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High-Throughput Metabarcoding Characterizes Fungal Endophyte Diversity in the Phyllosphere of a Barley Crop

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ABSTRACT

Over the last decade, the microbiome has received increasing attention as a key factor in macroorganism fitness. Sustainable pest management requires an understanding of the complex microbial endophyte communities existing symbiotically within plants and the way synthetic pesticides interact with them. Fungal endophytes are known to benefit plant growth and fitness and may deter pests and diseases. Recent advances in high-throughput sequencing (HTS) have enabled integrative microbiome studies, especially in agricultural contexts. Here, we profile the fungal endophyte community in the phyllosphere of two barley (*Hordeum vulgare*) cultivars exposed to two systemic foliar fungicides using metabarcoding, an HTS tool that constructs community profiles from environmental DNA. We studied the fungal nuclear ribosomal large subunit D2 and internal transcribed spacer 2 (ITS2) DNA markers through a

bioinformatics pipeline introduced here. We found 88 and 128 unique amplicon sequence variants (ASVs) using the D2 and ITS2 metabarcoding assays, respectively. With principal coordinate analysis and permutational analysis of variance, ASV diversity did not change in response to barley cultivar or fungicide treatment; however, the community structure of unsprayed plants did change between two collection times 8 days apart. The workflow described here can be applied to other microbiome studies in agriculture and we hope it encourages further research into crop microbiomes to improve agroecosystem management.

Keywords: agronomy, endophyte, fungicide, *Hordeum vulgare*, metabarcoding, microbiome, phyllosphere, rhizosphere and phyllosphere

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Broadacre cropping leans heavily on chemical control measures to protect grain crops from disease, despite growing support for increased adoption of integrated pest management strategies (Walters et al. 2012). Pathogenic microorganisms cost the grain industry not only through lost yield potential but also through expensive crop protection regimes. Approximately one-third of the global trade of barley grain (*Hordeum vulgare*) is exported from Australia (ABARES 2017). The 2016–17 growing season in Australia produced 13.4 million metric tons of malting and feed barley, 9.5 million tons of which were exported (ABARES 2017). Despite these volumes, pathogenic microorganisms cause significant yield losses every year, leading Australian farmers to spend an estimated A\$33.4M on synthetic fungicides for barley protection every year (Murray and Brennan 2009, 2010). Fungi are the most economically damaging pathogens of barley in Australia; thus, synthetic fungicides such as demethylase inhibitors (DMI) and succinate dehydrogenase

inhibitors (SDHI) have become an important disease management tool (Mair et al. 2016; Murray and Brennan 2010).

Among grain producers, the broadly held attitude toward pest control tends toward “zero tolerance” of pathogens (Newton et al. 2010). However, most conventional fungicides have broad-spectrum activity, and their inhibitory effects do not discriminate between pathogenic fungi and nontarget organisms, including beneficial endophytes of broadacre and horticultural crops (Abdelfattah et al. 2016; Karlsson et al. 2014).

Endophytes are a ubiquitous group of microbes that asymptotically inhabit living plant tissues for at least some part of their life-cycle (Schulz and Boyle 2005; Stone et al. 2000). They form highly dynamic relationships thought to function along a continuum from mutualistic to (latent) pathogenic, where environmental stressors influence the nature of the symbiosis (Carroll 1988; Davitt et al. 2011; Saikkonen et al. 1998; Schulz and Boyle 2005). The phyllosphere, or crop canopy, provides a vital habitat for microbes that serve many ecological functions (Hawkes and Connor 2017); however, it also comes into direct contact with foliar fungicides.

Beneficial endophyte associations are known to support plant fitness (Brundrett 2006), deter insect and vertebrate herbivores (Cheplick and Clay 1988), and facilitate plant adaptation to environmental stress such as heat and drought (Rodriguez and Redman 2008; Rodriguez et al. 2010). For example, some fungal endophytes of the genera *Epichloë* and *Acremonium* are known to increase drought tolerance in wild cereals (Llorens et al. 2019; Schardl and Leuchtmann 2005). Endophytes also confer increased resistance to pathogenic microorganisms through various mechanisms, including chemical antagonism, niche overlap, and pathogen exclusion (Arnold et al. 2003; Blumenstein et al. 2015; Christian et al. 2017; O’Hanlon et al. 2012; Steinrucken et al. 2016). In an agricultural context, for example, *Aureobasidium pullulans* is a phyllosphere endophyte that is antagonistic to gray mold (*Botrytis cinerea*) in grapevine (Pinto et al. 2014) and strawberry (Sylla et al. 2013), and powdery mildew (*Blumeria graminis* f. sp. *tritici*) in durum wheat (De Curtis et al. 2012). Thus, endophyte inoculation as a form of biocontrol against microbial pathogens is gaining momentum (Abdelfattah et al. 2018; Murphy et al. 2018). Chemical management approaches with the aim of pathogen elimination are believed to disturb the health and resilience of complex microbial communities and, thus, whole agricultural ecosystems (Newton et al. 2010; Walters et al. 2012).

Until recently, studies into the effects of agricultural pesticides on plant endophyte communities have been limited by culture-dependent methodologies. Because most endophytes require living tissue to survive, relatively few can be isolated in pure culture and morphologically identified. Their presence was often misrepresented or missed entirely (Handelsman 2004; Wilson and Lindow 2000). This bias has been minimized with the uptake of culture-independent molecular identification approaches, particularly metabarcoding (Dissanayake et al. 2018).

Metabarcoding is a sensitive, high-throughput sequencing (HTS) method well-suited to exploratory investigations of biodiversity assemblages at the community or ecosystem level (Bálint et al. 2016; Porter and Hajibabaei 2018). From complex environmental DNA samples such as soil, water, and feces, entire microbial assemblages can be sequenced simultaneously (Taberlet et al. 2012). To delineate the sequences produced into taxa, they are traditionally clustered into operational taxonomic units (OTUs) based on a certain similarity threshold (Blaxter et al. 2005). Alternatively, amplicon sequence variants (ASVs) delineate sequences without clustering and, therefore, are preferred for generating finer sequence resolution and detecting rare sequences (Callahan et al. 2017).

Extensive research initiatives such as the Human Gut Microbiome Project and Metagenomics of the Human Intestinal Tract have deployed HTS techniques to study host-microbiome symbioses of the gastrointestinal system (Ley et al. 2006; Qin et al. 2010). Results from these efforts have linked microbiome disturbances to autoimmune diseases, inflammation, and obesity (Belkaid and Hand 2014; Hooper et al. 2012). HTS techniques have been recently adopted to investigate a similar theory in the plant microbiome: that plants suffering microbiome disturbances are more vulnerable to various biotic and abiotic stresses (Rastogi et al. 2013). In an agricultural context, this situation may arise due to certain management practices, particularly the application of chemical pesticides (Abdelfattah et al. 2016; Karlsson et al. 2014; Perazzolli et al. 2014; Prior et al. 2017; Siebers et al. 2018; Sommermann et al. 2018). Thus, sustainable pest management strategies should consider the effects of systemic pesticides on resident microbial communities. To our knowledge, only one study has investigated effects of fungicide on the phyllosphere community of cereal crops (Karlsson et al. 2014); however, endophytes were not sequenced independently of epiphytes.

Here, we use metabarcoding to sequence the fungal internal transcribed spacer 2 (ITS2) and D2 ribosomal DNA markers to investigate the endophyte diversity of a barley crop in the Western Australian grain belt. We aim to examine how the community of phyllosphere endophytes responds to exposure to two broad-spectrum foliar fungicides, a widely deployed DMI fungicide and a recently commercialized fungicide containing a mixture of an SDHI and another DMI. We also compared the endophyte communities hosted by two malting-grade barley cultivars, the highly disease-susceptible cultivar Baudin and the more resistant cultivar Granger.

MATERIALS AND METHODS

Trial design. Barley leaves were sourced from a field trial located in the Shire of Northam in the central wheatbelt region of Western Australia (−31.72929, 116.69671). The trial was sown into moist soil on 16 June 2018, after a late-season break. Two factors, barley cultivar and fungicide treatment, were randomly assigned to 18 plots measuring 1.44 by 10 m (Supplementary Fig. S1). The field trial was managed throughout the season following a conventional commercial program detailed in Supplementary Table S1.

Two barley cultivars were sown: the typically disease-susceptible Baudin, and Granger, which is more resistant to barley powdery mildew, barley leaf rust, and net type net blotch (DPIRD 2017). Two systemic foliar fungicides and water used as a control were sprayed when the crop reached the early stem elongation growth stage (GS31) (Zadoks et al. 1974). Fungicides were applied by professional operators in accordance with the label instructions. Folicur 430 (SC tebuconazole at 430 g/liter; Bayer CropScience) was applied at a rate of 290 ml/ha, while Aviator Xpro (EC bixafen at 75 g/liter and prothioconazole at 150 g/liter; Bayer CropScience) was applied at 500 ml/ha. Both fungicides have a broad spectrum of activity, though Folicur has a single mode of action (DMI) and Aviator has two (SDHI and DMI). The aforementioned treatment combinations are henceforth labeled with B (Baudin), G (Granger), C (control), F (Folicur), or A (Aviator). Due to treatment administration errors beyond our control, the six treatment combinations were replicated two (BC and GA), three (BF and GF), or four (BA and GC) times (Supplementary Fig. S1).

Sampling. All plots were sampled 1 day prior to the first fungicide application at GS31 and 8 days after the spray (including control plots that were not sprayed with fungicide). Each sample consisted of a pool of 10 leaves (the second fully emerged leaf from the youngest leaf) collected from 10 randomly selected plants in each plot. Additionally, to evaluate the magnitude of variability

in endophyte community compositions between leaves within the same plot, 10 leaves were randomly collected from one control plot of each cultivar at the second collection time and were processed individually rather than pooled.

Plant tissue preparation. Epiphytic microorganisms were removed from leaf surfaces by surface sterilizing the leaves. This was carried out in a UV-sterilized laminar flow cabinet to minimize environmental contamination, within 6 h of field sample collection. The sterilization protocol was adapted from Arnold (2002) as follows; a sterile scalpel was used to trim the raw leaf edge and halve leaves crossways; 10 leaves per sample were submerged and agitated in 70% ethanol for 15 s, 0.125% sodium hypochlorite for 30 s, and 70% ethanol for 15 s, then rinsed in sterile deionized water three times. Samples were placed in sterile 50-ml falcon tubes with two sterile 10-mm steel ball bearings before being snap frozen in liquid nitrogen. Samples were stored at -80°C for 48 h, then lyophilized for 16 to 24 h.

DNA extraction. To homogenize each pooled sample (from all trial plots), the freeze-dried material was mechanically ground using a Mixer Mill MM 400 (Retsch, Germany) for 5 min at 25 Hz. Subsamples of 40 to 50 mg of homogenized tissue were weighed into 2-ml microfuge tubes for each extraction. Leaves processed individually (from selected control plots) required a different tissue lysing procedure due to their sample volumes being too small for the Mixer Mill method. Instead, the single leaf samples were roughly fragmented using sterile forceps, and subsamples of 300 mg were placed in 2-ml Precellys lysing tubes with 1.4-mm ceramic beads (Bertin Technologies, France). They were mechanically homogenized in a Minilys instrument (Bertin Technologies, France) at top speed for 30 s.

DNA extraction was performed using the DNeasy Plant Mini Kit (Qiagen, The Netherlands) following the manufacturer's instructions but adapted for a larger sample volume by doubling reagent volumes (800 μl of buffer AP1, 8 μl of RNase A, and 260 μl of buffer P3). An equal mass of lysed plant material was used in DNA extractions regardless of the tissue lysing procedure used above. Extraction was continued with 500 μl of lysate, using QIAcube (Qiagen) following the standard "plant cells and tissues" protocol.

Fusion-tag quantitative PCR amplification. Two ribosomal DNA regions were amplified for HTS using universal fungal primers (Table 1). A 214-base portion of the D1/D2 hypervariable ribosomal large subunit (LSU) region of the 28S gene (Arbefeville et al. 2017) was amplified using the U1 forward (Putignani et al. 2008) and NL4 reverse (Kwiatkowski et al. 2012) primers. A second amplicon, the 122-to-245-base ITS2 region of the fungal barcode ribosomal ITS marker, was amplified using the ITS7f and ITS4 primer pair (Ihrmark et al. 2012; White et al. 1990). Two hypervariable regions were amplified to increase the diversity of taxa captured.

Amplification efficiency was determined with quantitative (q)PCR performed on the sample (neat) and a 1:10 dilution with ITS2 primers (Table 1). Amplification curves and cycle threshold

(C_T) values informed the optimal sample concentration with which to proceed with fusion-tag PCR that incorporates Illumina adapters in a single-step PCR (Supplementary Table S2).

Fusion-tag qPCR amplification was conducted with D2 and ITS2 primers modified with sequencing adapters and unique 6-to-8-base multiple identifier (MID) sequences with which to identify sequence reads (Supplementary Table S3). The master mix was prepared in a dedicated (PCR-free) clean lab using a QIAgility instrument (Qiagen) to minimize the risk of cross-contamination between fusion tags. Each PCR was duplicated to minimize stochasticity, and no-template (negative) controls were included to detect cross-contamination and background fungal contamination. No-template controls were carried through to account for contamination.

Reaction volumes were made up to 25 μl with 2 μl of MgCl_2 (Applied Biosystems, U.S.A.), 2.5 μl of 10 $\times$ PCR Gold buffer (Applied Biosystems), 0.25 μl of deoxynucleotide triphosphate (Astral Scientific, Australia), 1 μl of bovine serum albumin (Fisher Biotic, Australia), 0.6 μl of SYBR Green nucleic acid stain (Lonza, Switzerland), 0.2 μl of Ampli-Taq Gold DNA polymerase (Applied Biosystems), and 10 μM each of forward and reverse primer. In a separate laboratory, 2 μl of template DNA was added and PCR assays were carried out on a StepOne Plus real-time PCR instrument (Applied Biosystems). Cycling conditions consisted of one denaturation cycle of 95°C for 5 min; 45 cycles of 95°C for 30 s, annealing (52°C for D2 and 54°C for ITS2) for 30 s, and 72°C for 45 s; and final extension of 72°C for 10 min.

Amplicon library preparation and sequencing. The concentration of amplicons in each MID-tagged sample was evaluated based on qPCR Δ -normalized reporter value and C_T values to construct approximately equimolar minipools (Supplementary Tables S4 and S5). Amplicon minipool concentration was then assessed using the QIAxcel fragment analyzer (Qiagen) and samples were pooled accordingly to build a single sequencing library representing DNA from each sample. Fragments were size selected for 250- to 600-base lengths using the Pippin Prep instrument (Sage Sciences, U.S.A.).

The library was purified using the QIAquick PCR purification kit (Qiagen), yielding 50 μl of library. The DNA concentration of the purified library was determined using the Qubit Fluorometric Quantitation instrument (Thermo Fisher Scientific, U.S.A.) and diluted to 2 nM DNA. Finally, the library was spiked with the PhiX Control v2 library (Illumina, U.S.A.) to increase sequence diversity within the library.

Paired-end sequencing was carried out on the Illumina MiSeq platform (Illumina) (2×260 -base sequencing cycles) using the standard flow cell and MiSeq Reagent Kit 500 cycle v2 (Illumina) according to the manufacturer's instructions, except with custom forward and reverse sequencing primers.

Sequence analysis and taxonomic assignment. Paired-end reads were merged together using the StitchReads command in MiSeq Reporter v2.3, with a minimum overlap of 10 bases. The stitched sequences were demultiplexed using Geneious v10.2.6, allowing for no barcode mismatches, before primer trimming. Unidentifiable

TABLE 1
Primers used in this study

Primer	Target region ^a	Sequence (5'-3')	Temp ($^{\circ}\text{C}$) ^b	Reference
U1f	D2, forward	GTGAAATTGTTGAAAGGGAA	52	Putignani et al. 2008
NL4r	D2, reverse	GGTCCGTGTTTCAAGACGG	52	Kwiatkowski et al. 2012
ITS7f	ITS2, forward	GTGARTCATCGAATCTTTG	54	Ihrmark et al. 2012
ITS4	ITS2, reverse	TCTCCGCTTATTGATATGC	54	White et al. 1990

^a ITS = internal transcribed spacer.

^b PCR annealing temperature.

sequences and those with a missing barcode or primer were discarded. Data were imported as joined demultiplexed sequences into the software package Quantitative Insights Into Microbial Ecology 2 (QIIME 2) (Bolyen et al. 2019). Sequences with a median quality (Phred) score below 33 were discarded. Those remaining were truncated to a uniform length for denoising with Deblur (Amir et al. 2017; Bokulich et al. 2013); D2 amplicons were trimmed to 212 bases and ITS2 amplicons to 235 bases, and all shorter amplicons were discarded. The Deblur algorithm resolved unique sequences into ASVs (Callahan et al. 2017) based on custom ITS and LSU databases. A biological observation matrix format feature table was compiled in QIIME 2 with the ASV output (McDonald et al. 2012). De novo chimera filtering removed chimeric and borderline chimeric ASVs from the feature table using the UCHIME algorithm (Edgar et al. 2011) in accordance with the VSEARCH pipeline (Rognes et al. 2016).

Taxonomically redundant and erroneous ASVs were also removed using the LULU algorithm ('lulu' package) in R (Frøslev et al. 2017). This involved constructing a database of representative D2 and ITS2 sequences using VSEARCH v11 (Edgar 2010), from which match lists were created with VSEARCH (Rognes et al. 2016). These match lists and the ASV feature tables were input into the LULU algorithm to produce curated ASV tables. The final sets of D2 and ITS2 ASVs were analyzed as two independent datasets. Treating D2 and ITS2 ASVs separately in downstream analyses addressed database inconsistencies and alleviated the chance of taxonomic assignment error.

ASVs were compared with entries in GenBank using the Nucleotide Basic Local Alignment Search Tool (BLASTn) 2.7.1 (Altschul et al. 1997). The BLAST parameters included a maximum E-value of 0.001 and maximum target sequences of 500 hits (Shah et al. 2019).

Taxonomic assignment was then performed according to a lowest common ancestor algorithm which was applied manually to the BLAST matches. The reliability of different matches was evaluated with consideration of E value (less than 0.0001), percent similarity, year of entry, and the level of detail describing the entry. Any nonfungal ASVs were removed from the data set. The remaining matches for a given ASV were compared and assigned to the finest possible taxonomic level such that all valid entries agreed. ASV sequences that returned matches to the effect of "uncultured fungus" in GenBank were queried against the UNITE database (Nilsson et al. 2019) for further investigation. To identify UNITE database species hypothesis (SH) codes, ITS2 ASVs were queried against the UNITE database (version 8.2) using BLASTn (version 2.7.1), and the SH code (and corresponding DOIs) assigned to the highest scoring match by sequence identity was selected for the ASV. Taxonomic assignments from both assays were visually represented using Krona charts (Ondov et al. 2011) (Supplementary Files S1 and S2).

Statistical analysis. Sequencing depth was evaluated with rarefaction analysis in R (version 3.5.1) using the *rarecurve* function in the 'vegan' package (version 2.5-3) (Oksanen et al. 2018). Species accumulation curves were created with the *specaccum* function in vegan with the 'random' method to explore the relationship between the number of samples analyzed and the number of ASVs discovered.

Patterns in fungal community composition were explored using the PRIMER v7 program (Clarke and Gorley 2015). First, the data were transformed into presence-absence matrices, then expressed as Jaccard distance matrices (Schaalje and Beus 1997). Principal coordinate analysis (PCoA) was used to examine the data for ordination based on cultivar, fungicide treatment, sample type (pooled and unpooled), and collection time. Permutational analysis of variance (PERMANOVA) (Anderson 2017) was performed on Jaccard distance matrices to evaluate the statistical significance of detected

differences between these factors, as well as interaction effects between factors. This was followed up with PERMDISP analysis (Anderson 2017) in PRIMER to test for homogeneity of group dispersions.

RESULTS AND DISCUSSION

Improved metabarcoding workflow for profiling endophytes.

This study aimed to profile the fungal endophyte community of the barley phyllosphere using metabarcoding and observe its response to fungicide exposure and host cultivar. To this end, we developed a workflow integrating both experimental and computational phases. It is built on established technologies where possible such as the QIIME 2 bioinformatics pipeline (Bolyen et al. 2019).

Although diverse metabarcoding pipelines are described for the analysis of microbiomes from different ecological contexts (Giauque and Hawkes 2013; Lau et al. 2013; Oono et al. 2015), to our knowledge, no workflows have been established for the metabarcoding analysis of phyllosphere endophytes of crops. Few published workflows involve sample processing steps that focus specifically on endophyte communities in agricultural contexts (Prior et al. 2017), and fewer still employ HTS methods. Several studies have examined foliar microorganisms at a community level using HTS but endophytes and epiphytes are commonly studied simultaneously (Abdelfattah et al. 2015, 2016; Gdanetz and Trail 2017; Karlsson et al. 2014, 2017). Others sequence material from leaf washings, thus excluding endophytes altogether (Moulas et al. 2013; Perazzolli et al. 2014). Targeting endophytes in this study gives weight to those microorganisms that are more likely to be involved in close symbioses with the host plant (Hawkes and Connor 2017; Rodriguez et al. 2009). Thus, the workflow in this study involved surface sterilization to reduce "background noise" from epiphytic organisms in downstream processes. In developing this workflow for agricultural systems, we integrated processes from leaf washing for endophyte separation through to bioinformatics processing to sequence communities that are most likely to affect crop growth and productivity.

Sequence data. The fungal endophyte community of the barley phyllosphere was profiled using Illumina sequencing of two fungal hypervariable barcode regions, to increase the diversity captured and evaluate their relative suitability. Sequencing yielded more than 12 million raw sequences (deposited to NCBI BioProject PRJNA693133), of which 64% were demultiplexed into 56 samples per primer assay. Of the demultiplexed reads, 68% passed quality filtering stages and D2 and ITS2 amplicons were clustered into 88 and 128 unique ASVs, respectively. ITS2 ASVs are linked with SH accession codes and DOIs through the UNITE community to ensure reproducibility and long-term stability of our dataset over time (Supplementary Table S6). ASVs, also known as exact sequence variants or zero-radius OTUs, can delineate taxa at a higher resolution than the standard of 97% OTU clustering. As such, resolving ASVs is preferred over OTU clustering to a standard threshold (Callahan et al. 2017).

Given that this analysis was based on presence or absence rather than abundance, ASVs composed of single sequences were retained based on the assumption that they represent actual taxa. One ITS2 ASV matching to *H. vulgare* was discarded due to its nonfungal origin. Rarefaction analysis indicated that sequencing depth for both assays was sufficient with samples approaching saturation (Fig. 1). However, ASV accumulation curves did not approach saturation, indicating that community diversity was not sufficiently captured within the samples analyzed (Fig. 2).

Across both D2 and ITS2 assays, a greater number of unique ASVs were detected from samples collected before fungicide treatment than after. In general, the ITS2 assay detected significantly

more ASVs than D2, and showed little sign of plateauing as the number of samples increased (Fig. 2). It follows that sampling more leaves would detect more of these rarer ASVs. The taxonomic diversity of ASVs sequenced was visually represented for each assay with Krona charts (Supplementary Files S1 and S2). The D2 assay represented species of Ascomycota and Basidiomycota almost equally, while ITS2 represented a relatively larger proportion of Ascomycota and in greater depth. Further, Sordariomycetes and Dothideomycetes, two fungal classes that represent significant groups of endophytes and pathogens, are represented in greater depth by ITS2. In fact, D2 did not sequence taxa of the class Sordariomycetes. For this reason, we would only use the ITS2 region in future studies. Although D2 detected some different taxa within division Basidiomycota, for the purpose of studying agriculturally important pathogens and endophytes, we do not believe this loss of detail is important.

A heatmap depicting the variation in assemblages across single-leaf samples (unpooled, collected from control plots only) suggested that each leaf housed generally similar assemblages but, although some ASVs were present in all leaves, others were found in just one or two (Supplementary Fig. S2). In future studies, sample pooling may require more replicates (>10 leaves), depending on the size of the sample area, to increase biodiversity coverage.

Factors affecting fungal endophyte community. The effects of cultivar, fungicide treatment, and collection time on the fungal endophyte community was evaluated with PERMANOVA and PCoA. The effect of cultivar on community composition was not significant, consistent across both D2 and ITS2 assays ($\text{Pseudo-}F_{(1,48)} = 0.927$, $P = 0.534$ and $\text{Pseudo-}F_{(1,48)} = 1.05$, $P = 0.370$, respectively). Similarly, fungicide treatment did not have a significant effect on ASV fungal community composition ($\text{Pseudo-}F_{(2,48)} = 0.650$, $P = 0.950$ and $\text{Pseudo-}F_{(2,48)} = 0.967$, $P = 0.511$, respectively). Analysis and ordination of the data using PCoA detected no discrete grouping of the samples of any treatment compared with controls (Fig. 3), also indicating that neither the cultivars nor fungicides used here significantly affected fungal endophyte communities of barley.

Even so, the topic of pesticide disturbance to nontarget microorganisms in agricultural ecosystems is gaining prominence as knowledge

of microbiome functional roles increases (Hawkes and Connor 2017; Nettles et al. 2016). The premise that pathogen-resistant and -susceptible host cultivars support different microbiomes was investigated by Kwak et al. (2018) in tomato plants. The study focused on a soilborne pathogenic bacterium and, thus, the plants' rhizosphere microbiome. Resistant and susceptible tomato cultivars were found to host different microbiomes. Furthermore, inoculating susceptible tomato plants with certain microorganisms associated with the resistant cultivar suppressed disease symptoms in the typically susceptible host. However, it was noted that rhizosphere communities differed more between sampling times that were 14 days apart than between cultivars (Kwak et al. 2018).

Similarly, the present study found that fungal community composition changed significantly between the first and second collection times, according to data from D2 and ITS2 amplicons ($\text{Pseudo-}F_{(1,48)} = 7.431$, $P < 0.001$ and $\text{Pseudo-}F_{(1,48)} = 4.853$,

Fig. 2. Amplicon sequence variant (ASV) accumulation curves with 95% confidence interval show the relationship between sampling depth and the number of unique ASVs discovered. ITS = internal transcribed spacer.

Fig. 1. Rarefaction curves presenting the relationship between sequencing depth (number of reads per sample) and the number of unique amplicon sequence variants (ASVs) identified using **A**, D2 and **B**, internal transcribed spacer 2 primer assays.

$P < 0.001$, respectively). This is supported by PCoA that shows clear object separation between control samples collected at different time points, indicating that metabarcoding is capable of detecting change in community assemblage (Fig. 4). Among “After”

data, the control (Fig. 4, black outline) and fungicide-treated objects do not show distinct grouping from each other, indicating that collection time, more than fungicide treatment, is driving community composition. Despite the distinct before-and-after grouping,

Fig. 3. Community composition ordinations based on barley cultivar and fungicide treatment. Fungal community composition was explored on presence-or-absence Jaccard resemblance matrices. Amplicon sequence variants (ASVs) from **A**, D2 amplicons and **B**, internal transcribed spacer 2 amplicons do not show strong ordinations with principal coordinate (PCO) analysis based on the barley cultivar and fungicide treatment factors. BC = Baudin-Control, BF = Baudin-Folicur, BA = Barley-Aviator, GC = Granger-Control, GF = Granger-Folicur, and GA = Granger-Aviator.

Fig. 4. Community composition ordinations based on sample collection time. Fungal community composition was explored on presence-or-absence Jaccard resemblance matrices. Amplicon sequence variants (ASVs) from **A**, D2 amplicons and **B**, internal transcribed spacer 2 amplicons show significant object separation ($P < 0.001$) with principal coordinate (PCO) analysis between sample collection time (Before = before fungicide spray and After = 8 days after fungicide spray). Objects with black outlines are unsprayed (all data before fungicide spray and control data after spray).

considerable variation was unexplained by the first two axes of the ordinations (Fig. 4) and is perhaps explained, in part, by the need for more replication.

Similar studies investigating fungicide effects on crop microbiomes have also found that secondary factors have a greater impact on community composition than the fungicide treatment in question such as plant organ and growth stage in wheat (Gdanetz and Trail 2017), geographical location (e.g., proximity between paddocks in wheat [Karlsson et al. 2014] and grapevines [Perazzolli et al. 2014]), climatic conditions in grapevines (Perazzolli et al. 2014), and sampling date in tomato (Kwak et al. 2018). This highlights the inherent variability and transience of microbiome assemblages across time and space but, at the same time, it reveals a level of resilience in resident microbial communities against chemical disturbance (Perazzolli et al. 2014). Heightened tolerance to fungicides in the endophyte community could be a natural adaptation resulting from exposure to toxic molecules such as those produced by the plant to defend itself, those produced by other fungi, metabolic waste products, and other environmental toxins. Analogous studies have detected significant impacts of pesticides on phyllosphere community composition, particularly in the relative abundance of certain taxa rather than overall species richness (Abdelfattah et al. 2016; Karlsson et al. 2014; Nettles et al. 2016).

In this study, when the relative presence of individual ASVs in treated and untreated samples collected on the same day was compared, notable changes were not detected (Supplementary Tables S7 and S8). It is possible that we did not observe fungicide effects on the fungal endophyte community due to epistemic limitations. The length of time following exposure to a foliar fungicide in which it is considered to have acted varies between chemicals, formulations, application methods, and environmental conditions. Furthermore, the time taken for DNA to break down in the phyllosphere is not well studied. Microorganisms that are dead leave “legacy” (postmortem) DNA fragments which could be sequenced if amplified from plant tissue. To capture a narrower time-frame of “live” molecular data, a combination of DNA and RNA can be extracted for metabarcoding because RNA degrades faster than DNA and, thus, could be a more useful proxy for living organisms (Pochon et al. 2017). However, other studies that investigate fungicide effects on the microbiome do not appear to account for this. It is also possible that microbiome fungi rapidly repopulated the phyllosphere in the time before resampling occurred. Therefore, the sampling window, which must account for fungicidal activity, the rate of molecular degradation, and recovery of the microbiome, requires further investigation. Finally, fungicide treatment may have altered the proportions of ASVs, rather than their presence or absence. The multicopy nature of these markers makes it difficult to explore relative abundance, and this is an area where metabarcoding of single-copy genes may be of more value.

To study species richness across sample collection times, the total number of ASVs detected in control samples at each time was compared. We found that species richness did not vary significantly between sampling dates, which may be due to the relatively short 8-day sampling difference. Of 48 unique D2 ASVs, 35 were identified from samples obtained during the first collection compared with 33 ASVs from the second sampling collected 10 days later. Similarly, 55 of 78 ITS2 ASVs were identified from the first sampling date and 53 from the second. Although the total number of ASVs between sampling times is relatively consistent, certain ASVs did vary considerably in their relative presence and prevalence (Table 2). Also of note is that >7% of ASVs matched uncultured samples or could not be matched to a taxonomic rank beyond class in GenBank or UNITE. It is known that fungal biodiversity is immense and highly unknown, with accepted estimates ranging up

to 3.8 million species when considering the vast numbers of unknown plant-associated fungi (Hawksworth and Lücking 2018). Although molecular methods are increasing rates of discovery, it is unsurprising that unidentified genotypes have been sequenced here. However, the extent of novel diversity in a dryland monoculture system is intriguing. As endophytes continue to be studied and entries accumulate in databases such as GenBank and UNITE, the quantity, quality, and reliability of sequence match data will improve.

Biological significance. Considerable fungal diversity was found; however, the most frequently detected fungal taxa were Dothideomycetes, Tremellomycetes, Leotiomycetes, and Sordariomycetes. Other studies sequencing fungal endophytes of wheat also found that Dothideomycetes, Leotiomycetes, and Sordariomycetes were dominant taxa (Gdanetz and Trail 2017; Karlsson et al. 2017). Pleosporales was the most represented fungal order, though many ASVs were not assigned at the order level. Members of Pleosporales included common plant pathogens of genera *Pyrenophora*, *Alternaria*, *Parastagonospora*, and *Leptosphaeria* and the known endophytes *Preussia* and *Sporormiella*. Other common plant pathogens outside of Pleosporales include genera *Blumeria*, *Puccinia*, *Fusarium*, and *Botrytis*. Although technically still endophytic, taxa that belong to other functional groups, including saprophytes (*Aureobasidium*, *Alternaria*, and *Cladosporium*) and yeasts (*Filobasidium* and *Cystofilobasidium*), were also detected.

Pleosporales, the most represented fungal order, showed a mixed response with respect to detection between sampling times. Some ASVs were detected less frequently at the later collection date (e.g., D2 ASV35 and ASV31, and ITS2 ASV21 and ASV30) whereas the detection of others increased at the later collection (e.g., D2 ASV56, ASV16, and ASV30, and ITS2 ASV51). Other members of the order Pleosporales didn't change over the time period, regardless of the assay employed. For example, the genera *Alternaria*, *Sporormiella*, *Preussia*, and *Pyrenophora* were prevalent regardless of sampling date (Supplementary Tables S9 and S10). It is interesting that *Alternaria* and *Pyrenophora* spp., which represent common cereal pathogens, are among the most persistent in the community. Conversely, endophytic *Preussia* spp., including Australian taxa, are known to produce bioactive secondary metabolites that are antimicrobial against bacteria and yeasts (Mapperson et al. 2014). Persistence in the environment, as observed here, is a favorable trait in screening for biological control agents because it may indicate a robust longer-lasting effectivity if a practical application is designed. Other taxa became rarer among control plots over time such as the genus *Filobasidium* (D2 ASV 36, ITS2 ASV118, and ITS2 ASV37) and orders Tremellales (D2 ASV40 and ITS2 ASV107, ASV60, and ASV87), Dothideales (D2 ASV38), Helotiales (D2 ASV4), Leotiomycetes (D2 ASV88), and Cystofilobasidiales (ITS2 ASV15) (Table 2). Because these samples came from plots not exposed to fungicide treatment, we suggest that changes in community composition can be attributed to succession, the ecological processes that drive natural community progression.

Niche overlap, whereby resource-based competition results in the exclusion of less competitive taxa, is one ecological process that can alter endophyte community composition over time (Hawkes and Connor 2017). Resource availability can be driven by climatic conditions and ensuing abiotic stress (Giauque and Hawkes 2016), though this is unlikely to drive significant variation within the sampling period in this study. The availability of physical niche space as the barley plant grows is more likely to spur competition between endophyte species. The life cycle and mode of intergenerational transmission may also affect community progression (Rodriguez et al. 2009). Most endophytes do not inhabit plant tissues for their whole life

TABLE 2
Taxonomic assignment of amplicon sequence variants (ASVs) that differed most in prevalence between collection times

ASV ^a	Plots (n) ^b		Taxonomic assignment				Id (%) ^c	E value
	22A	31A	Order	Family	Genus	Assignment		
D2								
ASV36	5	1	Filobasidiales	Filobasidiaceae	Filobasidium	<i>Filobasidium</i> sp.	100	2.18E-105
ASV40	5	1	Tremellales	Cryptococcaceae	Cryptococcus	<i>Cryptococcus</i> sp.	100	2.18E-105
ASV38	4	1	Dothideales	Sacrotheciaceae	Aureobasidium	<i>Aureobasidium</i> sp.	100	2.18E-105
ASV4	4	0	Helotiales	–	–	Helotiales 1	97	5.00E-96
ASV35	4	0	Pleosporales	Didymellaceae	–	Didymellaceae 1	100	2.18E-105
ASV12	3	0	–	–	–	Dothidiomycetes	100	2.18E-105
ASV31	3	0	Pleosporales	–	–	Pleosporales 1	100	2.18E-105
ASV88	3	0	Leotiomycetes	Helotiales	Tetracladium	<i>Tetracladium</i> sp.	100	2.18E-105
ASV56	3	6	Pleosporales	Sporormiaceae	Preussia	<i>Preussia similis</i>	100	2.18E-105
ASV16	2	6	Pleosporales	Sporormiaceae	Preussia	<i>Preussia</i> sp.	100	2.18E-105
ASV21	1	4	Pucciniales	Pucciniaceae	Puccinia	<i>Puccinia</i> sp.	100	2.18E-105
ASV30	0	3	Pleosporales	–	–	Pleosporales 2	100	2.18E-105
ITS2								
ASV21	6	3	Pleosporales	Pleosporaceae	Alternaria	<i>Alternaria eureka</i>	99.576	1.88E-116
ASV99	5	1	–	–	–	Uncultured Ascomycota	100	4.03E-118
ASV118	5	1	Filobasidiales	Filobasidiaceae	Filobasidium	<i>Filobasidium oeirense</i>	100	4.03E-118
ASV37	4	1	Filobasidiales	Filobasidiaceae	Filobasidium	<i>Filobasidium</i> sp.	100	4.03E-118
ASV107	4	0	Tremellales	Bulleribasidiaceae	Vishniacozyma	<i>Vishniacozyma dimennae</i>	100	4.03E-118
ASV15	3	0	Cystofilobasidiales	Cystofilobasidiaceae	Cystofilobasidium	<i>Cystofilobasidium macerans</i>	100	4.03E-118
ASV30	3	0	Pleosporales	Didymellaceae	–	Didymellaceae 2	100	4.03E-118
ASV60	3	0	Tremellales	Tremellaceae	Tremella	<i>Tremella</i> sp.	100	4.03E-118
ASV87	3	0	Tremellales	Bulleribasidiaceae	Vishniacozyma	<i>Vishniacozyma</i> sp.	100	1.00E-117
ASV51	3	6	Pleosporales	Sporormiaceae	–	Sporormiaceae 1	100	4.03E-118

^a ITS = internal transcribed spacer.

^b Number of plots in which ASV was present. Data are from untreated plots only (of maximum six). Collection times as indicated are 22 August (22A) and 31 August (31A) 2018.

^c Identity match.

cycle (Hawkes and Connor 2017), further suggesting that the occupation of phyllosphere tissue is transient.

Conclusions. Using our metabarcoding workflow, we were able to profile a community of fungal endophytes in barley and develop a workflow which lays a foundation for further investigation into phyllosphere endophytes in agricultural ecosystems. We set out to investigate the impacts of host cultivar and foliar fungicides on the fungal endophyte community. Although we did not detect a significant response to these factors, the community changed significantly between collection times, which were 8 days apart. Further investigation into the capacity of DNA metabarcoding, compared with combined DNA and RNA metabarcoding, to detect live organisms but exclude dead ones may be required to verify the nil response to fungicides found here. Also, sampling at more frequent time points would be useful to further dissect the response of the endophyte communities to fungicide treatment.

Our current understanding of the plant microbiome in a production context is significantly lacking, though the growing accessibility of genetic sequencing platforms is changing this. Further research into phyllosphere microbiomes will increase knowledge about integrated pest management and improve the capacity for producers to enhance the health and resilience of not only crop plants but also agricultural ecosystems as a whole.

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