



# Exploring the natural microbiome of the model liverwort: fungal endophyte diversity in *Marchantia polymorpha* L

Jessica Nelson<sup>1,2</sup>  · A. Jonathan Shaw<sup>1</sup>

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## Abstract

Within their tissues, plants host diverse communities of fungi, termed fungal endophytes. These fungi can affect plant growth, competitiveness, and resistance to stressors, thereby influencing plant community structure. Research characterizing fungal endophyte communities has so far mostly focused on seed plants, but information on the endophytes of other plant lineages is needed to understand how plant microbiomes impact whole ecosystems and how major changes through land plant evolution have affected plant-microbe relationships. In this study, we assess the fungal endophyte community of the model liverwort *Marchantia polymorpha* L. by both culturing and Illumina amplicon sequencing methods. We detect a very diverse fungal community that is distinct between *M. polymorpha* patches and only shares a few core fungi between populations across the United States. We also show low overlap in taxa detected by the different methods. This study helps build a foundation for using *M. polymorpha* and other *Marchantia* species as models for the ecology and dynamics of bryophyte microbiomes.

**Keywords** Bryophyte · Liverwort · Fungal endophytes · *Marchantia polymorpha* · Plant microbiota

## 1 Introduction

Plants harbor diverse microbiomes of bacteria, archaea, fungi, and other eukaryotic microbes (Berg et al. 2015). The microbes living inside healthy plant tissues are termed endophytes (Stone et al. 2000). In the case of fungi, this broad definition includes mycorrhizal fungi, latent pathogens and saprobes, and a diverse array of other fungi with functions across the spectrum from mutualist to pathogen (Porrás-Alfaro and Bayman 2011; Schulz and Boyle 2005). Fungal endophytes have a wide range of effects on host success including changes in growth, competitive ability, and resistance to pathogens or stressors (Porrás-Alfaro and Bayman 2011). The many effects of fungal symbionts make them important, if inconspicuous drivers of plant community structure and therefore broader ecosystem function (Afkhami and Strauss 2016; Aguilar-Trigueros and Rillig 2016; Rudgers et al. 2004).

Fungal endophytes have been surveyed across a growing number of host plants and habitats, revealing diverse and complex communities that assemble based on host identity, geographic location, elevation, season, and plant tissue (Arnold 2007; Kivlin et al. 2017; Peršoh 2015). The focus, however, has been on seed plants, with much less work on ferns, lycophytes, and the non-vascular bryophytes (mosses, liverworts, and hornworts). The differences in evolutionary history, life strategies, life cycles, and ecology between seed plants and the rest of the land plant lineages may lead to distinct microbiome dynamics. Indeed, sampling that has assessed bryophyte and vascular plant culturable endophytes from the same sites has hinted at separation between their communities (U'Ren et al. 2012).

Associations with fungi are also thought to have been important in allowing plants to initially colonize land (Delaux et al. 2012; Heckman et al. 2001; Taylor and Krings 2005), making bryophyte fungal symbionts evolutionarily important to study because of the bryophytes' early-branching positions in the embryophyte clade (Shaw and Renzaglia 2004). Mycorrhiza-like fungal symbioses have been found in hornworts and liverworts, but mosses appear to lack them (Desirò et al. 2013; Pressel et al. 2010). Experiments in liverworts have demonstrated symbiotic nutrient exchange with mycorrhizal fungi belonging to Glomeromycotina, Mucoromycotina, and Ascomycota (Field et al. 2012; Field et al. 2016; Kowal et al.

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✉ Jessica Nelson  
nelson.bryo@gmail.com

<sup>1</sup> Department of Biology, Duke University, Durham, NC, USA

<sup>2</sup> Boyce Thompson Institute, Ithaca, NY, USA

2018). Research on microbes associated with mosses has instead focused mostly on parasites and pathogens (Döbbeler 1997; Davey and Currah 2006; Akita et al. 2011). In contrast to such work on individual symbionts, only a few researchers have attempted to characterize the diversity of the whole fungal communities found in liverworts (Davis et al. 2003; Davis and Shaw 2008; Knack et al. 2015) or mosses (Davey et al. 2013; Davey et al. 2012; Davey et al. 2014; Davey et al. 2017).

In this study, we explored the diversity of fungal endophytes in wild populations of the liverwort *Marchantia polymorpha* L. This widely distributed liverwort has been used as a model for botanical research for well over a century and is now emerging as a molecular model system with a recently published genome sequence (Bowman et al. 2016; Bowman et al. 2017). This species appears to contain three genetic groups having distinct ecologies and, based on morphology and isozyme assessment, these have been designated as *M. polymorpha* subsp. *polymorpha*, *ruderalis*, and *montivagans* (Bischler-Causse and Boisselier-Dubayle 1991; Boisselier-Dubayle et al. 1995). The subspecies used for the recent genome sequence and molecular work is *ruderalis*, but resequencing to investigate the diversity of the broader species is underway. The broad range and variable ecology of *M. polymorpha* make it an attractive candidate for studies on how microbiome assembly depends on geography, disturbance, and other ecological factors. The availability of genetic tools and the ease of growing *M. polymorpha* and other *Marchantia* species axenically in the lab also make the genus a promising model for experimental investigations of microbial interactions (Bowman et al. 2016).

Much of the mycorrhizal symbiosis work on liverworts thus far has not focused on *M. polymorpha* itself since it is not often found to harbor arbuscular mycorrhizal fungi (Ligrone et al. 2007; Pressel et al. 2010). Differences in fungal colonization between the three subspecies of *M. polymorpha* have been reported, with Glomeromycotina associations only detected in subsp. *montivagans* (Ligrone et al. 2007). On the other hand, *M. paleacea*, the sister species of *M. polymorpha*, does form mycorrhizal symbioses and is being used as a model for these interactions (Field et al. 2012; Humphreys et al. 2010; Radhakrishnan 2017). The comparison of the unusual lack of mycorrhizal association in some *M. polymorpha* to other *Marchantia* species promises to provide interesting insights into the effects of this dominant symbiosis on microbiome dynamics in bryophytes.

The microbiomes of *Marchantia* liverworts beyond mycorrhizal fungi are only just beginning to be investigated. Hipol et al. (2015) isolated a few culturable fungal endophytes from *M. polymorpha* in Baguio City, Philippines. Bacterial communities have been assessed by amplicon sequencing for *M. polymorpha* and *M. paleacea* near Oxtlapa, Mexico (Alcaraz et al. 2018) and *Marchantia inflexa* from Trinidad (Marks et al. 2018).

In this study, we collected *M. polymorpha* plants of all three subspecies as defined by Bischler-Causse and Boisselier-Dubayle (1991), and surveyed their endophytes both by culturing fungi and using direct Illumina amplicon sequencing across a broad geographic area in the United States. This dataset provides a broader sampling of the microbiome of this model species and will serve as a starting point for developing experiments on microbial interactions using *M. polymorpha*, as well as allowing future assessments of patterns of microbiome composition between major plant lineages.

The specific goals of this study were (1) to characterize the fungal endophyte communities of *M. polymorpha*, (2) to determine how its fungal endophyte communities vary between geographic locations, and (3) to directly compare the results of culturing and amplicon sequencing methods.

## 2 Materials and methods

### 2.1 Collections

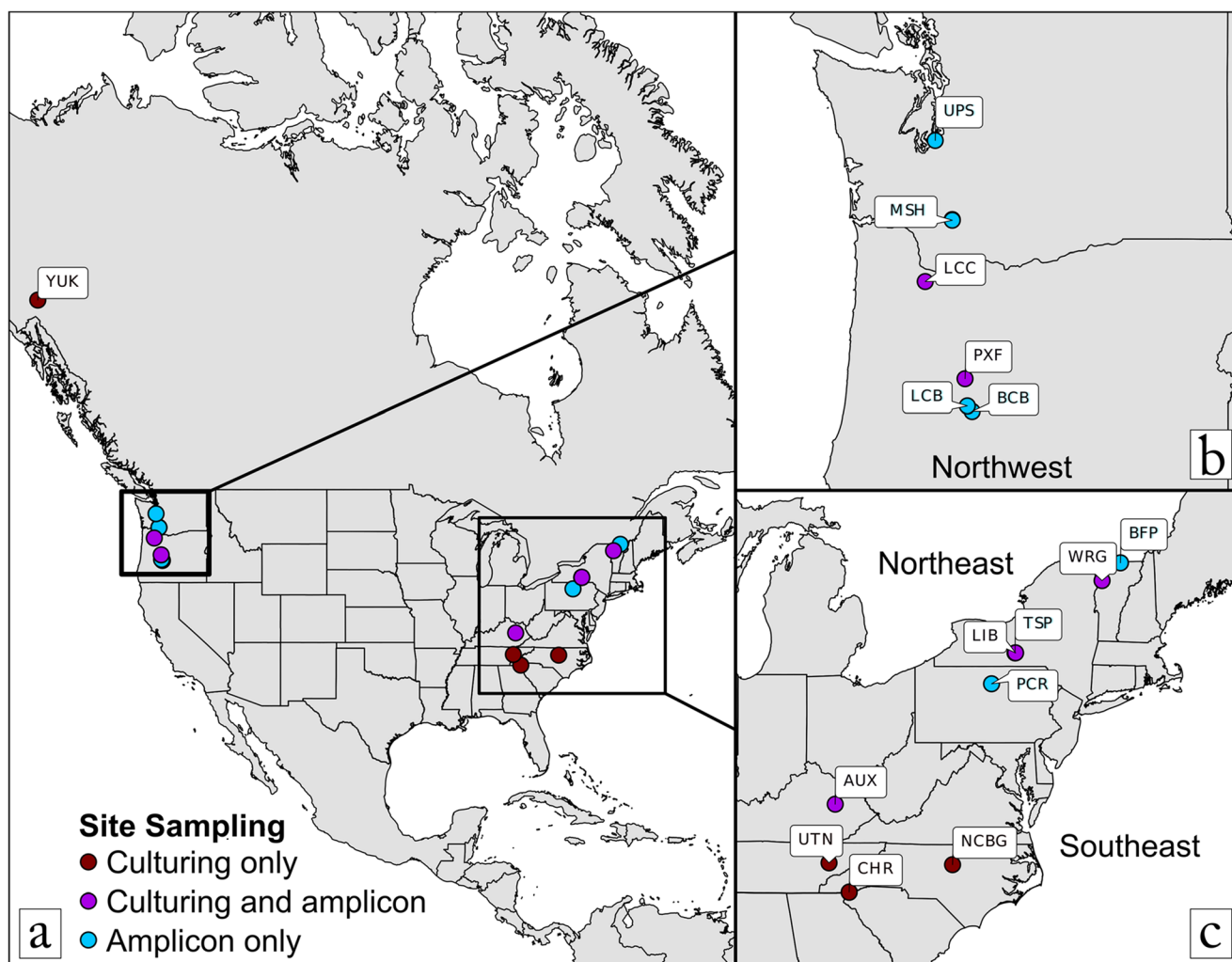
Twenty-four patches of *M. polymorpha* were collected from 16 sites in eight US states and one Canadian territory (Fig. 1) from October 2013 to August 2015. At each site, one to four patches, each approximately 8 cm in diameter, were collected (Table 1). One of the sites (AUX) was sampled in both 2014 and 2015; all other sites were only sampled once. All three *M. polymorpha* subspecies were collected: subsp. *polymorpha* was found at the two Vermont sites (BFP, WRG), subsp. *montivagans* was found at three Oregon sites and one Washington site (BCB, LCB, PXF, MSH), and all other collections were subsp. *ruderalis* (Table 1).

For testing hypotheses about broad geographic patterns, sampling sites were grouped into three major regions: southeast (North Carolina, Tennessee, Kentucky) with four sites, northeast (Pennsylvania, New York, Vermont) with five, and northwest (Oregon, Washington, the Yukon) with seven (Fig. 1). All plant voucher specimens were deposited into the L.E. Anderson Bryophyte Herbarium at Duke University (DUKE).

After collection, plants with adhering soil were refrigerated in plastic bags until being cleaned and processed, no longer than 2 days (except the Canadian sample which was shipped live first). Plants were washed in sterile deionized water then surface sterilized. Surface sterilization was based on the methods of Arnold (2002). Plant fragments were immersed in 95% ethanol for 30 s, 10% bleach for 2 min, and 70% ethanol for 2 min, then dried on an ethanol treated Kimwipe.

### 2.2 Endophyte isolation and identification

Briefly, fungi were isolated on 1.5% malt extract agar (MEA) from plant fragments approximately 2 mm<sup>2</sup> in area. Fungi



**Fig. 1** Map of sampling locations. Sampling sites were grouped into three regions: Northeast which included sites in Pennsylvania, New York, and Vermont; southeast with North Carolina, Tennessee, and Kentucky; and Northwest with Oregon, Washington, and the Yukon

were isolated from three tissue types: thallus, rhizoids (unicellular holdfasts), and gametangiophores (gametophytic receptacles for reproductive structures). A few additional cultures were isolated from tiny fungal fruiting bodies found on *M. polymorpha* thalli. Isolation and culturing of endophytes was conducted as previously described (Nelson et al. 2018).

DNA was extracted from pure fungal cultures with a cetyl trimethylammonium bromide (CTAB) protocol as previously described (Nelson et al. 2018). A segment of the rRNA gene including the internal transcribed spacer region (ITS) and part of the large subunit (LSU) was then amplified by PCR using the primers ITS1f (Gardes and Bruns 1993) and LR3 (Vilgalys and Hester 1990) and the amplicons were Sanger sequenced, as previously described (Nelson et al. 2018). Fungal isolates were identified by comparing their ITS1f-LR3 sequences to the National Center for Biotechnology Information (NCBI) GenBank database with BLAST searches using a 97% similarity threshold. When multiple isolates were at least 97%

similar to each other, they were considered the same species. If multiple isolates formed a grade such that all samples were 97% similar to some but not all isolates in the grade with no independent groupings that could be split out, the whole grade was treated as one species.

### 2.3 DNA extraction from *M. polymorpha*

Whole *M. polymorpha* thalli were surface sterilized using the same protocol described above for plant fragments. Sterilized plants were stored in sterile 2 mL tubes at  $-80^{\circ}\text{C}$ . Frozen whole liverwort thalli were ground in 1.2 mL tubes using two 3.5 mm stainless steel balls and a mix of 2 mm and 0.7 mm zirconia beads on a Geno/Grinder. 500  $\mu\text{L}$  of 2% CTAB buffer with 2%  $\beta$ -mercaptoethanol was added to each sample. Thereafter, the extraction followed the same protocol as used for fungal culture extractions. DNA extract concentrations were determined using a Qubit 2.0 fluorimeter (Thermo

**Table 1** Collection details for *M. polymorpha* used in this study

Patch	Habitat	Date	Subspecies	Locality notes
AUX1	Soil, under log	5/20/16	<i>ruderalis</i>	Near Auxier Ridge trailhead, Daniel Boone National Forest Burned October 2010 Max distance: 65 m
AUX2	Soil, among leaf litter			
AUX3	Soil, in shade of log			
AUX4	Soil, between two logs, among litter			
AUX5	Soil, downslope side of log	7/11/15		
BCB	Along side channel of stream in thick bryophyte mat	8/5/15	<i>montivagans</i>	Deschutes National Forest, Browns Creek Burned July 2013
BFP	River bank in bedrock gorge	7/1/15	<i>polymorpha</i>	Big Falls State Park
CHR	Edge of roadside ditch	5/21/14	<i>ruderalis</i>	Beside Chatooga River Gorge
LCB	Edge of tiny water channel in bog	8/5/15	<i>montivagans</i>	Near Little Cultus Lake
LCC	Among edging stones of gravel path	8/15/15	<i>ruderalis</i>	Lewis & Clark College campus
LIB	On bedrock ledge in middle of stream	6/27/15	<i>ruderalis</i>	Lick Brook lower bowl
MSH1	Edge of small stream side channel	7/31/15	<i>montivagans</i>	Mount St. Helens pumice plain Max distance: 960 m
MSH2	Tiny bog, on ~24 cm of moss and <i>Marchantia</i> peat			
MSH3	Soil, shaded edge of path			
NCB	Weed mat below nursery benches	10/24/13	<i>ruderalis</i>	North Carolina Botanical Garden
PCR	Edge of ditch beside gravel path	6/25/15	<i>ruderalis</i>	Pine Creek Rail Trail
PXF	On log, in mist from waterfall	8/4/15	<i>montivagans</i>	Proxy Falls
TSP	Soil, bank near waterfall	6/27/15	<i>ruderalis</i>	Robert H. Treman State Park
UPS	Bare soil, in shade of building	8/10/15	<i>ruderalis</i>	University of Puget Sound campus
UTN	Bryophyte garden in shade of building	3/2/14	(not determined)	University of Tennessee Knoxville campus
WRG1	Sandy soil (females)	6/30/15	<i>polymorpha</i>	Winooski River Gorge, just below dam in Overlook Park Distance: 8 m
WRG2	Sandy soil, over rocky ledge (males)	6/30/15	<i>polymorpha</i>	
YUK1	Flooded area by seepage stream	6/13/14	(not determined)	Near Fish Lake, Yukon Distance: 3.78 km
YUK2	Seepy ground near lake shore			

Please refer to Fig. 1 map for geographic locations of sites and fungal sampling methods applied to each. For sites where multiple patches were collected, maximum distances between sampling points are listed in the locality notes column

Fisher) with the High Sensitivity DNA assay. All extracts with a concentration higher than 8 ng/ $\mu$ L were diluted to 5 ng/ $\mu$ L before PCR amplification.

## 2.4 Amplicon sequencing

Mock community positive controls were created to run with the samples for assessing quality and biases in the library preparation and sequencing process (Nguyen et al. 2015). Mock communities included DNA from a set of 19 taxonomically diverse endophytes isolated from *M. polymorpha* (<https://doi.org/10.6084/m9.figshare.7560434>). Two mock communities were constructed: the first with equal ng amounts of raw fungal DNA extract and the second with equal ng amounts of the amplicon from ITS1f to LR3 in order to remove possible discrepancies from differences in copy number of the rRNA genes between fungi.

Mock community DNA mixes, extracts from whole *M. polymorpha* plants, and negative controls (water) were used for PCR targeting two fungal rRNA gene regions: ITS

(ITS1, 5.8S, ITS2) using the primers ITS1f (Gardes and Bruns 1993) and ITS4 (Innis et al. 2012) and part of the LSU using primers LROR (Bunyard et al. 1994) and LR3 (Vilgalys and Hester 1990). Two replicate libraries were prepared in parallel for each sample.

Libraries of amplicons were prepared in three separate PCR stages using frame-shifted primers to improve Illumina data quality by increasing sequence diversity, based on the methods of Lundberg et al. (2013). In the first stage, all samples were amplified using standard primer pairs for 15 cycles to enrich for the target region. In the second stage, Illumina sequencing adaptors were added in 10 cycles of PCR using 2  $\mu$ L of enriched sample. Adaptor primers in this step were mixes of six frameshifted primers to increase sequence diversity for Illumina sequencing. In the third stage, unique barcodes and Illumina adaptors were added to each sample in a final 10 cycle PCR. This final barcode step was set up in a sterile flow hood to prevent cross-contamination. For details of PCR recipes and programs, see Nelson (2017).

After library preparation, concentrations of all samples were determined with a Qubit fluorimeter. Samples were used if they had at least 5 ng/ $\mu$ L concentration. This resulted in 94 usable amplicon samples from the original 140 plants, of which all samples had at least one replicate for LSU and 73 samples had at least one for ITS. Amounts of DNA from all samples and controls were normalized and pooled to 50 ng per sample based on Qubit readings. The pooled amplicons were cleaned using Agencourt AmpPure XP beads at a 1.2:1 ratio of bead solution to sample to remove sequences shorter than the target amplicons. The prepared library was sequenced on an Illumina MiSeq at the Duke Center for Genomic and Computational Biology.

## 2.5 Amplicon data analysis

Reads obtained from the MiSeq library were demultiplexed using QIIME (Caporaso et al. 2010), and split by primer and cleaned using cutadapt (Martin 2011). Adaptors were removed and reads were quality filtered using Trimmomatic (Bolger et al. 2014). The remaining processing steps were conducted with VSEARCH (Rognes et al. 2016): reads were filtered by length and quality and dereplicated, singletons and chimeras were removed; operational taxonomic units (OTUs) were clustered at 95% similarity for ITS and 96% for LSU; OTUs with fewer than 10 reads were removed; and reads were assigned to OTUs using a 95% threshold for ITS reads and 96% for LSU reads. Clustering thresholds were selected by running the data processing with multiple thresholds and comparing the accuracy of the mock community recovery. Only forward reads were used because reverse reads were low quality and poorly recovered the mock community. Code for data processing pipeline is provided at <https://doi.org/10.6084/m9.figshare.7544852>.

For ITS reads, taxonomy was assigned with mothur implemented in QIIME (Schloss et al. 2009) using the UNITE database (Abarenkov et al. 2010). For LSU reads, taxonomy was assigned with the RDP classifier web portal (Wang et al. 2007). Identifications for taxa of interest were refined using individual BLAST searches of the NCBI database.

To filter out unreliable data, the number of reads appearing in sequenced negative controls was checked. The maximum number of reads for an OTU in any negative control was four, so all cells in the OTU table with four or fewer reads were changed to zero. Any OTUs with no reads remaining after this filtering step were removed. Based on automated taxonomy assignments and BLAST searches, non-fungal OTUs were removed. The LSU amplicon detected many non-fungal organisms and these sequences were separated out and their identifications refined with BLAST searches. Any samples with no reads remaining after restricting the data to fungi were removed. Replicate PCR samples for the same plants were pooled by adding read counts for each OTU. After pooling,

samples with too few reads were removed using a threshold of 500 for LSU samples and 1000 for ITS samples because the LSU samples resulted in fewer fungal sequences on average. The OTU tables were normalized by converting all read counts into percent of total reads for the sample. OTUs with mean abundances under one per million after normalization were removed.

To determine similarities between the sequenced fungal communities, Pearson correlation coefficients were calculated in R 3.4.4 (Team RC 2018) and Bray-Curtis and Jaccard indices were calculated with the R vegan package (Oksanen et al. 2017). Downstream results from both Bray-Curtis and Jaccard indices were similar and we chose to present the Jaccard index which does not use OTU abundances. This was based on the observation that read counts did not accurately reflect known starting amounts of fungi in the mock community data. Principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) using the MASS package (Venables and Ripley 2002) were conducted in R and visualized with the ggplot2 (Wickham 2009) and plyr (Wickham 2011) packages. Significance of separation between communities from different sites and from the three North American regions sampled (northwest, northeast, and southeast) was determined with a permutational analysis of variance (PERMANOVA) using the vegan package in R. The R code used for all data analyses and visualization is provided at <https://doi.org/10.6084/m9.figshare.7542095>.

## 2.6 Fungal phylogeny

A phylogenetic analysis of the sequences from the fungal cultures and LSU amplicons was conducted using the online T-BAS portal applying maximum likelihood with RAxML and default settings to place query sequences into a reference fungal phylogeny using the nuLSU dataset (Carbone et al. 2016).

## 2.7 Estimating minimum total species richness

To estimate how many species or OTUs would be likely to be found with sufficient sampling in each method, the chao1 index was calculated using the vegan command `specpool()` in R. For amplicon datasets, mock community reads were removed before calculating chao1. For culture data, cultures from the Yukon samples were removed since their initial handling differed from the other samples.

## 2.8 Identifying core endophyte community members

We identified endophytes that were common across our sampling to determine core members of the *M. polymorpha* fungal community both at local (site) and broad (full study) scales. For individual sites in the amplicon datasets with data for at

least five plants, core members of the local community were defined as fungi found in all plants from that site with an average abundance over 1%. For the culture data set, because so few fungi were isolated multiple times, we defined core members of the *M. polymorpha* fungal community as ones isolated from more than one site. For the full amplicon datasets, fungi were considered core members if they were detected in at least 50% of sites and had an average read abundance over 1%.

### 3 Results

#### 3.1 Diversity of fungi isolated in culture

A total of 93 fungal isolates were obtained from *M. polymorpha* plants (Genbank accessions MG004722-MG004798 and MK359672-MK359687), seven from surficial fruiting bodies and the remainder from surface-sterilized tissues. Fungi were obtained from 29 of 43 plants sampled. Pieces from a single plant yielded up to six fungal species. Fungi were isolated from 14.5% of the 592 surface-sterilized plant fragments. This isolation rate varied among sites, from 1.9% recovery from one patch in Vermont (WRG2 with only one isolate), to 68.4% isolation rate from one Kentucky collection. Plant tissues also had differing isolation rates with gametangiophores yielding the highest (27.9%), thalli intermediate (14.1%), and rhizoids the lowest (7.6%). This is reflected in the observation that fertile material often yielded higher total isolate numbers. Isolation rates and identities show no clear correlation with time of sampling or sex of host plant.

The isolated fungi represented at least 50 distinct species and only 15 species were isolated more than once. For the endophytes, calculation of *chao1* indicated 35.3% completeness of species sampling. Six of the isolated species could not be identified past class. The vast majority of the fungal cultures belonged to Ascomycota; only two Basidiomycota cultures were obtained. Six classes of Ascomycota were represented: Eurotiomycetes, Pezizomycetes, Saccharomycetes, Leotiomycetes, Dothideomycetes, and, most abundantly, Sordariomycetes. Sordariomycetes were found in all *M. polymorpha* patches that yielded more than a single isolate, and at two sites they were the only isolated class (NCB, UTN) (Fig. 2). At finer taxonomic scales, no individual taxon predominated across all or most sites. As indicated by the small number of species found at more than a single site (nine), there was little species overlap between sampling locations. Only *Phoma herbarum* and *Candida sake* were found in both western and eastern North America (although the western isolate of *P. herbarum* came from the Canadian sample which had

longer before refrigeration); all other isolates found at multiple sites were restricted to one sampling region (northeast, southeast, or northwest).

Some repeatedly isolated fungi were only found in a particular tissue. *Phoma herbarum* and *Colletotrichum* spp. only came from gametangiophores. Species isolated from different tissues were mostly non-overlapping and only one fungus was isolated from rhizoids, gametangiophores, and thalli: *Xylaria cubensis*.

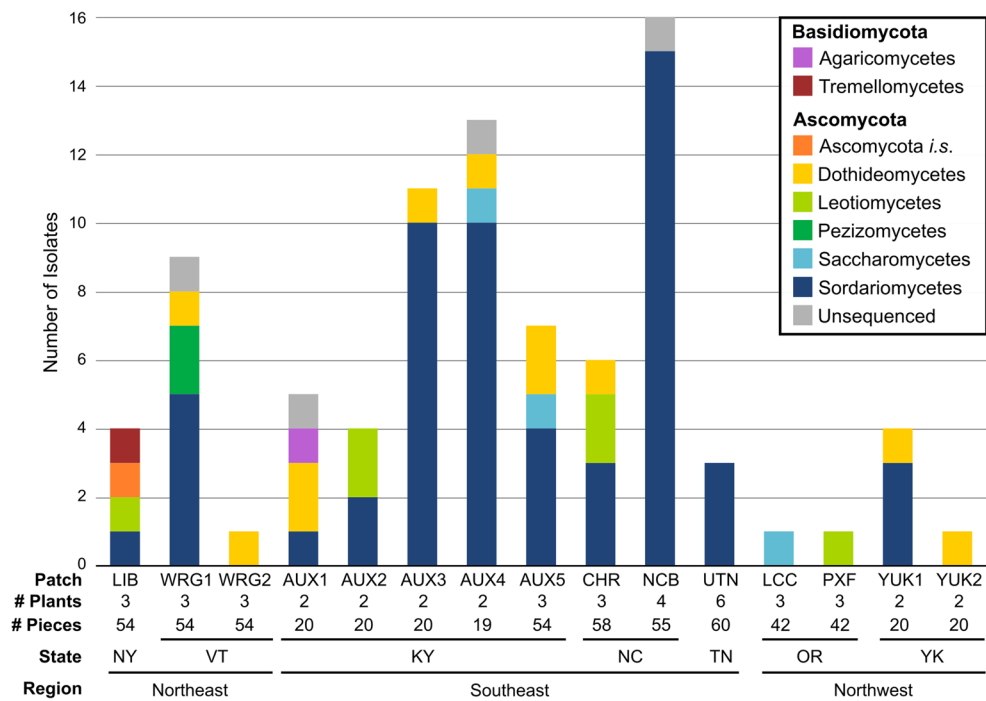
#### 3.2 Amplicon sequencing diversity

The Illumina MiSeq run yielded 22,837,264 raw paired-end reads (SRA BioProject PRJNA514318). After filtering, the LSU and ITS datasets contained 463,809 and 1,636,556 reads, respectively. After all filtering, the LSU data yielded 367 OTUs and the ITS data 891 (OTU tables available at <https://doi.org/10.6084/m9.figshare.c.4352507>). The final sampling included data from 69 plants, seven of which only had LSU data and eight only had ITS, with each site having data for one to nine plants. The OTU accumulation curves for both amplicon data sets show only slight leveling (data not shown), and calculation of *chao1* indicated that the sequencing recovered 72.4% (ITS) and 78.2% (LSU) of predicted OTUs detectable for the sampled area. Pearson correlations indicated in almost all cases that PCR replicates of the same plant samples had communities most similar to their paired replicate (data not shown).

Both ITS and LSU datasets were dominated by Ascomycota fungi, followed by Basidiomycota; some earlier-diverging fungi also were detected (Table 2, Fig. 3). Among the Ascomycota, the most abundant classes were Dothideomycetes, Leotiomycetes, and Sordariomycetes. Agaricomycetes were the most abundant Basidiomycota (Table 2).

PERMANOVA analyses comparing individual sites and regions (northwest, northeast, and southeast) indicate significant separation in fungal communities between both geographic units ( $p < 0.01$ ), but Pearson correlations between all samples and PCoA and NMDS analyses show that differences between sites did not strongly correlate with geographic distance between them (Fig. 4). For example, both ordination methods place the Kentucky site (AUX) as most similar to sites in Washington (MSH, UPS) rather than the much closer sites in Pennsylvania or New York. Differences between sites did not clearly correlate with bioclimatic variables, subspecies of host plant populations, time of sampling, nor level of human influence on the habitat.

Core endophyte community members of individual sites varied, but all sites with enough plants sampled showed at least one core OTU found in all plants at that site. Core fungi for individual sites are listed in Table 3.



**Fig. 2** Diversity of endophytes cultured from *M. polymorpha* across sampling locations. Heights of bars show total numbers of fungal isolates obtained from sites and colors indicate how many of those belonged to the various fungal classes. Unsequenced isolates were those for which multiple attempts at DNA extraction and PCR failed. Note that sampling effort differed between sites: numbers of plants sampled (#

plants) and tissue fragments for which isolation was attempted (# pieces) are shown below site codes. Patch codes match the location codes used in Fig. 1. US states where plants were obtained include New York (NY), Kentucky (KY), North Carolina (NC), Tennessee (TN), and Oregon (OR). Two specimens from the Canadian Yukon territory (YK) were also used

### 3.3 Comparison of fungal communities inferred from culturing and sequencing datasets

The fungi detected in *M. polymorpha* come from across the fungal tree of life with few major groups lacking representatives (Fig. 3). Fungal orders frequently recovered by both culturing and amplicon methods include Xylariales, Helotiales, and Pleosporales (all Ascomycota). The culture collection was greatly enriched in Sordariomycetes (Table 2), especially Xylariaceae which have been abundantly isolated from other liverworts (Davis et al. 2003), but Xylariaceae were rare in our amplicon data. There was little overlap in the core fungi identified from the full culturing and sequencing datasets, with the exception of *Phoma* (Table 4). This fungus was isolated multiple times and from the most widespread set of sites of any species in the culture dataset (Kentucky, Vermont, and the Yukon). It was also persistent over time, as demonstrated by its isolation from the Kentucky population in two subsequent years. Comparing amplicon OTUs to the culture sequences indicates that some of the OTUs consistently detected at high abundances are likely this same species of *Phoma*. One OTU of *Phoma* from the ITS dataset was found in every site with an average of over 20% of each site's reads.

Sites for which both culturing and sequencing were conducted on plants collected at the same time provide a

direct comparison of taxa recovered by the two methods. Collection of both data types was attempted at five sites: LCC and PXF in Oregon, AUX in Kentucky, LIB in New York, and WRG in Vermont. LCC yielded only one culture and too few reads to analyze. PXF only yielded one culture, but sequencing detected mostly sebacinales and chytrids in these plants, neither of which would have been likely to grow in the culture conditions used. The other three sites had enough data to compare, but showed little overlap between cultured and sequenced fungi, with the exception of *Phoma herbarum* at AUX. Of the OTUs that contributed on average more than 2% of reads at AUX, only two were putatively detected by both ITS and LSU: Agaricomycetes sp. and Sebaciales sp. Similarly, ITS and LSU at LIB shared *Plectosphaerella* sp., *Tetracladium* sp., and Pleosporales sp. WRG showed the greatest correspondence between abundant OTUs from ITS and LSU with half (five) of the OTUs overlapping: Agaricales sp., Botryosphaeriaceae sp., Helotiales sp., *Tetracladium* sp., Sebaciales sp., and *Lachancea kluyveri* (data not shown).

### 3.4 Non-fungal eukaryotes

Non-fungal taxa detected by LSU sequencing included oomycetes, chlorophyte algae, animals such as mites and

**Table 2** Comparison of fungal taxa recovered from *M. polymorpha* by culturing and amplicon sequencing

Taxon	Dataset		
	ITS OTUs	LSU OTUs	Isolates
<b>Ascomycota Total</b>	<b>413 (46.8%)</b>	<b>179 (50.4%)</b>	<b>87 (93.5%)</b>
Dothideomycetes	131 (14.8%)	48 (13.5%)	13 (14%)
Eurotiomycetes	13 (1.5%)	14 (3.9%)	
Lecanoromycetes	2 (0.2%)	4 (1.1%)	
Leotiomycetes	68 (7.7%)	32 (9%)	10 (10.8%)
Orbiliomycetes	6 (0.7%)		
Other Ascomycota	91 (10.3%)	7 (2%)	1 (1.1%)
Pezizomycetes	6 (0.7%)	21 (5.9%)	2 (2.2%)
Saccharomycetes	7 (0.8%)	4 (1.1%)	3 (3.2%)
Sordariomycetes	87 (9.9%)	49 (13.8%)	58 (62.4%)
Taphrinomycetes	2 (0.2%)		
<b>Basidiomycota Total</b>	<b>148 (16.8%)</b>	<b>85 (23.9%)</b>	<b>2 (2.2%)</b>
Agaricomycetes	83 (9.4%)	59 (16.6%)	1 (1.1%)
Cystobasidiomycetes	3 (0.3%)	4 (1.1%)	
Exobasidiomycetes	5 (0.6%)	3 (0.8%)	
Malasseziomycetes	2 (0.2%)		
Microbotryomycetes	12 (1.4%)	3 (0.8%)	
Other Basidiomycota	3 (0.3%)	3 (0.8%)	
Pucciniomycetes	5 (0.6%)	1 (0.3%)	
Tremellomycetes	35 (4%)	12 (3.4%)	1 (1.1%)
<b>Mucoromycota Total</b>	<b>22 (2.5%)</b>	<b>3 (0.8%)</b>	
Glomeromycetes	9 (1%)	3 (0.8%)	
Mortierellomycetes	13 (1.5%)		
Chytridiomycota	<b>5 (0.6%)</b>	<b>27 (7.6%)</b>	
Rozellomycota	<b>14 (1.6%)</b>		
Other/Unidentified Fungi	<b>281 (31.8%)</b>	<b>61 (17.2%)</b>	<b>4 (4.3%)</b>

Phyla are shown in bold with classes indented beneath. Taxa are shown if they contain more than one OTU in an amplicon dataset. Phylum classifications follow Spatafora et al. (2016)

nematodes, alveolates including *Paramecium* and *Stentor*, and other microbial eukaryote groups (Fig. 5). Two OTUs likely representing mites even appeared in the more fungal-specific ITS data set. LSU reads from trebouxiophycean algae, alveolates of the class Colpodea, and nematodes were detected across all sites. None of the non-fungal OTUs appeared at every site, but five were found in at least three quarters of the sites: one nematode, two chlorophyte algae, and two ciliophorans.

## 4 Discussion

Our sampling of *M. polymorpha* endophytes indicates a diverse community of fungi with high variation across space and even between habitats close to one another. Some of the

taxa repeatedly cultured or frequently detected by sequencing have no close relatives in sequence databases, making *M. polymorpha* a potential source for discovering new fungi.

### 4.1 Dominant and notable members of the *M. polymorpha* mycobiome

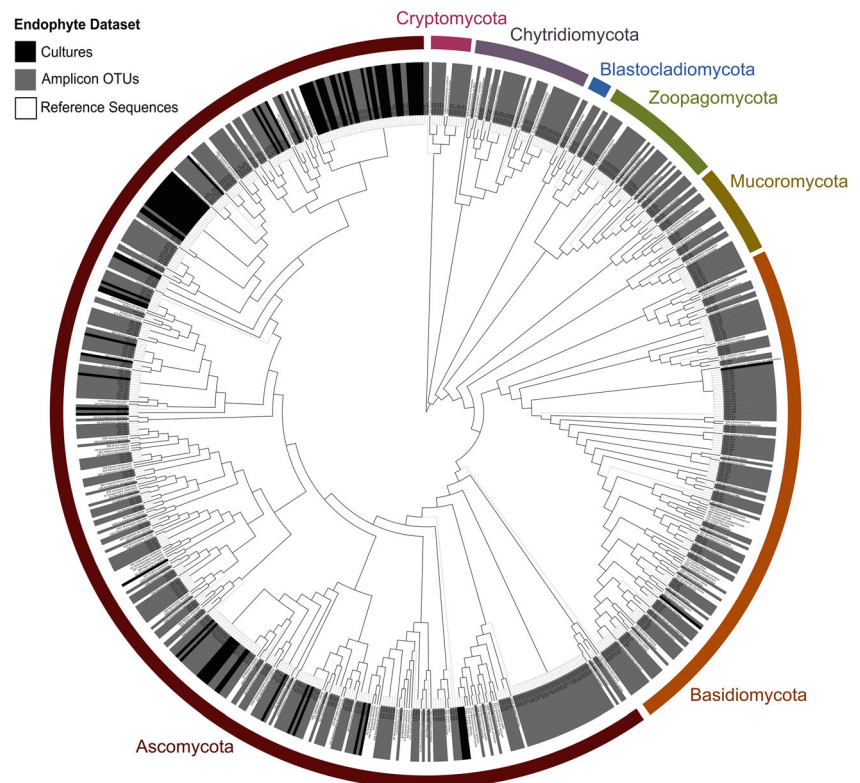
One fungus stood out as common between culturing and amplicon data sets: *Phoma herbarum*. This fungus is widespread in habitats ranging from terrestrial plants to marine fish (Ross et al. 1975). A strain of this species isolated as a soybean endophyte promoted growth of rice and soybean (Hamayun et al. 2009). A close relative of *P. herbarum*, *Phoma muscivora*, is a pathogen identified in boreal mosses (Davey and Currah 2009; Davey et al. 2009). *Phoma muscivora* produces enzymes capable of degrading bryophyte cell walls and may function as an opportunistic saprobe as well as a pathogen of live mosses (Davey and Currah 2009). Tests of the effects of *P. herbarum* isolates on *M. polymorpha* under controlled laboratory conditions do not indicate strong benefits or detriments (Nelson et al. 2018), but it may be that this widespread associate of *M. polymorpha* has effects only induced under natural conditions or certain stressors.

All datasets indicated that fungi from the order Helotiales were common (Table 4). Many of these could not be further identified, but one that could be identified to species stood out for its previously known association with *M. polymorpha*: *Pezoloma marchantiae* (Egertová et al. 2015; Garcia and Van Vooren 2005). Fruiting bodies with the macroscopic morphology of *P. marchantiae* were observed on plants collected from the Kentucky site in 2015 (Nelson 2017), which was reflected in the high recovery of *P. marchantiae* OTUs in the LSU dataset. This fungus is a close relative of the ericoid mycorrhizal fungus *Pezoloma ericae* and various *Pezoloma* species are also known to associate with bryophytes (Stenroos et al. 2010). These bryophyte-associated *Pezolomas* have been hypothesized to be saprobes specializing on bryophytes (Stenroos et al. 2010), but the recent demonstration of growth enhancement and nutrient transfer by *Pezoloma ericae* in two leafy liverwort hosts indicates that some fungi in the group could also function as mutualists (Kowal et al. 2018). Formation of fruiting bodies on plant hosts is sometimes interpreted as evidence of a parasitic function (Döbbeler 1997), but experiments with our *P. marchantiae* isolates under controlled conditions indicate no detrimental effects and even some growth enhancement of *M. polymorpha* (Nelson et al. 2018). If *P. marchantiae* has a mycorrhizal mutualism with *M. polymorpha*, this would likely only manifest in nutrient limiting conditions unlike those used in these growth experiments.

Another fungus previously known to associate with *M. polymorpha* is *Loreleia marchantiae* (Bresinsky and Schotz 2006). Mushrooms matching the macroscopic characteristics of *L. marchantiae* were found in the MSH2 site in



**Fig. 3** Phylogeny of fungal endophytes detected in *M. polymorpha*. Black tips are fungi isolated in culture, gray tips are OTUs from the LSU amplicon sequencing dataset, and white tips are reference taxa. ITS OTUs are not included because ITS is too variable for use in phylogenetic placement at this scale. Fungal phyla are marked by colored arcs



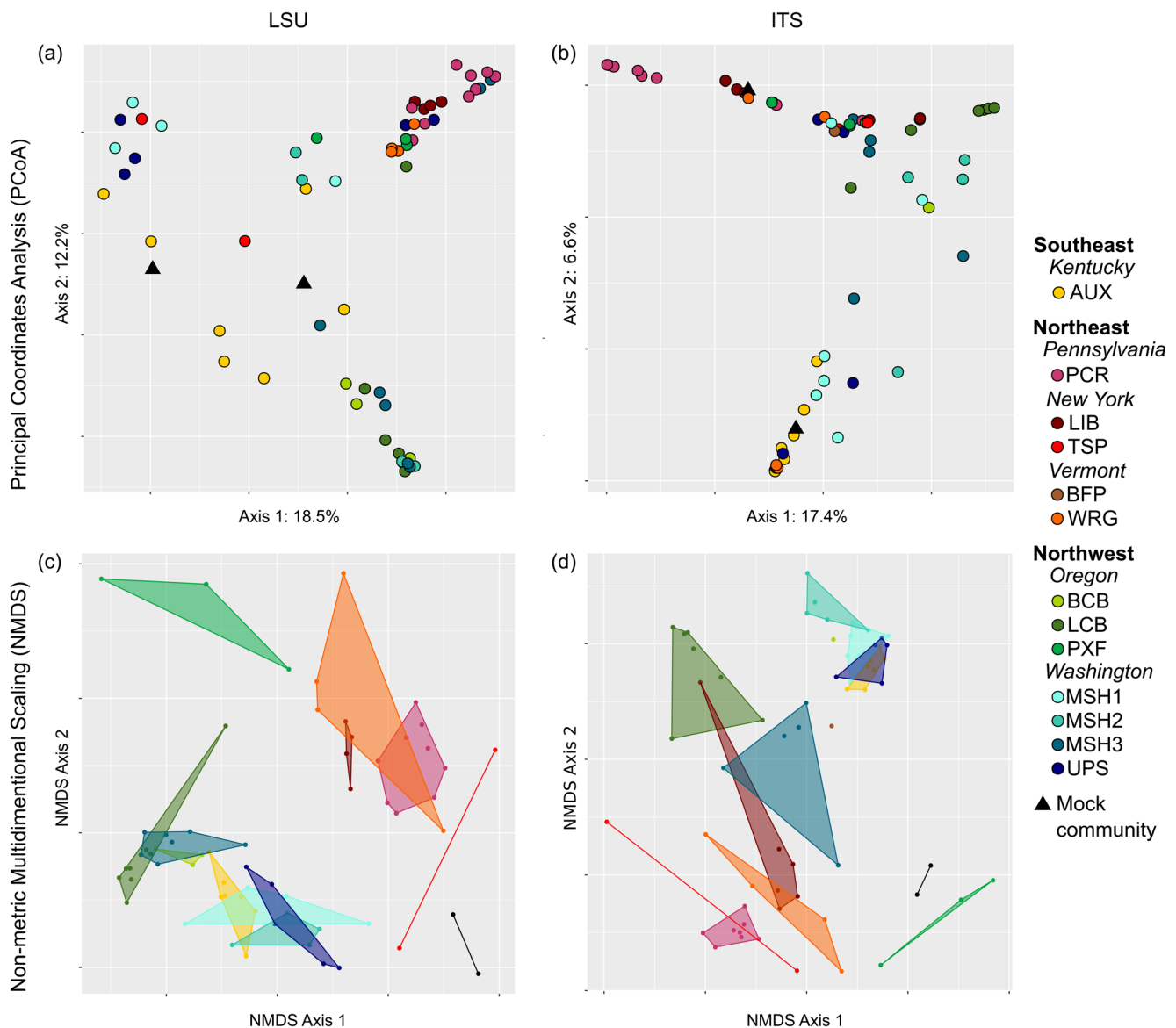
Washington at the time of collection (Nelson 2017), and Mt. St. Helens was one of the locations where it was abundant in the ITS amplicon dataset. It can be found fruiting on a number of complex thalloid liverworts and has been observed to infect both types of *M. polymorpha* rhizoids: smooth (live at maturity) and pegged (dead at maturity) (Bresinsky and Schotz 2006; Guminska and Mierzenska 1992). *Marchantia polymorpha* plants infected with *L. marchantiae* have been observed to form more oil cells and more smooth rhizoids and to attract colonies of cyanobacteria to gather among their rhizoids (Guminska and Mierzenska 1992).

Consistent with previous reports that *M. polymorpha* does not form arbuscular mycorrhizal associations (Ligrone et al. 2007), the few Glomeromycetes we detected by amplicon sequencing were in very low abundance. But contrary to previous findings (Ligrone et al. 2007), we detected these Glomeromycete OTUs in all three subspecies of *M. polymorpha*. Methods specifically targeting arbuscular mycorrhizal fungi would be needed to confirm this result. Our sequencing did, however, detect OTUs of other mycorrhiza-forming fungi in high abundance at certain sites, notably fungi belonging to Sebaciales, Russulaceae, and Thelephoraceae. Kowal et al. (2016, 2018) demonstrated that the ericoid mycorrhizal fungus *Pezoloma ericae* forms mutualisms with leafy liverworts and can provide an effective source inoculum for ericaceous plants; it is thus possible that associations of various mycorrhizal fungi with *M. polymorpha* could provide similar services.

A number of common endophyte genera frequently cultured from diverse vascular hosts were conspicuously absent from our culture collections. These included *Alternaria*, *Fusarium*, *Penicillium*, and *Trichoderma*. *Alternaria* and *Penicillium* were detected in relatively low abundance by the amplicon sequencing and OTUs of *Fusarium* were abundantly detected in the LSU data set at the Pennsylvania site which had no culture sampling. In contrast, no *Trichoderma* were identified in the amplicon data. *Trichoderma* cultures might have been discarded as contamination due to their fast-growing “green mold” morphology, but this would not have changed the amplicon sampling and the absence of this genus that is common in soils and plants is notable (Harman et al. 2004).

#### 4.2 Factors organizing fungal communities in *M. polymorpha*

The amplicon sequencing data indicate that *M. polymorpha* plants collected from a single patch usually had fungal communities most similar to other plants from the same patch. However, there was still significant variation within a patch, with few fungal OTUs being found in every plant from one patch. This could indicate stochasticity in the assembly of the fungal community for each separate *M. polymorpha* thallus, adjacent plants at different developmental stages having distinct communities, or inflated differences from incomplete sampling. Although there was some signal of differentiation



**Fig. 4** Variation in fungal communities detected in *M. polymorpha* by amplicon sequencing. PCoA (a, b) and NMDS (c, d) ordinations of fungal communities assessed by sequencing LSU (a, c) and ITS (b, d) amplicons. Each point represents the fungal community found in a single

*M. polymorpha* plant. Points are colored by sampling site and the color key applies to all four graphs. The ordinations reveal some separation in fungal communities between various sites and only weak separation of communities by geographic region

by geographic region, distance between sites did not correlate strongly with differentiation in fungal communities. For example, NMDS analyses show two of the Mt. St. Helens (MSH) sites having fungi more similar to those found in Kentucky (AUX) and elsewhere in Washington (UPS) than the third MSH site. This spatial heterogeneity is consistent with previous studies of endophyte communities of a single host at multiple sites (Arnold et al. 2001; Glynou et al. 2016), though caution should be used in interpreting this variability since accumulation curves for the fungal diversity collected from *M. polymorpha* were not saturated. If the variability does reflect reality rather than incomplete sampling, it suggests that local environmental factors on a relatively small scale or

stochastic processes are determining the pool of fungi available to colonize *M. polymorpha*. Local environmental controls may include soil characteristics, moisture, and the community of surrounding plants (Peay et al. 2016; Peršoh 2015).

Although we saw no clear correlation of sampling time with fungal community, it is likely that differences in seasonal conditions contributed to variability in the fungi detected, especially in the culture dataset which spanned multiple seasons and years. The site that we isolated cultures from in two consecutive years showed some continuity over time with three of the same taxa detected the first year also found the second year. The impacts of time and seasonal change on endophyte communities are so far underexplored, but the studies that

**Table 3** Core members of fungal communities in individual populations of *M. polymorpha*

Region		Northwest						Southeast		Northeast	
State		Oregon		Washington				Kentucky		Pennsylvania	
Site		LCB		MSH3		UPS		AUX		PCR	
Taxon	Dataset	ITS	LSU	ITS	LSU	ITS	LSU	ITS	LSU	ITS	LSU
Agaricomycetes sp.								1	1		
Ascomycota sp.		1								1	1
<i>Boeremia</i> sp.										1	
Cladosporiaceae sp.		1		1	1					1	2
Didymellaceae sp.							2		1	2	2
Fungus sp.										1	
Helotiales sp.		1	3		3	1			3		
Hypocreales sp.										1	
<i>Microdochium</i> sp.										1	
<i>Mortierella</i> sp.				1							
Mycosphaerellaceae sp.				1							
Orbiliaceae sp.											1
<i>Pezoloma marchantiae</i>		1	1	1	1		1		1		
<i>Phialophora</i> sp.										1	
<i>Phoma</i> sp.				1		1	1	1	1		
Pleosporales sp.										2	1
Sebacinales sp.								1	1		
Sordariales sp.				1							1
Sporocadaceae sp.										1	1
Stachybotryaceae sp.											1
<i>Stagonospora</i> sp.		1									
Thelephoraceae sp.				1	1						

Data are used for sites where at least five plants were included in the final amplicon dataset. Core fungi are those that are detected in all plants at the site at over 1% average read abundance. Numbers indicate how many OTUs for that taxon were core fungi for that site

have taken these variables into account show sometimes dramatic differences between years and even at scales as short as a month (Cordier et al. 2012; Davey et al. 2012; Junker et al. 2012; Peršoh 2015). Future repeated sampling of the same *M. polymorpha* populations over time would be needed to define the patterns and scale of its fungal endophyte community turnover.

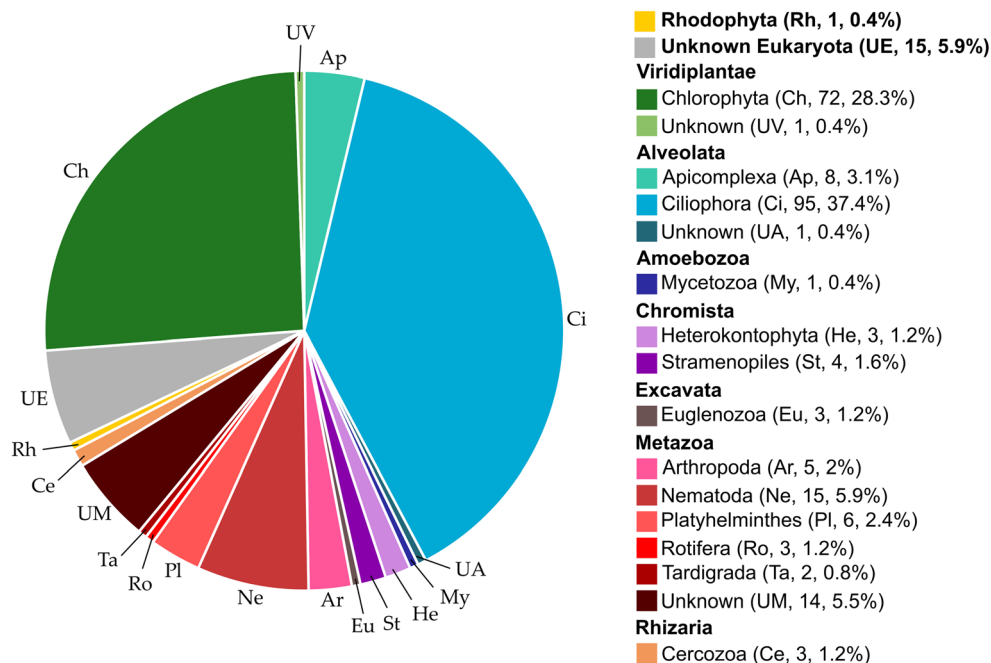
Another factor that can structure endophyte communities is tissue specificity. Previous studies have observed tissue specificity in culturable fungi from conifer and angiosperm hosts, documenting differences between fungal assemblages of stems, bark, leaves, fruits, roots, and flowers (Arnold 2007; Junker et al. 2012; Wu et al. 2013; Yuan et al. 2009). None of these sporophytic structures are present in haploid-dominant

**Table 4** Core members of the *M. polymorpha* fungal community

Cultures	%S	#	ITS OTUs	%S	AA	LSU OTUs	%S	AA
<i>Candida sake</i>	20%	3	<i>Cladosporium</i> sp.	92.3%	1.3%	Didymellaceae spp. (2)	91.7%	6.6%/ 7.5%
Helotiaceae sp.	20%	2	Helotiales sp.	53.8%	1.4%	Helotiales spp. (6)	58.3%/ 66.7%	1.2% - 3.9%
<i>Hypoxylon</i> sp.	20%	2	Hypocreales spp. (2)	61.5%	2.8%/ 4.8%	<i>Pezoloma marchantiae</i>	66.7%	24.5%
<i>Hypoxylon submonticulosum</i>	20%	3	Leotiaceae sp.	53.8%	1.9%	<b><i>Phoma</i> sp.</b>	66.7%	5.8%
<i>Nemania</i> sp.	20%	3	<i>Phialophora</i> sp.	53.8%	3.4%	Pleosporales spp. (2)	58.3%/ 83.3%	1.2%/ 3.6%
<i>Phoma herbarum</i>	30%	5	<b><i>Phoma</i> sp.</b>	100.0%	20.6%	<i>Tetracladium</i> sp.	66.7%	3.0%
Sordariomycetes sp.	20%	2	<i>Pyrenochaeta</i> sp.	53.8%	6.5%			
<i>Xylaria cubensis</i>	30%	8	Tremellomycetes sp.	69.2%	1.7%			

For cultures, all fungi isolated at more than one site are shown with the percent of sites they were found at (%S) and the number of isolates obtained (#). For the amplicon datasets, OTUs are shown if they were detected in at least 50% of sites and had an average normalized abundance of at least 1% of reads. Numbers in parentheses indicate how many OTUs with the listed name were present. When these multiple OTUs had different values for percent of sites or average abundance (AA), they are shown with a slash (for two values) or a dash to show the range of values represented. *Phoma*, the only core taxon shared across all three datasets, is highlighted in bold

**Fig. 5** Non-fungal eukaryotes detected in *M. polymorpha*. A total of 254 OTUs from non-fungal eukaryotes were detected with at least 20 reads in the LSU dataset. Kingdom names are shown in bold, phylum names in regular text. Parentheticals next to plotted taxa show two letter abbreviations used to label the pie chart sections, number of OTUs, and percent (rounded to one decimal place) of the total 254 non-fungal OTUs



bryophytes and tissue specificity of microbes has not been assessed for bryophytes. In our culturing, we separated *M. polymorpha* thalli, rhizoids, and gametangiophores and found some indication of tissue-specificity for certain fungi. Even without the complex, highly differentiated tissues found in tracheophytes, bryophytes may present multiple niches to their fungal inhabitants. Our observations suggest that differences even in the simpler tissues of a liverwort may be significant for structuring fungal communities at the small scale of bryophytes.

### 4.3 Comparison of culture and amplicon sampling of *M. polymorpha* endophytes

The enrichment of Sordariomycetes we observed in cultures relative to sequencing data has been observed previously using similar methods (Chen et al. 2018) and suggests either that the sequencing library preparation is biased against Sordariomycetes or that MEA culturing selects for them (or both). Overall, the extreme differences in abundance and limited overlap between culturing and sequencing data, and even between sequencing of two different gene regions, indicates that multiple methods must be combined to detect anything approaching the full fungal communities of plants. In addition, despite the improvements in estimated sampling completeness possible with amplicon sequencing over culturing, more intensive sampling is needed for all methods to sufficiently circumscribe the communities. Even with the combination of multiple sampling methods, many questions about the community composition remain. Our mock community sequencing control indicated that read abundances correlate

poorly to initial DNA content from different taxa, making interpretation of dominance of certain taxa questionable. In order to address this issue, future work on plant microbiomes can employ direct sequencing metagenomics or taxonomically specific in situ hybridization approaches.

### 4.4 Non-fungal associates of *M. polymorpha*

The most abundant non-fungal eukaryotes detected by LSU amplicon sequencing include chlorophyte algae, animals such as nematodes and mites, and alveolates. Such organisms are known to be common in the limnoterrestrial habitats provided by bryophytes (Glime 2017). It is possible that some of these organisms were not inside the liverwort tissues, but rather adhering to the surface strongly enough to resist the washing and sterilization treatments. Stalked ciliates like *Stentor* have been reported to adhere to bryophyte surfaces (Glime 2013a), microarthropods could shelter in the air pores on the liverwort's surface, and organisms like tardigrades and rotifers form resistant resting stages that might withstand washing. But it is also possible that some of the detected organisms could be inside the plant tissues. Nematodes might be inside galls formed in the plant (Glime 2013b) and some of the green algae may also be internal. Intracellular *Coccomyxa*-like algae have been reported to be widespread in *Ginkgo biloba* (Trémouillaux-Guiller and Huss 2007) and green algal endophytes have also been reported from a number of mosses (Reese 1981; Reese 1992). Little is known about the potential for most other non-fungal eukaryotic microbes to live as endophytes. So far, oomycete communities have been the main non-fungal eukaryotes assessed in plants, but Cercozoan

diversity has also been detected in the rhizosphere and phyllosphere of *Arabidopsis thaliana* and other Brassicaceae (Ploch et al. 2016; Sapp et al. 2018). In addition, instead of being inside *M. polymorpha* directly, some of the detected taxa may be living inside each other. For example, trebouxiophycean algae can be intracellular symbionts of ciliates like *Colpoda* and *Paramecium* (Glime 2013a; Hoshina and Kusuoka 2016).

#### 4.5 Concluding remarks

The global distribution of *M. polymorpha* and other *Marchantia* species makes this genus an attractive model for future research on host specificity and ecological factors determining microbiome assembly. Since *M. polymorpha* is often found in disturbed sites and is an early colonizer after fire (Bradbury 2006; Graff 1936), investigating its fungal communities along disturbance and successional gradients could elucidate how plant microbiomes respond to disturbance. The ease of growing axenic clones of *Marchantia* in the laboratory makes this genus a promising system for controlled experiments investigating the dynamics of complex endophytic communities that include not only of fungi and bacteria, but many other eukaryotes as well. Our study provides a foundation for such further investigations of liverwort microbiomes and use of the model *M. polymorpha* to answer questions about the ecology of plant microbiomes.

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