture, 64 sequences from cloning, and 198 471 sequences from MiSeq, of which only 63 reads (0.03%) were from negative controls. We compared endophyte communities from each host and processing method via analyses of similarity (ANOSIM) based on the Morisita Index, with visualization by non-metric multidimensional scaling and unconstrained UPGMA clustering in PAST (https://folk.uio.no/ohammer/past). Sequence data obtained from the three pathways (culturing, cloning and MiSeq) were deposited in GenBank (Sanger sequencing: Banklt2155927, MK034363–MK034427, and Illumina MiSeq: SAMN10218193–SAMN10218202).

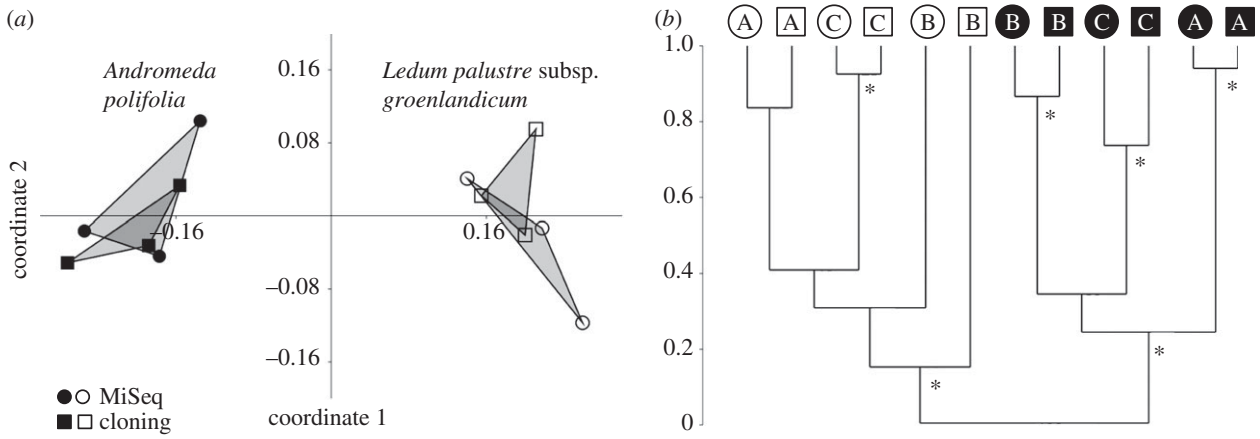

Figure 2. Comparison of fungal communities in herbarium specimens of *Andromeda polifolia* and *Ledum palustre* subsp. *groenlandicum* as inferred by culture-independent methods (cloning and next-generation amplicon sequencing via the Illumina MiSeq platform). (*a*) Non-metric multidimensional scaling shows that communities of fungi differed between hosts but less so as a function of method (cloning versus MiSeq). ANOSIM $p < 0.0001$, driven by differences between host species. Stress less than 0.10. (*b*) Pairwise UPGMA clustering analysis shows that communities of fungi are separated more by host species than by method or sterilization approach (A–C). Circles, MiSeq; squares, cloning; open symbols, *Andromeda;* closed symbols, *Ledum.* Asterisks indicate bootstrap values greater than or equal to 70 based on 1000 replicates.

3. Results

(a) Culture-based approach

From 1920 leaf segments plated on 2% MEA, we obtained one isolate in culture (electronic supplementary material, table S3). It was obtained from a leaf of *A. polifolia* that was treated with the least-stringent sterilization method (method C; immersed and agitated for 30 s in each sterilant). The isolate is a member of the Pezizomycetes, a common lineage among endophytes of boreal plants [44]. Its top BLAST match is to an endolichenic fungus associated with freshly collected lichen in the boreal zone (electronic supplementary material, table S3).

Although the culture-based method yielded only one fungal isolate, this approach was insightful in that we observed no surface-contaminating fungi in culture. This suggests that even the least-stringent sterilization method (method C) was effective in removing surface contaminants and that the pools of samples sterilized by this and method B might be considered to consist of endophytic fungi. However, even if the leaf surface did not contain viable fungi after the less-stringent sterilization treatments, it is possible that remnant DNA could persist and be amplified via cloning or MiSeq analyses. Therefore, we considered the sterilization method in subsequent analyses, with the expectation that those treated with the most stringent method (method A) are probably endophytic fungi, and those treated with the intermediate and least-stringent methods (B and C) probably include endophytic fungi, in part, validated by the taxonomic distribution of the fungi obtained in our culture-independent approaches (see below and the electronic supplementary material, table S3).

(b) Culture-independent approaches

Culture-independent approaches were more successful than culturing in identifying an endophytic mycota within herbarium samples. We observed 3–5 OTUs (mean = 4.0) among the 9–12 clones (mean = 10.7) examined in each species/sterilization treatment combination (electronic supplementary material, table S3). We observed 25–58 OTUs (mean = 35.5) among the ca. 17 400–56 900 MiSeq reads examined per species/sterilization treatment (electronic supplementary material, table S3). From cloning, we obtained eight OTUs from *A. polifolia*, and seven OTUs from *L. palustre* subsp. *groenlandicum*, all of which were observed in the MiSeq dataset (electronic supplementary material, table S3). In general, the MiSeq dataset included approximately four times the species richness as the cloning dataset (electronic supplementary material, table S3).

The number of reads obtained via MiSeq from the most stringently sterilized tissues (method A: 48 241 reads from *A. polifolia*; 20 377 from *L. palustre* subsp. *groenlandicum*) fell within the range observed from less stringently sterilized samples of each host species (methods B and C; electronic supplementary material, table S3). Reads obtained from leaf tissue treated with method A represented 38.4% of the total reads from *A. polifolia*, and 27.7% of those from *L. palustre* subsp. *groenlandicum*. In both species, the OTUs found in tissues treated with method A were similar to those in tissues treated with less-stringent sterilization methods. Among the 114 OTUs found in the entire analysis, 22 were found only in tissues treated with method A; 26 were found in tissues treated with B or A and B; and the remainder were found in tissues treated with C, C and B, C and A, or A, B and C (electronic supplementary material, table S3). Together these results suggest that leaf samples treated with less-stringent sterilization methods were not covered in dead cells or DNA from epiphytic fungi or other contaminants.

When the OTU composition of each sample was compared via ANOSIM, communities of fungi were separated more strikingly by host species (*A. polifolia* versus *L. palustre* subsp. *groenlandicum*) than by the sterilization method (A, B or C) or the analysis method (cloning versus MiSeq) (figure 2). If the contamination of superficial fungi from a shared herbarium environment, laboratory artefacts or other aspects of a shared history after collection were an issue, we would expect the less stringently sterilized samples to group together regardless of host species, but this was not observed (figure 2).

The phylogenetic distribution of OTUs is shown in figure 3, with their taxonomic placement from T-BAS and top BLAST matches listed in the electronic supplementary material, table S3. Ascomycota were particularly common,

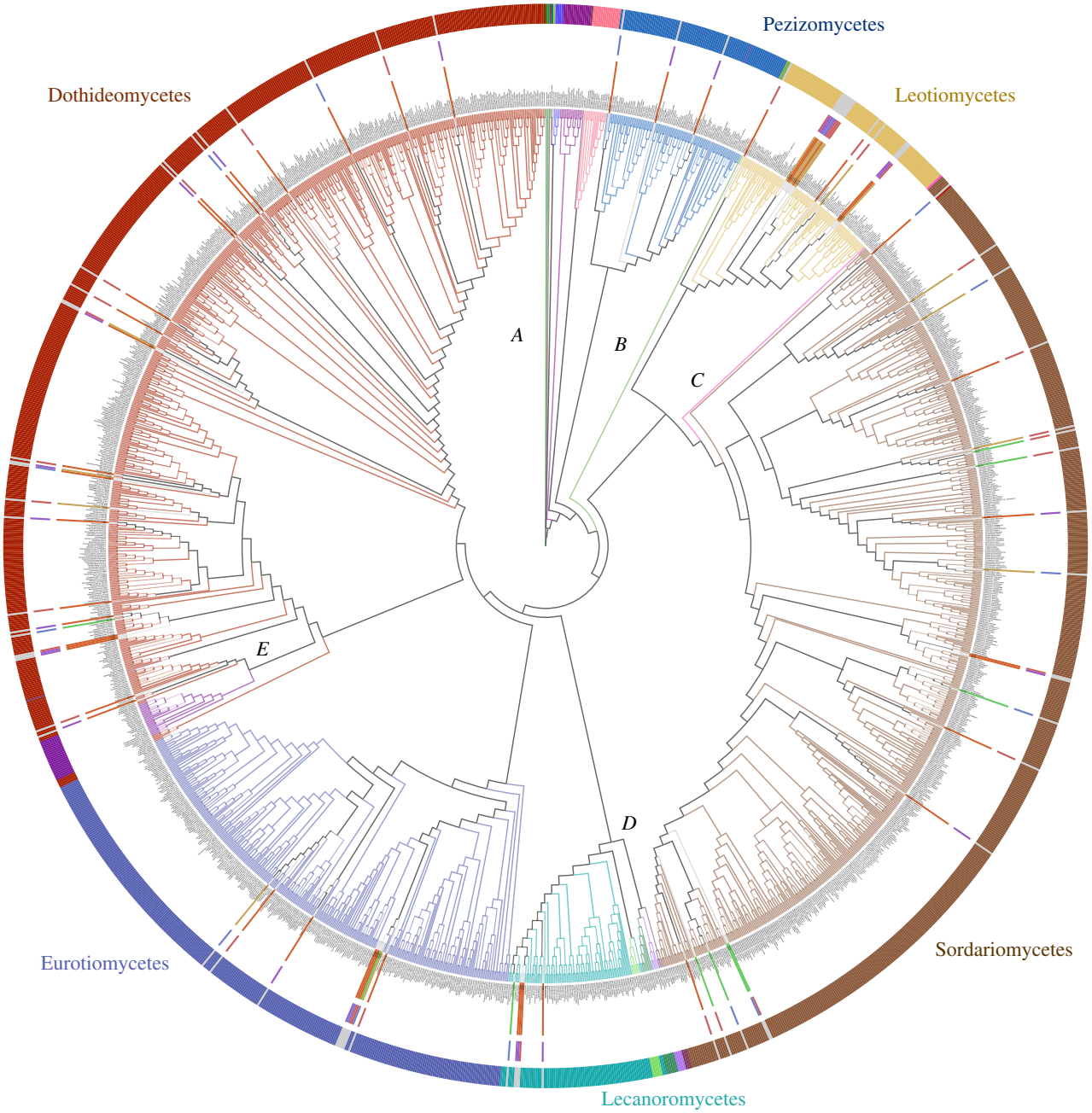

Figure 3. Phylogenetic diversity of Ascomycota observed by culturing, cloning and MiSeq analyses of herbarium specimens of *Andromeda polifolia* and *Ledum palustre* subsp. *groenlandicum,* as inferred in T-BAS [86]. Branches are coloured by class; classes in which fungi were most frequently observed in the present study are labelled in colours that match the branches and outermost ring (Pezizomycetes, Leotiomycetes, Sordariomycetes, Lecanoromycetes, Eurotiomycetes, Dothideomycetes). The middle ring indicates the host range observed for each OTU (observed in *Andromeda,* blue; *Ledum,* red; both species, purple; electronic supplementary material, table S3). The inner ring indicates the sterilization method(s) by which each OTU was observed (method A only, green; method B, or A and B, tan; method C, A and C, B and C, or A, B and C, ochre; electronic supplementary material, table S3). Clades or groups of taxa that were not observed, or were rarely observed in this study are marked with letters: A, outgroups, Schizosaccharomycetes, Taphrinomycetes, Saccharomycetes and Orbiliomycetes. B, Geoglossomycetes. C, Laboulbeniomycetes. D, Lichinomycetes, Coniocybomycetes, Xylonomycetes. E, Arthoniomycetes. T-BAS tentatively placed some strains in the Arthoniomycetes and Xylonomycetes (electronic supplementary material, table S3), but with low confidence and conflicting placement in the Lecanoromycetes, such that they are not depicted here.

accounting for ca. 85 of the 94 OTUs that could be placed to phylum by the union of T-BAS and BLAST results (electronic supplementary material, table S3). We also observed sequence data consistent with Basidiomycota (eight OTUs) and Chytridiomycota (one OTU) (figure 3; electronic supplementary material, table S3). We were successful in recovering sequence data for all strains used in the mock communities, suggesting that the suite of methods used in the MiSeq analysis was appropriate for capturing a phylogenetic breadth of fungi (electronic supplementary material, table S2).

Within the Ascomycota, the classes Pezizomycetes, Leotiomycetes, Lecanoromycetes, Eurotiomycetes and Dothideomycetes were particularly common (figure 3; electronic supplementary material, table S3). One OTU was placed in the Xylonomycetes (obtained from leaves treated with method B, but with conflicting matches in T-BAS to representative Lecanoromycetes) and two were placed in the Arthoniomycetes (both obtained only from leaves treated with method C, and placed with low confidence). Overall these results echo previous studies of endophytes of fresh tissue from the boreal zone and

similar ecosystems, which highlight the high phylogenetic richness of Pezizomycotina in the phyllosphere (see [44]). At least 24 orders and 38 families were predicted by T-BAS to be present among the Ascomycota observed here, with approximate genus- and species matches listed in the electronic supplementary material, table S3.

Each of the commonly represented classes except Pezizomycetes contained fungi observed only in tissues treated with method A (figure 3; electronic supplementary material, table S3). Each clade has been reported to contain endophytes previously (but see section Discussion for an evaluation of the Lecanoromycetes). The broad distribution of classes observed here is consistent with previous studies of angiosperms freshly collected in the boreal biome with respect to the high phylogenetic diversity of Ascomycota as a whole, and the especially high frequency of Dothideomycetes, Sordariomycetes, Leotiomycetes, Eurotiomycetes and Pezizomycetes (figure 3).

4. Discussion

(a) Perspectives on herbarium specimens for studies of endophytic fungi

Natural history collections are increasingly appreciated for their rich potential to complement studies centring on systematics and taxonomy. These include studies of climate change relevant to plant species distributions, phenology and functional traits [10,15–18]. Although herbarium specimens were not initially collected for these new uses and often present challenges with respect to sampling biases in geographical, taxonomic, temporal and phylogenetic dimensions [87,88], the relevance of herbarium specimens is growing as habitat alteration, climate shifts and other human impacts change the natural world. Such relevance also grows concomitantly with technological advances that allow herbarium specimens to be used in new ways. For example, large efforts to digitize and mobilize collections allow researchers to ask novel, emergent questions at large scales (e.g. with respect to biogeography or regional patterns of phenology [89]). In parallel, new technologies have increased the power of herbarium specimens with respect to accessing their DNA content, which can be used to address enquiries in evolutionary biology, genomics, and increasingly, microbial ecology [89,90].

Here, we report a proof-of-concept approach to explore the diversity of endophytes from plant specimens preserved in the manner of a typical herbarium. We show that culturing was not effective in capturing a diversity of endophytes, but that cloning and especially next-generation amplicon sequencing can reveal biodiversity of endophytic fungi within preserved plant tissues. Through the use of careful controls and comparison with the literature, we anticipate that the majority of the fungi observed here through their molecular signatures are endophytic fungi that were preserved in the form of DNA in the process of archiving plant material in a herbarium collection. If broadly applicable, such an approach could shed light on a hidden dimension of biodiversity currently housed in herbaria, while also setting the stage for questions regarding the historical biogeography and evolutionary history of plant-associated microbes.

Overall, the prevalence of fungi from the most stringent surface-sterilization method in clades such as the Leotiomycetes, Sordariomycetes, Eurotiomycetes and Dothideomycetes is consistent with that expected for boreal angiosperms [44]. We anticipate that OTUs which were present in leaves treated with method A, and also observed in less stringently sterilized samples, might also include endophytes in these clades and in the Pezizomycetes [44,45]: these taxa are rare as surface contaminants compared to the prolifically conidiating fungi in other lineages that often occur incidentally in herbaria, laboratories and other built environments (electronic supplementary material, table S3).

We focused on material that was dried and maintained in a herbarium collection following standard methods. In general, next-generation studies of freshly collected material rely on either processing material immediately upon collection or preservation via drying in silica gel or archiving in buffers or ethanol [45]. Our results suggest that the standard drying protocols used by herbarium-based collectors may be amenable to downstream microbial ecology studies, albeit with important caveats pending further work (see Biases and future directions).

Our study focused on leaves, highlighting a resource common in herbarium specimens. As herbarium specimens often lack roots, questions that might be addressed in terms of rhizosphere ecology may be somewhat limited. However, the presence of roots in specimens of many grasses, for example, raises the possibility for such future work with herbarium specimens. We collected entire leaves from herbarium specimens for the work described here, but small fragments—such as those that break from dried specimens with time or handling—could be used instead. Thus, the potential to perform this work without destructively sampling or actively damaging specimens is promising.

(b) Biases and future directions

Although we demonstrated a positive proof of concept in capturing evidence of the endophytic fungi in dried herbarium specimens, biases and uncertainties in our study should be explored before wide application of this approach. These concerns fall into three broad categories.

First, to study endophyte biodiversity in the historical framework of herbarium specimens requires careful attention to the nature of herbarium specimens. For example, choices made in the field to select particular material may not be documented with specimens, yet could impact perspectives on endophytes at various taxonomic and geographical scales (e.g. collections along forest edges versus interiors, or forest canopies versus understories, could correspond to different endophyte communities; similarly, collection of younger foliage could result in leaves with fewer endophytes than more mature leaves). Preservation methods for individual herbarium specimens also may be important: whether leaves were dried at high temperatures, quickly or slowly, or immediately after collection could impact DNA quality or the capacity of epiphytic fungi to colonize leaf interiors. Such issues could lead to spurious results regarding endophyte diversity in the broad sense, compounded by the multidimensional biases that herbarium collections represent and the fact that the original collections were intended for purposes other than those described here [87,88].

Second, our proof-of-concept work focused on only two species in one family, only on boreal plants, and only material that had been archived for four years. Further analyses are needed to determine whether the methods described here

will be successful for studying endophyte communities in older specimens, or in specimens from other plant lineages and biomes. We currently are exploring such methods and anticipate that for the oldest and most recalcitrant cases, 'ancient DNA' techniques may be needed to obtain high-quality data (see [65]). In that case, as in the present study, we emphasize the need for highly sterile work environments and careful attention to potential contamination, which could be especially important in the handling of dried plant material with only small quantities of fragmented fungal DNA. The temporal limitation of our study could be assessed by sampling across a time series of collections, with the first approaches perhaps benefitting most from focusing on specimens of a given host taxon from a focal region, collected and preserved with consistent methods over a long timeframe of collections. We have initiated such a study with a focus on ericaceous hosts in the northeastern USA, with results pending. In turn, limitations associated with biotic zones can be alleviated by comparing multiple specimens of the same species across broad geographical and environmental gradients. Finally, surveys of diverse taxonomic groups of plants collected contemporaneously would help inform the limits on studying endophyte communities given issues such as leaf chemistry, which impacts the efficacy of DNA extraction and PCR in many cases.

Third, a challenge with our study is that we do not have a 'positive control' in the sense of freshly collected leaf material with which to compare endophyte diversity. This is a challenging issue to overcome, as typically herbarium specimens will represent ecological and temporal contexts not exactly the same for freshly collected material. Similarly, we have limited inference with regard to the abundance of particular endophyte taxa, and thus the degree to which our analyses represent true endophyte diversity and community composition will require scrutiny. One useful approach would be to use probability-of-detection analyses on many subsets of the same accessions, allowing us to address statistically the probability of detecting a given endophyte if it is there. We also advocate the use of carefully constructed mock communities that, with known quantities of DNA for each endophyte strain, can help determine the validity of read abundance as a proxy for endophyte abundance (electronic supplementary material, table S2 for this study, and for an example of such an approach in a different study system, see Taylor *et al*. [91]).

(c) Conclusion

If broadly applicable and if tempered well by controls, the methods described here have the potential to unlock an exciting historical resource—that is, the holdings of endophytic fungi in herbaria worldwide. As we continue to test and improve these methods, we hope to scale up to questions that focus on those relevant to understanding major shifts at a global scale due to the human-driven changes that frame the Anthropocene. What are the historical associations of endophytes and plants? To what extent can each inform biogeographical and evolutionary questions of the other? How have patterns of diversity changed over time? What are the historic and modern ranges of endophytic fungi, and what forces—anthropogenic and otherwise—define their distributions and relationships with plants? By addressing these questions, we can contextualize plant and microbial ecology in a historic framework, inferring past shifts as a basis for predicting future changes in these diverse partners and the important symbioses they comprise.

Data accessibility. This article has no additional data.

Competing interests. We declare we have no competing interests.

Funding. We received no funding for this study.

Acknowledgements. We thank Jason M. Karakehian for plant samples, and Ashton Leo, Anyangatia Ndobegang, Ming-min Lee and Nathaniel Yang for laboratory assistance. Molecular analyses were supported by AEA and the Robert L. Gilbertson Mycological Herbarium at The University of Arizona (UA). We thank the UA College of Agriculture and Life Sciences, School of Plant Sciences, and the ARCS Foundation for supporting E.A.B. B.H.D. thanks Texas A&M University-Corpus Christi for logistic support. We thank three anonymous reviewers for comments that greatly improved this manuscript.

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