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Methodological Approaches Frame Insights into Endophyte Richness and Community Composition

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Abstract

Isolating microbes is vital to study microbiomes, but insights into microbial diversity and ecology can be constrained by recalcitrant or unculturable strains. Culture-free methods (e.g., next-generation sequencing, NGS) have become popular in part because they detect greater richness than culturing alone. Both approaches are used widely to characterize microfungi within healthy leaves (foliar endophytes), but methodological differences among studies can constrain large-scale insights into endophyte ecology. We examined endophytes in a temperate plant community to quantify how certain methodological factors, such as the choice of cultivation media for culturing and storage period after leaf collection, affect inferences regarding endophyte communities; how such effects vary among plant taxa; and how complementary culturing and NGS can be when subsets of the same plant tissue are used for each. We found that endophyte richness and composition from culturing were consistent across five media types. Insights from culturing and NGS were largely robust to differences in storage period (1, 5, and 10 days). Although endophyte richness, composition, and taxonomic diversity identified via culturing vs. NGS differed markedly, both methods revealed host-structured communities. Studies differing only in cultivation media or storage period thus can be compared to estimate endophyte richness, composition, and turnover at scales larger than those of individual studies alone. Our data show that it is likely more important to sample more host species, rather than sampling fewer species more intensively, to quantify endophyte diversity in given locations, with the richest insights into endophyte ecology emerging when culturing and NGS are paired.

Keywords Ascomycota · Diversity · Illumina · Media · Metabarcoding · Magnoliophyta · Pinophyta · Pteridophyta

Introduction

Isolating microbes on culture media is a traditional and informative technique to document richness of microbial communities and to identify their constituent species [1–6]. However, the expansion of molecular methods, especially over recent decades, has illuminated the challenges posed by recalcitrant microbes [7–9]: the great majority of microorganisms have not been isolated in culture due to specific affiliations with their hosts or growth requirements that can only be provided by their natural substrates, environmental conditions, or synergy with co-occurring microbes [10–13]. Following the development of diverse culture-free approaches such as amplicon cloning [7], high throughput, next-generation amplicon sequencing approaches in amplicon metabarcoding (hereafter NGS, such as that implemented on the Illumina platform) are considered a potential solution to capturing more complete snapshots of microbial diversity [14–16]. By amplifying DNA from targeted microbes preferentially and bypassing the culturing step, NGS often captures greater richness than culturing alone, even when the same amounts of tissue, or subsets of the same tissue, are used for each approach [17–20].

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In recent years, NGS as implemented via Illumina MiSeq and other platforms has provided unprecedented insight into the ecological and geographic distributions of microbes in diverse settings [21–27]. The relative efficiency of the NGS approach, both financially and in terms of effort, makes it appealing: cost and sequencing depth per sample are vastly improved over culturing alone. Additionally, with NGS data, researchers can adjust their culturing conditions to capture recalcitrant strains. However, the few cases in which both culture-based and NGS approaches have been applied to the same source material have indicated that, like other culture-free methods (e.g., [28]), NGS may not detect some species that are observed via culturing, in part due to primer bias or sequencing bias [17, 18, 29–32]. Thus culture-based methods can complement and extend insights gained from NGS by revealing the presence of microbes that may not be detected by NGS [11] as well as providing strains for diverse analyses of secondary metabolites, genomes and transcriptomes, functional traits, multi-locus datasets for phylogenetic and taxonomic studies, or laboratory experiments.

As such, culturing and NGS approaches can be complementary, but only rarely have they been applied together in ecological studies (see [33, 34]). Moreover, most studies that isolate microbes in culture use only a single culturing medium, leaving uncertainty about the capacity to recover communities more completely by using multiple types of media (but see [17]). How samples are preserved prior to DNA extraction may influence inferences from NGS [18], but the relevance of factors such as the time that elapses between collection and DNA extraction is not clear. Finally, there is uncertainty given the pools of taxa that might be observed with NGS, culturing in general, or culturing only on standard media [17]: if each method captures different taxa, then are ecological inferences based on any single method or approach broadly extendable to the focal microbial community as a whole?

We addressed these questions as they apply to the fungi that inhabit the interior of living leaves: foliar fungal endophytes (class 3 endophytes, sensu [35]; hereafter endophytes). Endophytes are microfungi that occur in healthy plant leaves without causing any symptoms of disease [35]. They exhibit high species richness and phylogenetic diversity at local to global scales and are known from all lineages of plants examined thus far [35]. Although their ecological roles are often not known, it is clear that some endophytes can protect host plants from pathogens, alter photosynthetic efficiency, and produce various secondary metabolites that are sometimes beneficial for medicinal purposes [36–38].

Due to such benefits and their associated impacts on natural and human-made ecosystems, endophytes have been studied over the past century with a strong tradition of culturing from surface-sterilized, healthy leaves (Supplementary Table 1). A review of the representative literature indicates that most culture-based studies have used only one cultivation medium (e.g., malt extract agar (MEA) or potato dextrose agar (PDA); Table 1, Supplementary Table 1). On these nutrient-rich media, fast-growing endophytes may grow preferentially, which could bias inferences regarding richness and endophyte community structure [11]. Although most studies focus on fresh leaves, the time between leaf collection and tissue processing (i.e., storage period) also varies across studies (Supplementary Table 1). When leaves are detached from plants, plant defenses are less responsive and gene expression profiles of leaves rapidly shift to signals relevant to senescence [45–47]. Many endophytes reproduce from senesced leaves [48–50] are closely related to pathogens and saprotrophs, or are thought to have pathogenic or saprotrophic life stages [31, 47, 51], such that distinctive subsets of the endophyte community may grow especially rapidly after leaves are collected, and thus could dominate the perceived community if leaves are not processed immediately. For these reasons, it is unclear

Table 1 Summary of studies that have considered the effect of culture media and storage periods for ecological studies of foliar endophytes

Media	Storage period	Host plant	Major findings	Reference
(a) Culture media				
MEA, CMA	< 36 h	Four desert plant species	No difference in isolation frequency, diversity, or composition	[39]
PDA, V8	N/A	*Gossypium hirsutum*	No difference in isolation frequency	[40]
PDA, ISP2, ISP3	Immediately	*Hevea brasiliensis*	No difference in isolation frequency	[41]
WA, PDA	N/A	*Vitis vinifera*	PDA yielded more endophyte colonies per plant tissue than WA *[1]	[42]
(b) Storage period				
MEA	6 h vs. 24 h	*Leptospermum scoparium*	No difference in overall infection rate. Infection rate of *Phyllosticta* sp. decreased at 24 h	[43]
PDA	< 7 days	*Nothofagus menziesii*	Up to 7 days showed no significant differences in fungal assemblages*[2]	[44]

References correspond to Supplementary Table 1, which contains the full list of papers considered for our literature review. *MEA*, Malt extract agar; *CMA*, Cornmeal agar; *PDA*, Potato dextrose agar; *V8*, cultivation medium based on V8 brand vegetable juice. *ISP*, International Streptomyces Project Medium; *WA*, water agar. *1 No data were shown. *2 Unpublished data

whether studies differing only in cultivation media or storage period thus can be compared to estimate endophyte richness, composition, and turnover at scales larger than those of individual studies alone.

Here we examine how cultivation media and storage time after leaf collection can influence inferences of endophyte abundance, richness, and composition in culture-based studies, and whether such effects differ among different plant hosts. We used foliage of representative species that co-occur in a temperate plant community, including a fern, a conifer, and an angiosperm; considered five different culture media with various carbon sources; and used leaf tissues stored for 1, 5, and 10 days after collection. We then examined how storage time influences inferences from NGS, using an amplicon metabarcoding approach on the Illumina platform with robust positive and negative controls. Finally, we compared insights from culturing and NGS for subsets of the same plant tissue.

Materials and Methods

We first performed a literature review to evaluate the frequency with which individual studies of foliar endophytes have compared insights from different culture media; to determine the degree to which storage period is considered in individual studies and differs among studies; and to identify individual studies that have used both culturing and NGS-based, culture-free approaches. In sum, we examined 73 studies. We obtained the papers via Web of Science and Google Scholar with keywords "fungi," "endophyte," and "culture" or "media (or medium)." We excluded papers that focused on non-fungal endophytes, fungal endophytes of tissues other than leaves, and those that did not describe culturing conditions. If the same data were used in multiple papers, we retained only one paper in the set. We focused only on papers that included a culture-based approach. For each paper, we recorded the host plant species, culture media, storage period, and if the paper used a NGS approach (Table 1, Supplementary Table 1).

Sampling

We established three 4 × 5-m plots in the native forest sector of Sarah P. Duke Botanical Gardens (Durham, NC, USA) in October 2017. The forest sector of the garden consists of a mature canopy of native loblolly pine (*Pinus taeda*) and an understory of native woody and herbaceous plants. The garden is located within 1.2 km of the edge of the > 2800 ha Duke Forest, which in turn is adjacent to the largely rural and wooded landscape to the immediate west, north, and east of Durham. We characterized environmental and vegetation characteristics in each plot following methods described in ref. [52] (Supplementary Table 2).

In each plot, we collected mature and apparently healthy foliage of plants representing three major plant lineages. We focused on *Thelypteris kunthii*, which grew naturally intermixed with *Dryopteris* sp.; respectively, Thelypteridaceae and Dryopteridaceae, Polypodiopsida), *Juniperus virginiana* (eastern redcedar: Cupressaceae, Pinopsida), and *Magnolia grandiflora* (southern magnolia, Magnoliaceae, Magnoliid) (Supplementary Table 2).

Tissue Preparation

We stored leaves in plastic bags at 4 °C immediately after collection. At each of three processing points (day 1, day 5, and day 10 after collection), we haphazardly selected sets of 2–3 leaves from each collection. We washed each set of leaves under running tap water to remove surface debris. We cut each set of leaves into at least 576 segments (each 1 × 2 mm) and surface-sterilized them by agitating them in 95% ethanol (10 s), 0.5% NaOCl (2 min), and 70% ethanol (2 min) [53]. Samples from the ferns represented both species in a roughly equal quantity. From each set of 576 segments, we selected 96 segments haphazardly for culturing on each of five media and the same quantity for NGS (below; see also Supplementary Fig. 1).

Culture-Based Approach

We used 96 surface-sterilized segments from each plant collection at each time point to establish cultures on each of five solid media [53]. We placed each segment into an individual sterile 1.5-mL tube containing 500 μL of 2% malt extract agar (MEA), 2% potato dextrose agar (PDA), molasses yeast agar (MYA) [54, 55], cellulose agar (CCA: carboxymethyl cellulose 5 g and agar 20 g) [50, 56], or Sabouraud's medium (SDA: glucose 40 g, peptone 10 g and agar 20 g) [57]. In sum, we prepared 2592 segments on each of five media (3 plots × 3 species × 3 storage times × 96 segments), for a total of 12,960 segments.

We incubated the culture tubes at room temperature with ca. 12 h light/dark cycles for 6 months. Each emergent isolate was subcultured onto 2% MEA. Subcultures were vouchered in sterile water and deposited as part of the living collection of endophytes at the Robert L. Gilbertson Mycological Herbarium at University of Arizona (culture accessions SO05500-SO10912, Supplementary Table 3). We calculated isolation frequency as the number of isolates obtained from each set of 96 leaf segments, expressed as a percent.

We used restriction fragment length polymorphism (RFLP) analysis to select representative strains for sequencing [31, 58]. Briefly, we grouped isolates into morphotypes based on

observable phenotypic traits on 2% MEA (i.e., colony color from above and below; growth rate; colony edge characteristics; coloration of the growth medium; density and characteristics of aerial hyphae) [59]. We used the RedExtract-N-Amp plant PCR kit (Sigma-Aldrich, St. Louis, Missouri, USA) to extract total genomic DNA from each isolate. We used primers ITS1F and LR3 [49, 60, 61] in PCR to amplify the nuclear ribosomal internal transcribed spacers (ITS) and 5.8S region (together, ITS rDNA) and an adjacent portion of the nuclear ribosomal large subunit (LSU rDNA). If amplification failed, we used primers ITS5 and ITS4 to amplify the ITS rDNA only [49, 60, 61]. PCR conditions followed methods described in [49]. PCR products were visualized by staining with SYBR Green I (Molecular Probes, Invitrogen; Carlsbad, CA, USA) after electrophoresis on a 1% agarose gel. We digested positive products with restriction enzyme MSPI (Thermo Scientific, ER0541, CCGG 5'-CG) and compared RFLP-based groups to morphotypes. MSPI was selected based on 22 randomly chosen cultures for which distinct RFLP patterns corresponded to distinct operational taxonomic units (OTUs) based on 95% sequence similarity [18, 49, 62, 63]. We selected up to 10 isolates from each representative RFLP-informed morphotype group for sequencing. We cleaned positive PCR products with ExoSAP-IT (Affymetrix, Santa Clara, California, USA) and sequenced them bidirectionally with primers ITS1F and LR3 or ITS5 and ITS4 on the Sanger platform at the University of Arizona Genetics Core.

We used *phred* and *phrap* [64, 65] to call bases and assemble sequences into contigs in Mesquite [66]. We manually checked the quality and edited each sequence with Sequencher v.5.1 (Gene Codes Corp., Ann Arbor, MI, USA). We clustered edited sequences into OTUs at 95% sequence similarity in Tree-Based Alignment Selector (T-BAS) v.2.1 [62, 67] and evaluated the relationship of RFLP-based groups to OTUs. We sequenced all isolates in RFLP groups that corresponded to more than one OTU. We successfully obtained sequences or used RFLP patterns to link isolates to OTUs for 4723 of 4934 isolates (95.7%) (Supplementary Table 3), which we analyzed as the culture-based data set. These represented 186 OTUs, of which 112 were found more than once (i.e., non-singletons).

NGS Approach

We partitioned each set of 96 surface-sterilized segments from each plant collection at each time point into four subsets of 24 segments each. Each subset was stored in 1 mL of sterile CTAB [18] in a sterile 2-mL tube (Supplementary Fig. 1). In total, we prepared 2592 segments for NGS (3 plots × 3 species × 3 storage times × 96 leaf fragments). We stored the tubes at − 80 °C until DNA was extracted.

We extracted total genomic DNA from each subset as described in [68]. Briefly, we drained CTAB from each tube and then homogenized the segments with pre-sterilized stainless steel beads for 45 s via bead-beating in a FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals, Solon, OH, USA). We extracted total genomic DNA with the Qiagen PowerPlant Pro-htp 96 Well kit (Qiagen, Valencia, CA, USA). We pooled the four DNA extractions for each individual before amplification (i.e., four subsets of 24 segments each; Supplementary Fig. 1).

We used a two-step PCR approach to prepare samples for NGS as described in [69]. Briefly, we amplified fungal ITS rDNA with primers ITS1F and ITS4 that were modified with universal consensus sequences CS1 and CS2 and 0–5 bp phase-shifting (Integrated DNA Technologies Inc., Skokie, IL, USA). Each sample was amplified in three parallel reactions that each contained 1–2 μL of DNA template. After visualizing these PCR products with SYBR Green 1 (Invitrogen, Carlsbad, CA, USA) on a 2% agarose gel, we pooled the PCR products per individual and diluted each amplicon to a similar concentration based on the band intensity. We used the diluted PCR products as template for a second PCR with barcoded adapters (IBEST Genomics Resource Core, Moscow, ID, USA). For details of the workflow, see Supplementary Fig. 1.

To limit contamination, we used sterile equipment and separate pipettes for DNA extraction and PCR. We used a sterile, dedicated PCR hood and always segregated PCR products from pre-PCR materials. We sequenced the pooled negative controls from DNA extractions with kit reagents and from PCR1 with sterile water to ensure no contamination prior to PCR2. We used them as a template for PCR2 and prepared 5 μL of each negative control as above for sequencing even though no visible bands were observed via gel electrophoresis.

The University of Idaho IBEST Genomics Resources Core provided Illumina MiSeq 300-bp paired-end sequencing and demultiplexing of raw reads. We analyzed reverse reads (ITS2) rather than ITS1 in analyses because of high variation in length of ITS1 ([70], see also [32]). We used FastQC [71] and USEARCH v10 [72] for post-sequencing quality control with criteria described in [52]. Briefly, we trimmed reads to a length of 200 bp and filtered them at a maximum expected error of 0.5. Our NGS data set consisted of 476,392 reads after quality control, which represented 258 OTUs when analyzed separately from the culture-based data set (below).

Mock Communities for NGS

To evaluate the quality of our NGS data set, we also processed and sequenced two mock communities using methods described above. Each mock community consisted of PCR product generated from DNA extractions of 31 phylogenetically

diverse fungi that represent the lineages typically observed as endophytes [52, 73] (Supplementary Table 4). One mock community (equimolar) consisted of equimolar concentrations of DNA from all 31 fungal taxa, which were used for the two-step PCR process (above). The second mock community (tiered) contained the same fungal taxa but in tiered DNA concentrations ranging from 0.94 to 23.6 ng/μL based on the order of abundant phyla as endophytes ("tiered" mock) [73] (Supplementary Table 4). We sequenced each mock community five times (i.e., as five replicates). Read abundance in tiered mock communities was associated positively with the expected read number (with replicate as a random factor: $R^2 = 0.35$, $P = 0.001$, Supplementary Table 4, Supplementary Fig. 2). Therefore we used read abundance as a meaningful proxy of biological abundance (see also [32, 74]).

Bioinformatics

We used ITSx [75] to extract the ITS2 nrDNA region from Sanger sequences from cultures. For NGS reads, we removed 40 bases downstream of the conserved region at the start of LSU rDNA and trimmed the 5′ end of NGS reads to 160 bp for comparison. We concatenated sequences from culturing and NGS at this step, dereplicated the reads with the command –fastx_uniques, and removed chimeras and reads with sequencing errors via the commands –unoise3 (parameters –zotus –minsize 1) in USEARCH v.10 [72, 73, 76]. We clustered dereplicated sequences into OTUs at 95% sequence similarity [18, 62, 63]. We performed taxonomic assignment for each OTU with the SINTAX algorithm [77] based on the UNITE database (version 7.2, [78] with a cut-off of 0.8 [79]. Taxonomic data for each OTU are provided in Supplementary Table. 5.

Prior to analyses of richness and community structure, we removed all singletons found only from cultures or NGS reads, all reads found only in NGS with read abundance < 8, and OTUs that were observed in controls if present in ≥ 10% of the number of reads observed from each sample. We removed spurious OTUs found only in NGS samples at the threshold of abundance < 0.15% per sample. We chose this threshold because OTUs with abundance < 0.15% for each sample appeared to reflect sequencing error based on evaluation of the number of OTUs in our mock community (Supplementary Fig. 2).

In the final analyses, we used all reads obtained from NGS after quality control and rare-OTU filtering because rarefaction curves for all samples reached asymptotes (slope = 0, Supplementary Fig. 3) and because we did not observe a significant decrease of read abundance after coverage-based rarefaction [80]. The final combined data set consisted of 4701 cultures and 495,155 NGS reads that together represented 334 fungal OTUs.

Statistical Analyses

We used a three-way analysis of variance (ANOVA) to examine how culture media and storage period influenced isolation frequency of endophytes obtained in culture from each host species. We used host species, culture media, and storage period as main effects and included all interaction terms in models. We used the same approach to evaluate how culture media and storage period influenced richness of endophytes isolated in culture from each host species.

For the NGS data, we examined how endophyte richness varied as a function of storage period for each host species, with the response variable represented by residuals of the number of OTUs in relation to the square root of read abundance of each sample to account for differences in sequencing depth [23, 32]. Residuals were associated positively with OTU richness (culturing: $R^2 = 0.36$, $P < 0.0001$; NGS: $R^2 = 0.95$, $P < 0.0001$).

We used permutational analyses of variance (PERMANOVA) to examine how endophyte community structure differed as a function of culture media and storage period for each host species. We calculated similarity with the Bray-Curtis index, which includes information on abundances, and the Jaccard index, which includes presence/absence data only. We visualized community structure via nonmetric multidimensional scaling (NMDS).

We used PERMANOVA and NMDS to compare the structure of communities obtained via culturing vs. NGS. We compared taxonomic composition based on the taxonomic assignment by the UNITE database. Recovery ratio was calculated as the number of OTUs found in both approaches (culture-based and NGS) divided by the number of total OTUs found in the approach, expressed as percentage.

In our analysis, we considered all fungal OTUs, as well as those OTUs representing only the Ascomycota, as this phylum accounted for the majority of OTUs (88.2% and 73.6% of culture-based and NGS approach, respectively). We used the *vegan* package for R [81] for similarity calculation, NMDS, and PERMANOVA. All other analyses were performed in JMP (version 12 and 13, SAS Institute, Cary, NC, USA).

Results

Endophytes were observed in culture from leaves of all host species and individuals, including those processed 1, 5, or 10 days after collection and those incubated on all five culture media (Supplementary Table 6). Overall we obtained 4934 cultures (total isolation frequency = 39.2%), including isolates from all host species, all culture media, and all storage times.

Isolation frequency did not vary with culturing medium, but did reflect the storage period in a host-specific manner (Storage period × host species interaction: $F_{4,86} = 5.00$, $P < 0.001$, Fig. 1, Supplementary Fig. 4, Supplementary

Table 7). Isolation frequency was similar at each storage period for *Dryopteris*/*Thelypteris*, but generally increased from day 1 to day 5 for *Juniperus* and *Magnolia*. However, in all three species, isolation frequency from samples evaluated at day 10 was indistinguishable from that on day 1.

Richness of endophytes observed in culture did not differ appreciably among host species, storage periods, or culture media (Table 2). Overall, we observed the same patterns when we considered all fungi as well as Ascomycota only (Supplementary Table 8).

Endophyte communities isolated in culture differed markedly among host species (Jaccard: partial $R^2 = 0.25$, $P = 0.001$; Bray-Curtis: partial $R^2 = 0.36$, $P = 0.001$, Fig. 2a and b). After accounting for variation among host species, endophyte communities differed as a function of storage period when we considered presence/absence data (partial $R^2 = 0.01$, $P = 0.037$) but not when we considered abundance data ($P > 0.05$) (Fig. 2a and b). On average, 29.4% ± 2.2 (standard error, SE) of OTUs per species were observed in all three storage periods. The mean percentage of OTUs per host species shared between day 1 and day 5 was 7.5% ± 0.9 (SE), day 5 and day 10 was 7.6% ± 2.0, and day 1 and day 10 was 8.8% ± 2.4 (Fig. 3). Overall 46.7% ± 5.6 of OTUs for each species were observed in only one storage period, and the remainder were observed in at least two storage periods. Overall, endophyte communities did not differ among culture media (Jaccard: $P = 0.18$; Bray-Curtis: $P = 0.44$). We observed the same patterns when we considered only Ascomycota (Supplementary Fig. 5).

We examined endophytes of *Dryopteris*/*Thelypteris* in detail because these collections represented the highest variation in species composition over the temporal and spatial structure

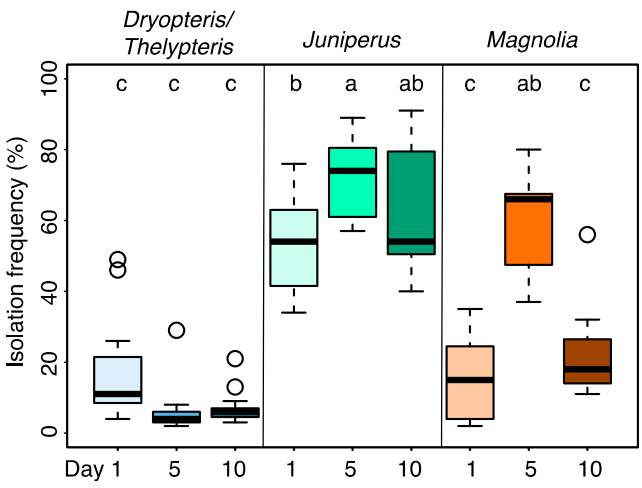

Fig. 1 Isolation frequency of endophytes varied as a function of storage period in a host-specific manner. Colors of each box plot are indicated in Fig. 2. Data from all media were pooled because isolation frequency did not vary with culture media (Supplementary Table 7). For analyses of Ascomycota only, see Supplementary Fig. 4. We used a post hoc Tukey's HSD test to compare across all groups indicated with letters above each box plot ($P < 0.05$, Supplementary Table 7)

Table 2 Species richness of endophytes observed by (a) culturing and (b) NGS did not vary with storage period or culture media

Source	DF	Sum of squares	*F*	*P*
(a) Culturing				
Storage period	2	44.96	1.73	0.18
Host	2	42.35	1.63	0.20
Media	4	3.19	0.06	0.99
Storage period × host	4	59.95	1.15	0.34
Storage period × media	8	48.91	0.47	0.87
Host × Media	8	30.62	0.29	0.97
Storage period × host × media	16	80.17	0.39	0.98
Error	86	1117.12		
(b) NGS				
Storage period	2	48.25	0.23	0.80
Host	2	14.09	0.07	0.94
Storage period × host	4	608.97	1.43	0.26
Error	18	1912.88		

Data were adjusted for sampling and sequencing artifacts as described in the text. DF represents degrees of freedom

of our study. We found that communities were more similar between days 1 and 5 relative to those on day 1 and 10 or day 5 and 10, with a particularly distinct community observed at day 10 (Fig. 2a and Supplementary Table 9). Shifts in species composition for endophytes of the other two hosts generally reflected presence/absence of a relatively small but detectable number of species.

After accounting for variation in sequencing depth among samples in the NGS data, species richness did not vary with storage period or among species ($P > 0.05$, Table 2, Supplementary Table 8). Endophyte communities differed among host species (Jaccard: partial $R^2 = 0.29$, $P = 0.001$; Bray-Curtis: partial $R^2 = 0.50$, $P = 0.001$, Fig. 2c and d, Supplementary Fig. 5), but after accounting for this variation, endophyte community structure did not differ with storage period (Jaccard: $P = 0.59$; Bray-Curtis: $P = 0.61$, Fig. 2c and d). We observed the same pattern when we considered only Ascomycota (Supplementary Fig. 5).

Comparison of Culture-Based and NGS Approach

Raw species richness obtained via NGS was greater than the richness observed by the culture-based approach in all three species regardless of the storage period (1.24 to 9.37 times, Supplementary Table 10, Supplementary Table 11). When analyzed together, both approaches reveal a strong signature of host species in defining endophyte community structure (Jaccard: partial $R^2 = 0.16$, $P = 0.001$; Bray-Curtis: partial $R^2 = 0.24$, $P = 0.001$, Fig. 2e and f), and no effect of storage period (Jaccard: $P = 0.07$; Bray-Curtis: $P = 0.13$, Fig. 2e and

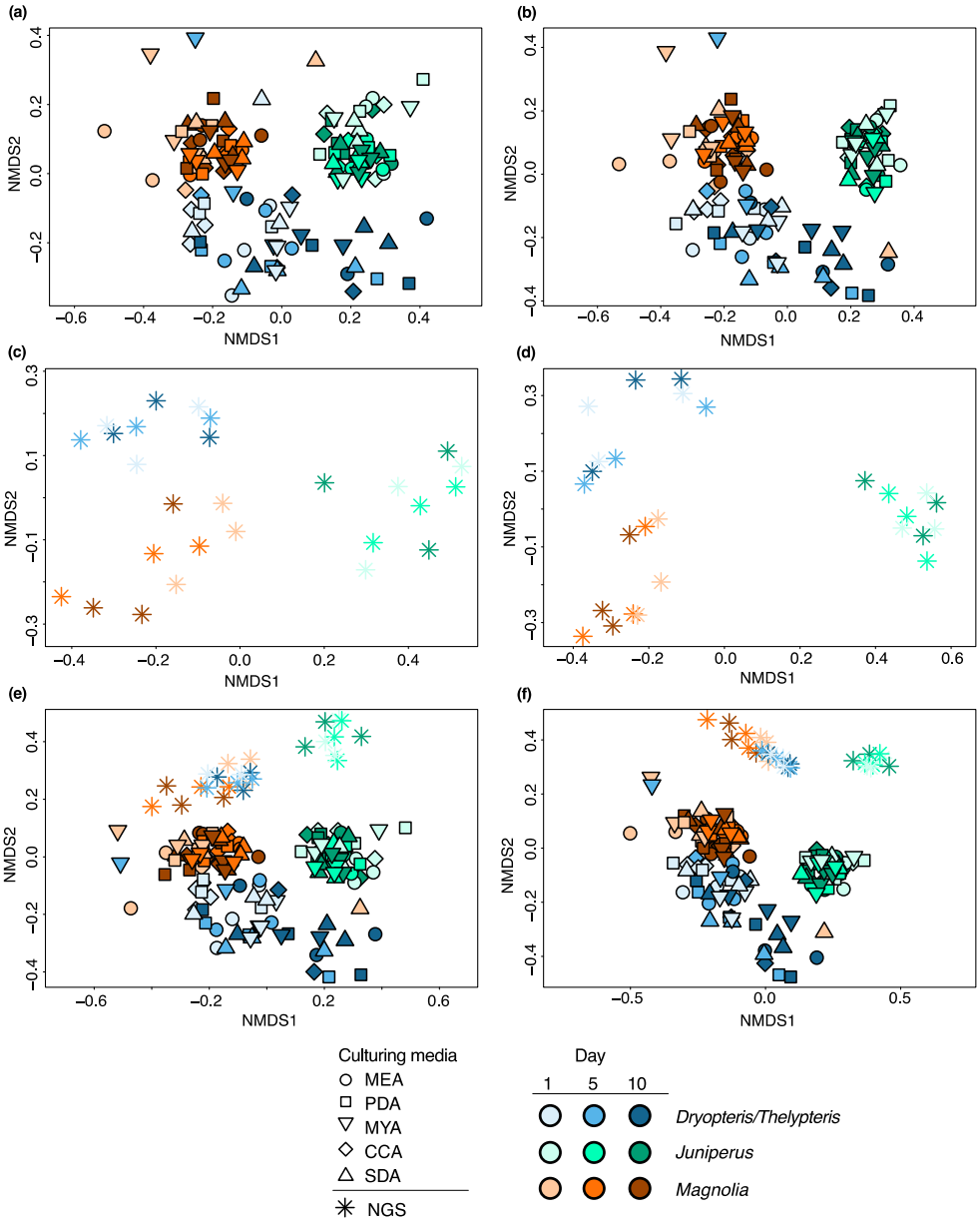

Fig. 2 Communities of endophytes differed among host species and between culturing and NGS approaches (see also Supplementary Table 9). For results for Ascomycota only, see Supplementary Fig. 5. Panels show NMDS based on the Jaccard index (**a**, culture-based; **c**, NGS; **e**, both data sets) and Bray-Curtis index (**b**, culture-based; **d**, NGS; **f**, both data sets). We observed a strong signal of host regardless of approach (**a**, Jaccard: partial $R^2 = 0.25$, $P = 0.001$; **b**, Bray-Curtis: partial $R^2 = 0.36$, $P = 0.001$; **c**, Jaccard: partial $R^2 = 0.29$, $P = 0.001$; **d**, Bray-Curtis: partial $R^2 = 0.50$, $P = 0.001$). We observed a signal of storage period for the culture-based data set when we considered only presence/absence data (panel a, Jaccard: partial $R^2 = 0.01$, $P = 0.037$), but not when we considered abundance data (panel b, Bray-Curtis: $P > 0.05$). We observed no effect of culturing medium (panel a, Jaccard: $P = 0.18$; panel b, Bray-Curtis: $P = 0.44$). In the NGS data set, we observed no effect of storage period (panel c, Jaccard: $P = 0.59$; panel d, Bray-Curtis: $P = 0.61$). Distinct endophyte communities were observed by culturing and NGS (panel e, Jaccard: partial $R^2 = 0.05$, $P = 0.001$; panel f, Bray-Curtis: partial $R^2 = 0.08$, $P = 0.001$)

f). However, endophyte communities differed markedly when evaluated via culturing and NGS, even though subsets of the same host tissues were used for each approach (Jaccard: partial $R^2 = 0.05$, $P = 0.001$; Bray-Curtis: partial $R^2 = 0.08$, $P = 0.001$, Fig. 2e and f). These differences were detectable both at the level of OTUs (Fig. 2e and f) and at higher taxonomic levels (Supplementary Table 1). Overall, we observed that more than 50% of OTUs (190 out of 334 OTUs) were detected only via the NGS approach (Supplementary Table 12). Basidiomycota were observed only in the NGS data set (Fig. 4a, Supplementary Table 12). In turn, 65 OTUs were observed only in culture-based data set (Supplementary Table 12). Within the Ascomycota, Sordariomycetes were more prevalent in the culture-based data set than in the NGS data set ($\chi^2_1 = 42.7$, $P < 0.0001$, Fig. 4b; Supplementary Fig. 6; Supplementary Table 12). Overall, more than 50% of Dothideomycetes observed in the culture-based data set were detected by NGS, in contrast with only a ca. 30% recovery rate of Sordariomycetes (Supplementary Table 12).

Discussion

Fungal endophytes have been found in all plant species and in all biomes examined to date [32, 73, 82, 83]. A growing appreciation of their diversity and importance in natural and human-dominated ecosystems, interest in their production of natural products [38, 84–87], and inquiries into their potential uses in agriculture, forestry, and industry, have converged with the development of efficient molecular methods to

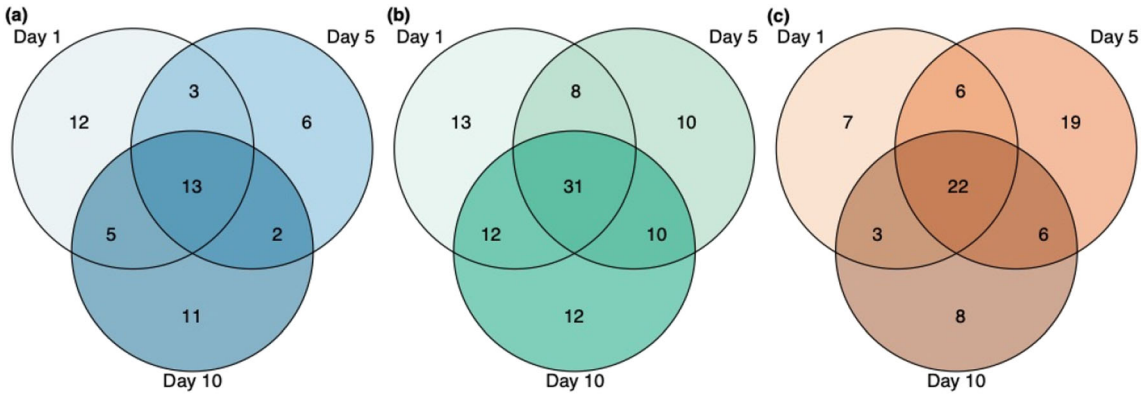

Fig. 3 The number of unique species at each storage periods and of shared species among storage periods in (**a**) *Dryopteris*/*Thelypteris*, (**b**) *Juniperus*, and (**c**) *Magnolia* in culture-based data set. Cultures from five media were pooled. Endophyte community structure based on presence/absence data differed among storage periods in *Dryopteris*/*Thelypteris* (PERMANOVA, $R^2 = 0.05$, $P = 0.003$) and *Juniperus* ($R^2 = 0.04$, $P = 0.022$), but not in *Magnolia* ($P = 0.168$) when we analyzed each host separately

evaluate endophyte communities in diverse plants. The majority of published studies are based on variations of several central methods, including the choice of cultivation media, the duration of storage between leaf collection and processing, and use of a culture-based or culture-free approach, particularly NGS (Table 1, Supplementary Table 1). This methodological variation raises the question: can the body of literature be used to inform large-scale inferences about endophyte diversity and distributions, or do these different methods preclude disparate studies from being compared meaningfully?

Our survey of endophytes associated with plants that co-occur in a temperate plant community provides insight to address this question. In our culture-based survey, we found that isolation frequency varied with storage period in a host-specific manner, but inferences about endophyte species richness were robust across five different culture media. This robustness was observed in all focal hosts, including representatives of three divisions of vascular plants (Pteridophyta, Pinophyta, and Magnoliophyta), and at storage periods ranging from 1 to 10 days. We found that endophyte communities isolated in culture differed with storage periods when evaluated with presence/absence data but not with abundance data. The difference due to storage period was comparatively small (partial $R^2 = 0.01$) relative to the one due to host species (partial $R^2 = 0.25$) and largely reflected the distinct community from day 10 in *Dryopteris*/*Thelypteris* (Fig. 2a and Supplementary Table 9). We found no signature of storage period in the NGS data set.

In most studies, leaf tissues are processed within a few hours of collection (Supplementary Table 1). Thus, we suggest that disparate studies that use culturing as the primary approach and differ only in cultivation medium or storage period might provide a basis for meaningful comparative analyses of endophyte richness and community structure. Moreover, even though endophyte richness and composition differed when we examined the same host tissue via culturing and NGS, the two approaches documented host-structured endophyte communities (see also [32]). This finding suggests

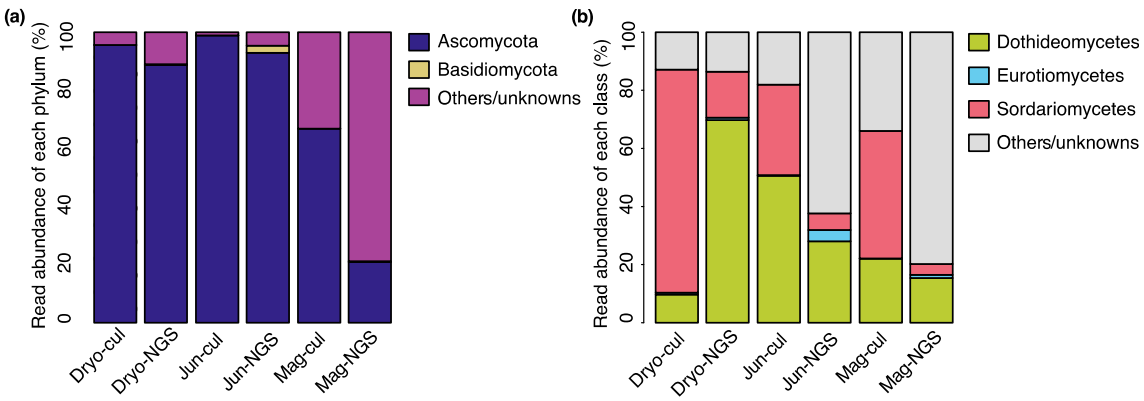

Fig. 4 Taxonomic abundance of endophytes for subsets of the same tissue from each host as inferred via culturing and NGS, showing (**a**) phylum and (**b**) class levels (for data for Ascomycota only, see Supplementary Fig. 6). Data from all media and storage periods were pooled due to their minimal effects on community structure. Others/unknowns represent OTUs assigned to other taxa or were not assigned with the SINTAX algorithm with a cut-off of 0.8. Abbreviations: Dryo, *Dryopteris*/*Thelypteris*; Jun, *Juniperus virginiana*; Mag, *Magnolia grandiflora*; cul, culture-based; NGS, NGS-based approach

that each approach is valid for ecological inferences such as host affinity, provided that the culturing effort is sufficiently extensive, and richness moderate enough, to allow culturing to produce a meaningful data set.

Overall, our findings join many other studies in showing that endophyte assemblages can differ markedly among co-occurring plant species. We suggest that to capture endophyte diversity in a region it is likely more important to sample more species, instead of sampling fewer species more intensively (as with multiple culture media or storage times).

Our data also suggest that it is reasonable to integrate data from culture-based studies of endophytes that may differ in storage time or isolation media, thus providing regional to global perspectives that may not be feasible in individual studies. We explored this in an initial fashion by compiling ITSrDNA data from culture-based surveys described in Supplementary Table 1 (a total of 20,473 sequences from 45 studies that deposited sequence data). We obtained the data from GenBank, extracted the ITSrDNA region via ITSx [75], and used T-BAS version 2.1 [67] to estimate the number of OTUs found overall, the novelty contributed by the culture-based portion of this study, and the phylogenetic relationships of this aggregated data set. The data from previous studies included 1658 OTUs. We selected one representative per OTU from the previously published data, as well as our full data set from culturing. Our study of three plant taxa in a small area, sampling only the equivalent of nine individuals, added 73 OTUs that were not observed in the body of work reviewed for this paper (with more stringent OTU definitions at 99% and 100% similarity, 118 and 304 OTUs, respectively). The distinctive OTUs represented Dothideomycetes (31 OTUs), Sordariomycetes, (26 OTUs), Pezizomycetes (3 OTUs), Eurotiomycetes (3 OTUs), and other taxa (10 OTUs) (Fig. 5). Several of the studies in Supplementary Table 1 were conducted in the same locality as this study (e.g., [31, 58]), such that we may expect to capture a larger number of distinct OTUs from one host species in an unexplored locality or region. Given the complementarity of NGS and culture-based methods, it is plausible that use of these two approaches will greatly expand our understanding of global endophyte diversity as large published data sets become more comparable through convergent methods, shared standards, or aggregation after factors such as storage time are ruled out as problematic.

Perspectives on Culture Media

Our results suggest that inferences from culturing are robust across five culture media that differ in their carbon sources. This result expands previous studies that examined two to three media (Table 1). The similarity in richness and community structure between nutrient-rich (e.g., MEA, PDA) and more nutrient-scarce media (e.g., CCA) suggests that endophytes may generally grow well on a wide range of nutrient concentrations and carbon sources, consistent with the substrate breadth of focal endophytes presented in [50]. It is plausible that even CCA provides a relatively rich or less recalcitrant environment relative to the nutrient-poor apoplast in which endophytes typically occur in living leaves [88, 89].

Given this wide substrate use, we anticipate that variation in endophyte communities among hosts more likely reflects factors such as secondary metabolites in host tissue rather than carbon content in leaves. This interpretation is supported by previous work in which leaf extracts were used in growth media for endophytes [90]. In that study, endophytes isolated frequently from particular hosts grew more rapidly on leaf extracts from that host vs. leaf extracts of other co-occurring plants, a result attributed to sensitivity to secondary metabolites [90]. In future work designed to capture the greatest diversity of endophytes, even more divergent nutrient sources or media chemistry could be employed with the aim to capture the taxa observed readily by NGS. It is plausible that including fundamentally different culture conditions could be fruitful as well. Previous work has shown that incubation of tissues at different temperatures may not be especially influential, at least for certain temperate endophyte communities [39].

Perspectives on Storage Time

Most studies describe the processing of leaf tissue, or its preservation, within hours to days of collection (Supplementary Table 1). We anticipated that some endophytes, particularly those with saprotrophic life phases or sensitivity to signals of leaf senescence [50], might grow rapidly during the storage period, which could account for changes in isolation frequency when subsamples of the same leaves are processed over time [90]. If endophyte communities vary among host species, as shown here and in previous work [63, 91, 92], then we might observe host-specific variation in isolation frequency as storage periods become longer. This prediction is consistent with the increase in isolation frequency from day 1 to day 5 in *Magnolia* (Fig. 1). Abundance of OTU5 (Blast top hit: 96% match to uncultured endophytic fungus, but not assigned to any taxon by UNITE, Supplementary Table 5) increased substantially from day 1 to 5 when data from all media were pooled (from 54 to 349 cultures in total, comprising 24.2% to 38.5% of total number of isolates at days 1 and 5). This suggests that OTU5 might be a candidate for gauging sensitivity to senescence and related signals [48, 50, 93], echoed by the observation that OTU5 increased in abundance in our NGS data set as well (65.7% to 80.0% of total read number at days 1 and 5). It is not clear why we observed a downward trend of isolation frequency from day 5 to 10 in *Juniperus* and *Magnolia*. It is possible that as leaf tissues aged after collection, they may have become more permeable to surface sterilants, even though we did not detect any observable changes

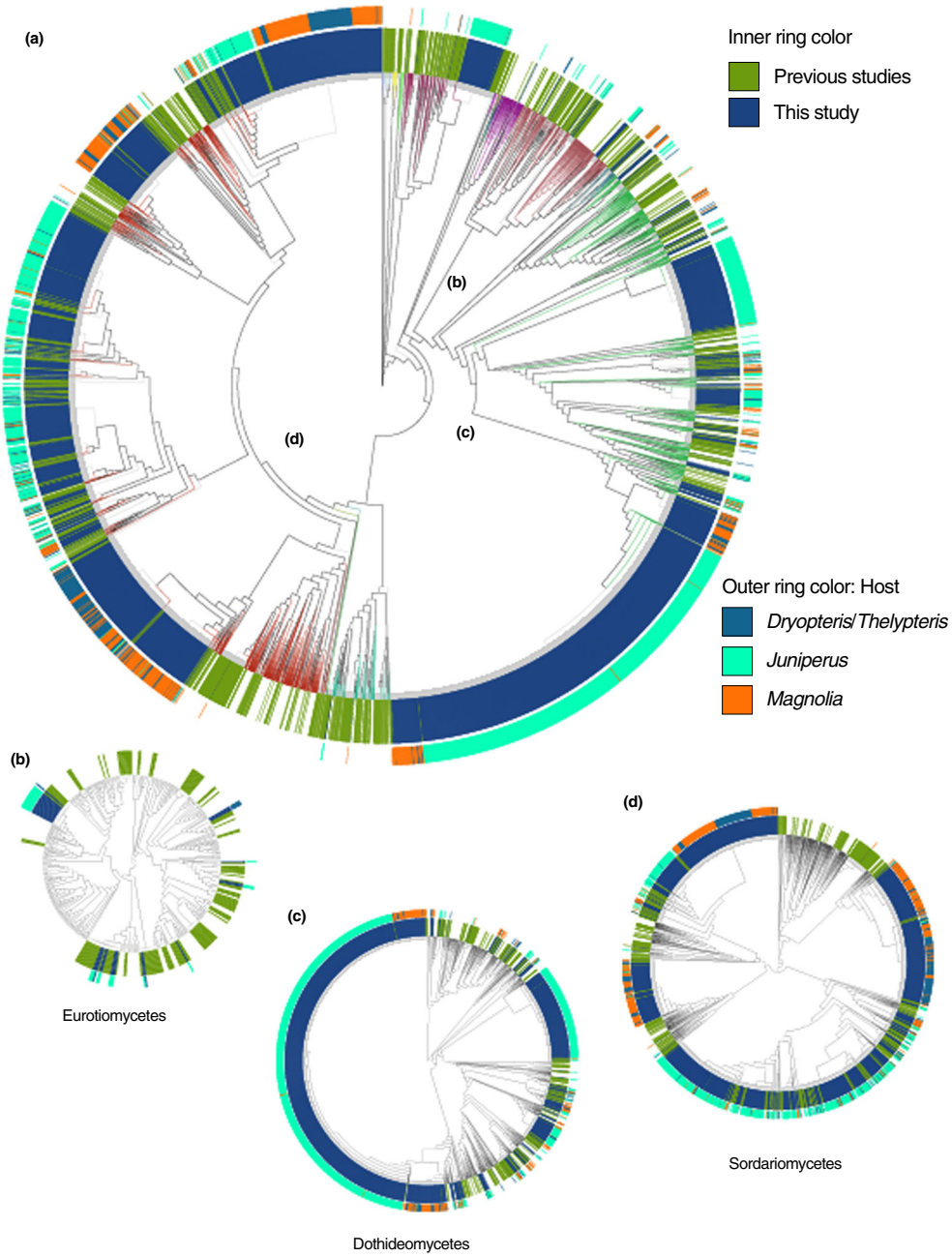

Fig. 5 Phylogenetic placement of cultured endophytes in this study with representative sequences from each OTU from 45 previous studies (Supplementary Table 1) and 1625 reference taxa in the Pezizomycotina, as determined using T-BAS [67]. All isolates of a given OTU obtained in this study and one representative per OTU from the previously published data are shown in tips of branches. OTUs are based on 95% sequence similarity. (**a**) Pezizomycotina tree, and (**b**–**d**) represent three major classes of Pezizomycotina that contained the majority of endophyte isolates from this study (See Fig. 4). The settings to place the sequences from this study and previous studies were UNITE filter engaged, no clustering for visualizing purpose, 10 standard deviation as genetic distance cutoff, and likelihood weights with the outgroup selected in RAxML options. Reference taxa are shown with colored branches (colored by class), with no color in the inner and outer ring in (**a**)

in leaf appearance or textures. This may account for the downward trend observed from day 1 to days 5 and 10 in the fern samples as well, as these were relatively fine leaf tissues that may have become permeable to surface sterilants more rapidly.

Although storage time may be associated with host-specific variation in isolation frequency, our data suggest that measures such as richness and composition are relatively robust over a period of 1–10 days, provided that leaves are kept under refrigerated (here, at 4 °C). We observed an effect of storage period on endophyte community composition, but only with presence/absence data and only for the culture-based data set. Host species explained ca. 25 times the amount of variation in community composition relative to storage time, which suggests that the effect of storage period is relatively small overall in when a survey contains phylogenetically diverse host species.

Our work complements the one previous study that, to our knowledge, explicitly evaluated storage period [43]. In that study, the timeframe was on the order of 6 h and 24 h, rather than the extended periods we evaluated here (Table 1, Supplementary Table 1). Important caveats are that leaves that are especially membranous might not be as robust to longer storage times.

Overall, our results suggest that in scenarios such as field studies in remote areas, there is some leeway in timing between collection and processing, provided that richness and community structure are the main measures of interest. Additionally, we documented unique species at each storage period for each host plant species, although these species had a little effect on the variation of endophyte community composition overall compared with the effect of host factors (Figs. 2 and 3). It may be useful to process plant tissues at two or three storage periods to maximize the number of OTUs isolated in culture.

Perspectives on Culturing and NGS

Culturing and NGS differed in values of richness and relative abundance of particular endophyte taxa, even though we analyzed subsets of the same tissue. The consistency of richness observed on each culturing medium for all species suggests that this is not an artifact of undersampling via culturing, but instead illustrates the oft-repeated pattern of detecting greater richness and different taxonomic structure with sensitive culture-free techniques [18, 30].

By expanding our survey to three storage periods and five media, we were unable to bridge the gap in richness between NGS and a culture-based approach. We observed greater richness in NGS than culture-based approach, which is consistent with previous studies (Supplementary Table 12, [18, 32]). Even though richness and taxonomic breadth detected by NGS were greater than those observed by culturing, we detected substantial portions of the endophyte community only via culturing (Supplementary Table 12). This disparity could be explained by primer bias in NGS [94] or by traits of the fungi themselves that could make them grow more readily, yet be detected via NGS less readily. Our analyses included mock communities and thus suggested that primer bias was not likely a concern, especially in Ascomycota. However, our mock communities provided evidence that we could amplify members of major lineages, not all species within those lineages. Therefore, multiple primer sets or degenerate primers also could be helpful to reduce this disparity [61, 70, 95], but it should be noted that these strategies costs more and still can cause some bias [70, 96]. Instead, it is plausible that certain endophytes occur at low biomass in leaves, but yet grow readily on rich media and are detected by culturing but not by NGS. In turn, some endophytes were detected here only by NGS. Such fungi may be prevalent or sufficiently abundant to be detected by NGS, yet grow poorly on synthetic media because they do not flourish with certain nutrients or nutrient compositions, they may require or have their growth enhanced by host secondary metabolites, or they may require synergy with co-occurring endophytes. These possibilities could be explored efficiently through careful inoculation experiments and studies with labeled endophytes that would permit quantification, visually or with molecular tools, to understand how each method might detect complementary components of the endophyte community in a given leaf, plant, or plant community.

Conclusions

In recent years, NGS has become the mainstream in microbial ecology, leading to the discovery of hidden diversity that a traditional culture-based approach has not yet found. Increasingly it is clear that culturing and NGS are complementary tools for documenting microbiome diversity [18, 63]. Here we find this to be true for communities of endophytes that inhabit representative plants: we detected high species richness of endophytes when evaluating only three plant species in each of three plots in a temperate habitat, showcasing the relevance of both culturing and NGS for future work. Based on our findings, we recommend that studies aiming to discover regional diversity of endophytes should use both culturing and NGS when possible, sample multiple plant species, and process material in a reasonable timeframe from 1 to 10 days after collection, provided that plant tissues are kept cool. For ecological inferences regarding host use and spatial distributions, either approach may be appropriate, but richness values and compositional data from either method may be incomplete.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00248-020-01654-y.

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Authors' Contributions S.O. and A.E.A. designed research; E.F.Y.H., I.C. collaborated to fund research; S.O., J.C., I.K., N.Y., A.I., A.E.A. performed research; S.O., A.E.A., and A.I. analyzed data; and S.O. and A.E.A. wrote the paper, with input from all authors.

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Data Availability Raw sequence data and metadata are deposited in GenBank under Sequence Raw Archive PRJNA649645 for Illumina reads. This Targeted Locus Study project with sequences by Sanger

sequencing has been deposited at DDBJ/EMBL/GenBank under the accession KEME00000000. The version described in this paper is the first version, KEME01000000. The other data supporting the findings of this study are available upon requests to AEA (arnold@ag.arizona.edu).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

Ethics Approval Not applicable to this study.

Consent to Participate Not applicable to this study.

Code Availability The code that supports the findings of this study is available from the corresponding author. Requests for code used in this study should be addressed to AEA (arnold@ag.arizona.edu).

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