



The emergence of beech leaf disease in Ohio: Probing the plant microbiome in search of the cause

David J. Burke¹ | Adam J. Hoke¹ | Jennifer Koch²

¹The Holden Arboretum, Kirtland, OH, USA

²The United States Forest Service, Delaware, OH, USA

Correspondence

David J. Burke, The Holden Arboretum, 9500 Sperry Road, Kirtland, OH, USA.
Email: dburke@holdenarb.org

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Abstract

American beech (*Fagus grandifolia*) is the target of a newly emerging disease in North America called beech leaf disease (BLD) that affects and disfigures leaves and which can lead to tree mortality. Beech leaf disease may be caused by a newly recognized subspecies of the anguinid nematode *Litylenchus crenatae* subsp. *mccannii*, but the associations of this nematode with bacterial and fungal taxa are unknown. We examined microbial communities associated with beech leaves affected by BLD in a 16-year-old American beech plantation using molecular methods. We detected *L. crenatae* subsp. *mccannii* in anywhere from 45% to 90% of leaves depending on the degree of visual BLD symptoms. Approximately 37% of asymptomatic leaves contained *L. crenatae* subsp. *mccannii*, whereas 90% of buds associated with symptomatic leaves contained *L. crenatae* subsp. *mccannii*. We found that fungal communities on leaves and buds were unaffected by BLD, but bud and leaves had significantly different fungal communities. Bacterial communities on buds also were unaffected by BLD, but bacterial communities were significantly different between symptomatic and asymptomatic leaves suggesting that the nematode could be altering the community of bacteria on the leaves. Clone libraries indicate that *Wolbachia*, an intracellular endosymbiont of arthropods, was found only on symptomatic leaves and buds associated with either symptomatic or asymptomatic leaves. In addition, only symptomatic leaves contained taxa in the genus *Mucilaginibacter*, which previous studies suggest could produce exopolysaccharides. These bacterial taxa could represent a marker for the vector of *L. crenatae* subsp. *mccannii* that enables spread between trees and a possible endosymbiont that could facilitate nematode feeding and establishment on nematode infested leaves. Our results are the first to examine changes to the leaf microbiome of this newly emerging pest and may aid identification of mechanisms associated with the spread and success of *L. crenatae* subsp. *mccannii*.

KEYWORDS

bacteria, beech leaf disease, *Fagus grandifolia*, fungi, leaf nematodes, *Litylenchus crenatae* subsp. *mccannii*, plant microbiome

1 | INTRODUCTION

American beech (*Fagus grandifolia*) is an important hardwood forest tree species in the northern United States and Canada. The species is an important source of nuts which are used as a food resource by wildlife including birds and mammals (Cale, McNulty, Teale, & Castello, 2013; Jakubas, McLaughlin, Jensen, & McNulty, 2005; Jensen, Demers, McNulty, Jakubas, & Humphries, 2012). In many regions where oak and hickory are relatively rare, *F. grandifolia* nuts may be the predominant source of hard mast available for wildlife (Jakubas et al., 2005). *Fagus grandifolia* also has commercial uses as a timber species. Despite its importance, *F. grandifolia* is threatened by an invasive disease, beech bark disease (BBD), which is caused by an interaction between the invasive beech scale (*Cryptococcus fagisuga*) which entered North America from Europe in the 1890s, and fungal pathogens in the genus *Neonectria* which invade the phloem and cambium of the tree weakening and even killing many trees with mortality rates reaching 50%–80% in the first wave of the disease (Cale et al., 2013; Koch, 2010). Although the feeding by *C. fagisuga* is considered relatively harmless to the tree, the scale acts as a vector for *Neonectria* which can enter the small fissures created by the scale to infect the tree. Beech bark disease has caused widespread mortality of *F. grandifolia* throughout much of the Northeastern United States and substantially changed forest composition towards beech *F. grandifolia* thickets in many areas, which develop as adult trees die from the disease and root sprouts are stimulated to grow (Cale et al., 2013). Despite BBD, beech remains an important forest tree, and mature stands are still found in many places including northern Ohio. The value of beech to both state and national forest managers is evidenced by investments in breeding programmes to produce BBD-resistant seed sources (Koch & Heyd, 2013; Koch et al., 2015).

Unfortunately, despite progress in producing BBD-resistant American beech, recently, a new emerging threat to *F. grandifolia* has developed in northern Ohio that leads to decline, senescence and mortality of *F. grandifolia* leaves and has come to be called “beech leaf disease” (BLD) (Macy & ODNR, 2019). First observed in Lake County, Ohio in 2012, BLD has spread to 24 counties in Ohio, western Pennsylvania, western New York and southern Ontario. At the Holden Arboretum in Lake County, Ohio, BLD was first noticed in 2014 and has since spread rapidly through the Arboretum's 1,250 ha of natural areas which are dominated by *F. grandifolia*. Beech leaf disease also now affects 97% of the accessioned beech trees in Holden's collection and in addition to *F. grandifolia* also affects European beech, (*Fagus sylvatica*), Chinese beech (*Fagus engleriana*) and Oriental beech (*Fagus orientalis*). Beech leaf disease is characterized by interveinal thickening and darkening of leaf tissue followed by blackening of tissue and leaf curling (Figure 1). Leaves often senesce by late summer, and lower and understory leaves and branches are often frequently affected. Although death of canopy trees at this point is rare, having been observed primarily in or near stands known to have had BLD symptoms for several years, declines in leaf canopy cover have been detected (increases in gap fraction, a measurement of canopy openness, from 4.5 to 9.1; personal communication Michael Watson), and understory beech mortality is frequent. This suggests that BLD has

the potential to negatively impact both the survival and regeneration of beech within hardwood forests, eventually leading to alteration of both canopy cover and environmental conditions within forests. Until recently, the cause of BLD was unknown.

However, new experimental work has determined that BLD is associated with the presence of a non-native nematode, *Litylenchus crenatae* subsp. *mccannii*, that seems to have originated from the Pacific rim (Carta et al., 2020). The vector by which the nematode accesses the leaves of canopy trees and the infection mechanism are poorly understood. However, experimental inoculation of tree buds with nematodes isolated from infected trees resulted in BLD symptoms on leaves that emerge from inoculated buds (Carta et al., 2020). Inoculation of fully developed leaves with nematodes has so far failed to initiate symptoms of BLD, suggesting that nematodes may need to colonize and inhabit bud tissue for a portion of their life cycle. Alternatively, secondary infections caused by fungi or bacteria may be involved in leaf senescence, or the nematodes may harbour symbiotic bacteria or fungi that could facilitate damage to leaf membranes and enhance feeding success. Entomopathogenic nematodes which kill insects often harbour symbiotic bacteria that kill the insect thereby allowing the nematode to feed on the resulting carcass (Murfin et al., 2012). A similar association between *L. crenatae* subsp. *mccannii* and bacteria could exist allowing for efficient feeding of the nematode, especially if bacteria assist in cell wall degradation. But very little is known about the life cycle of *L. crenatae* subsp. *mccannii*, and its microbial partners and additional study are greatly needed.

In this work, we examined the microbiome of *F. grandifolia* leaves affected to varying degrees by BLD to determine whether known pathogenic microbes could be detected within the leaves and possibly associated with *L. crenatae* subsp. *mccannii*. Although *L. crenatae* subsp. *mccannii* may be the proximate cause of BLD, the disease complex is not well understood, and other microbial partners or opportunistic microbes could be involved. Our objective was to narrow the range of potential microbial agents associated with BLD and gain a better initial understanding of the disease. Leaves and dormant buds were collected in October 2017 from a common garden established in 2006 to assess *F. grandifolia* resistance to the beech scale insect as part of a BBD-resistance breeding programme (Koch & Carey, 2014). Molecular methods were used to probe the leaf microbiome, and microscopy coupled to molecular methods was used to examine the presence of nematodes in leaves affected by BLD.

2 | METHODS

2.1 | Sample collection

We collected samples in October 2017 from 8 *F. grandifolia* trees previously selected for resistance to BBD and planted into a common garden for evaluation to BBD (Koch, 2010). Trees showed various levels of BLD from no visible symptoms (1 tree) to light (minute interveinal thickening and dark bands) to heavy (substantial thickening and banding along with chlorotic yellowing, desiccation and

FIGURE 1 Leaves affected by beech leaf disease (BLD). Photograph on left shows characteristic interveinal darkening on young leaf. This darkening appears in many cases just after leaf emergence in spring. Photograph on right shows leaves with more advanced symptoms of beech leaf disease, including leaf browning and yellowing



browning of the leaf tissue) (see Table 1). Leaves and adjoining buds were collected from each tree from branches with leaves affected by BLD (symptomatic leaves and buds) or from branches where leaves possessed no visible signs of BLD (asymptomatic leaves and buds). We also collected leaves and buds from 4 beech trees in a forest remnant adjoining the common garden, as well as 2 mature trees near the Holden collection and canopy walk, for a total of 14 trees examined. The leaf microbiome was described for a total of 56 leaf samples (see Table 1 for details).

2.2 | DNA extraction

DNA was extracted from approximately 0.15 g of leaf or bud tissue collected from each tree and branch type. DNA was extracted using a bead beating approach where tissue was transferred into a 1.5-ml bead beating tube which contained 300 mg of 400 μ M sterile glass beads (VWR) and 200 mg of 1 mm sterile glass beads (Chemglass). We

added 750 μ l of 2% CTAB (cetyltrimethyl-ammonium bromide) to each tube as the extraction buffer, and samples were bead beaten using a Precellys homogenizer (Bertin Technologies) for 80 s to lyse cells and release DNA. DNA was purified from the lysate using phenol-chloroform extraction (Burke, Smemo, López-Gutiérrez, & DeForest, 2012) followed by precipitation in 20% polyethylene glycol 8000 with 2.5 M NaCl. Precipitated DNA was subsequently dried and suspended in 50 μ l TE (Tris EDTA) buffer and stored in a 1.5-ml low retention microcentrifuge tube (Fisher Scientific) at -20°C until analysis.

2.3 | Leaf nematode analysis

Nematode specific ITS primers were used to detect nematodes within leaf and bud samples. We used forward primer TW81 (GTTTCCGTAGGTGAACCTGC) and reverse primer 5.8MS (GGCGCAATGTGCATTCGA) (Tanha Maafi, Subbotin, & Moens, 2003; Vovlas, Subbotin, Troccoli, Liébanas, & Castillo, 2008) for

TABLE 1 Beech tissue collection, Holden Arboretum, 26 October 2017

| Tree ID | Location | Tree size | Disease severity of tree | Symptomatic leaf | | | Asymptomatic leaf | | |
|------------------|-------------------|------------------|--------------------------|------------------|-----|------|-------------------|-----|------|
| | | | | Leaf | Bud | Twig | Leaf | Bud | Twig |
| 59B | LB beech orchard | | Light | x | x | x | x | x | x |
| 60D | LB beech orchard | | Light | x | x | x | x | x | x |
| 57M | LB beech orchard | | Heavy | x | x | x | NA | NA | NA |
| 60A | LB beech orchard | | Medium | x | x | x | x | x | x |
| 59D | LB beech orchard | | Medium | x | x | x | x | x | x |
| 94E | LB beech orchard | | Light | x | x | x | x | x | x |
| 57 C | LB beech orchard | | Light | x | x | x | x | x | x |
| 94H ^a | LB beech orchard | | No obvious symptoms | NA | NA | NA | x | x | x |
| FS-1 | LB natural forest | Small understory | Very heavy | x | x | x | x | x | x |
| FS-2 | LB natural forest | Large mature | Very heavy | x | x | x | x | x | x |
| FS-3 | LB natural forest | Small understory | Very heavy | x | x | x | x | x | x |
| FS-4 | LB natural forest | Large mature | Very heavy | x | x | x | x | x | x |
| FS-5 | Canopy walk | Small understory | Very heavy | x | x | x | x | x | x |
| FS-6 | Canopy walk | Small understory | Very heavy | x | x | x | x | x | x |

Abbreviation: NA, not available.

^aRepresents an asymptomatic tree. Only asymptomatic tissue was collected.

nematode amplification. Amplification reactions were performed in an MJ Research PTC-200 thermal cycler (Bio-Rad Laboratories, Inc.). An initial denaturation for 2 min at 94°C was followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 45 s at 55°C and extension for 3 min at 72°C, with a final extension of 10 min at 72°C (Esmaili, Heydari, & Ye, 2017). Clone libraries constructed from the PCR product confirmed that only nematode sequences were generated with this primer set (Note: LSU primers reported to be specific for nematodes were found to amplify *F. grandifolia* rDNA; these were primers D2A and reverse primer D3B; see Nunn, 1992). Since only one nematode sequence was observed, we did not complete nematode community analysis on the samples but used PCR results to determine presence/absence of nematodes. Nematodes from *F. grandifolia* leaves were later confirmed to be present within leaf samples through leaf extraction of nematodes. In brief, we used the “water soaking” method of Zhen, Agudelo, and Gerard (2012) to isolate nematodes as it was found to produce the largest number of nematodes. Leaves were collected from the lower Baldwin site in July 2018, cut into 1-cm² pieces and soaked overnight in sterile water for 24 hr at 22°C. After soaking, the liquid was passed through a Baermann funnel for final nematode collection. Nematodes collected in this fashion were examined microscopically for confirmation purposes. Direct sequence analysis on extracted nematodes was used to further confirm identity.

2.4 | Microbial community DNA fragment analysis

DNA fragment analysis (terminal restriction fragment length polymorphism-TRFLP) was used to examine microbial communities. TRFLP is a well-established method for examining differences in microbial communities and compares well to next generation sequencing approaches (van Dorst et al., 2014). For bacteria, a nested PCR approach was used that excluded amplification of leaf plastid DNA (Burke, Dunham, & Kretzer, 2008). We initially used primers 41f and 1223r (see Burke et al., 2008) which were specifically designed to prevent amplification of plastid rDNA in plant samples, and PCR products generated with these primers was subsequently amplified using general bacterial primers 338f and 926r (Muyzer, Teske, Wirsén, & Jannasch, 1995; Muyzer, Waal, & Uitterlinden, 1993) to target variable regions 3–5 of the 16S rDNA gene (Carrino-Kyker, Smemo, & Burke, 2012; Liu, Marsh, Cheng, & Forney, 1997). Primers 338f and 926r were labelled with HEX (4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein) or 6FAM (6-carboxyfluorescein), respectively, for TRFLP analysis. PCR product was digested with restriction enzymes *HaeIII* (Promega). PCR was completed in 50 µl reaction volumes using 1 µl of gel purified leaf or bud DNA (1:10 dilution), 0.2 µm of primers, 2.0 mM MgCl₂, 0.2 mM dNTP, 0.25 µg bovine serum albumin and 1.0 unit Taq DNA polymerase (Promega) on an PTC 100 Thermal Cycler (MJ Research). TRFLP analyses were completed through the Life Sciences Core Laboratories Center (Cornell University) using an Applied BioSystems 3730xl DNA Analyzer and the GS600 LIZ size standard. Profiles were subsequently analysed

using Peak Scanner™ Software (version 1.0, Applied Biosystems 2006).

For general fungi, we targeted the ITS2 region of the rDNA using the primers of Martin and Rygielwicz (2005). Primer pair 58A2F and NLB4 (Martin & Rygielwicz, 2005) amplify an approximately 550 bp region and are capable of amplifying all Dikaryomycota (both Ascomycetes and Basidiomycetes) and were specifically designed to amplify fungi in the presence of plant tissue. DNA was extracted from leaf and bud samples using standard phenol–chloroform extraction following bead beating with CTAB (Burke et al., 2012). Primers were labelled either with the fluorescent dye 6FAM (58A2F) or HEX (NLB4) and PCR carried out in 50 µl reaction volumes using 1 µl of purified DNA (approximately 100 ng), 0.2 µm of primers, 2.0 mM MgCl₂, 0.2 mM dNTP, 0.15 mg/ml bovine serum albumin and 1.0 unit FastStart Taq DNA polymerase (Roche Diagnostics Corporation) using a PTC 100 Thermal Cycler (MJ Research). For PCR, the initial denaturation step of 5 min at 94°C was followed by amplification for 35 cycles in the following conditions: 30 s at 94°C, 60 s at 60°C and 90 s at 72°C. A final 5-min extension at 72°C completed the protocol. PCR product was digested with restriction enzymes *HaeIII* (Promega) and TRFLP completed through the Life Sciences Core Laboratories Center (Cornell University) as noted above.

2.5 | Microbial community DNA sequence analysis

PCR was also completed for both bacteria and fungi using unlabelled primers for cloning and sequencing. Cloning and sequencing was carried out to confirm the specificity of the respective amplifications for the target groups, but also to qualify the fragment analysis and reveal dominant taxa within the communities. PCR conditions were as noted above, and PCR products were gel extracted prior to cloning using the Wizard SV Gel and PCR Clean Up System (Promega). Gel extracted fungal PCR product was cloned using the QIAGEN PCR Cloning Kit (QIAGEN), following the manufacturer's instructions. Gel extracted bacterial PCR product was cloned using the NEB PCR Cloning Kit (New England BioLabs), following the manufacturer's instructions. Randomly selected colonies were incubated overnight at 37°C in LB medium, and plasmids harvested using a Wizard Plus SV Miniprep DNA purification system (Promega). Cleaned PCR products were sequenced using Big Dye Terminator chemistry and a 3730 DNA Analyzer (Applied Biosystems Inc.). Taxa were identified by comparing the recovered sequences to EMBL/GenBank/DBJ database entries using the NCBI Blast tool through the European Bioinformatics Institute (<http://www.ebi.ac.uk/>). We used the TRFLP analysis to identify groups of samples for clone library construction. Since NMDS analysis suggested that fungal communities differed between leaves and buds, we constructed two libraries to qualify those contrasts. Similarly, since bacteria NMDS analysis found differences between leaves and buds, and between symptomatic and asymptomatic leaves, three clone libraries were constructed to explore those contrasts. Clone libraries consisted of a minimum of 50 clones per community type identified through TRFLP.

2.6 | Statistical analysis

TRFs detected within TRFLP profiles were used as operational taxonomic units for statistical analysis. The relative peak area of each TRF was used for non-metric multidimensional scaling (NMDS) analysis of community structure, and PERMANOVA was used to test for differences between groups. Both NMDS and PERMANOVA procedures were completed in the vegan package (v2.4-5) (Oksanen et al., 2013) of R (v2.15) (R Development Core Team, 2015). These analyses were performed following an arcsine square root transformation, which is appropriate for proportion data (McCune & Grace, 2002). A chi-square test was used for determining whether nematode detection (presence/absence) varied among plant tissue types or with disease severity.

3 | RESULTS

3.1 | Molecular analysis of nematodes

Nematodes were frequently detected on the leaf and bud samples (Figure 2). There was a trend towards significant differences in nematode detection across tissue types (chi-square $p = .06$) (Figure 2), with lower levels of nematodes found in leaves asymptomatic for BLD. However, 36% of asymptomatic leaves contained nematodes, whereas

the highest levels were found in buds from symptomatic branches where 91% of the buds contained nematodes. There were also significant differences in the detection of nematodes with BLD severity (chi-square $p = .02$), where trees with heavy disease incidence had a greater proportion of tissue samples colonized by nematodes (Figure 2). In trees with heavy BLD incidence, 89% of leaf and bud samples contained nematodes, whereas only 44% of leaf and bud samples in trees with light disease incidence contained nematodes.

Nematodes were extracted from subsamples of leaves collected at the lower Baldwin site in July 2018 and visually confirmed (Figure 3). Sequence analysis of extracted nematode ITS DNA identified the best match to the anguinid nematode *L. crenatae* (Accession number LC383724%-99% identity; 444 nucleotides match out of 446 base pair sequence length, only 2 gaps). Sequence accession numbers for recovered nematodes are MN625146-MN625161.

3.2 | Molecular analysis of bacterial communities

NMDS ordination produced a 3-dimensional solution with a stress value of 1.3. Leaf and bud tissue clearly separated in ordination space, with leaves affected and unaffected by BLD also separating in ordination space suggesting that BLD altered bacterial communities

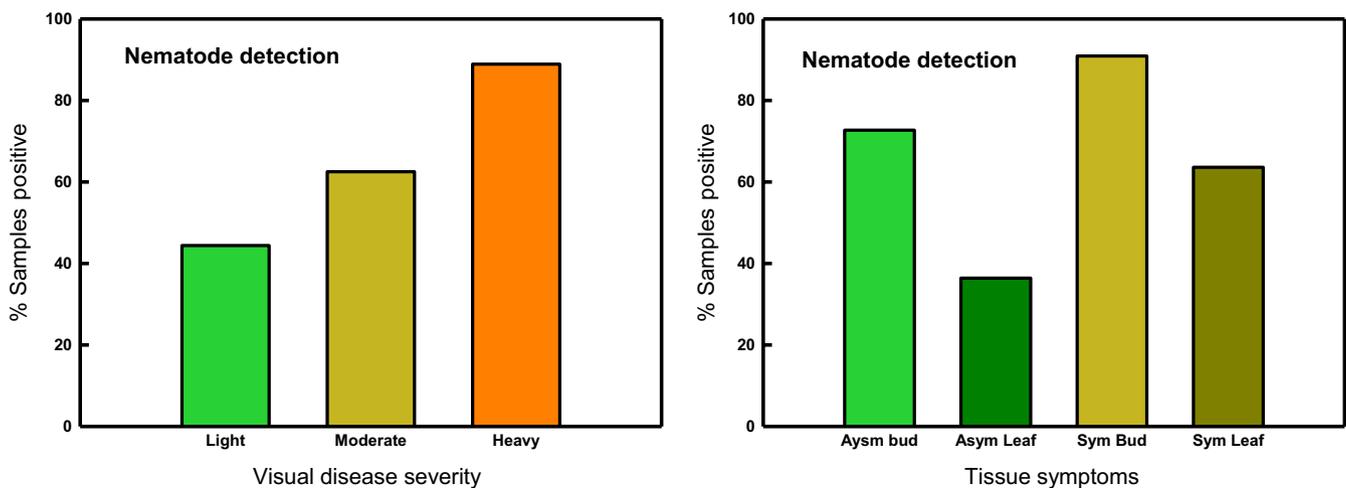
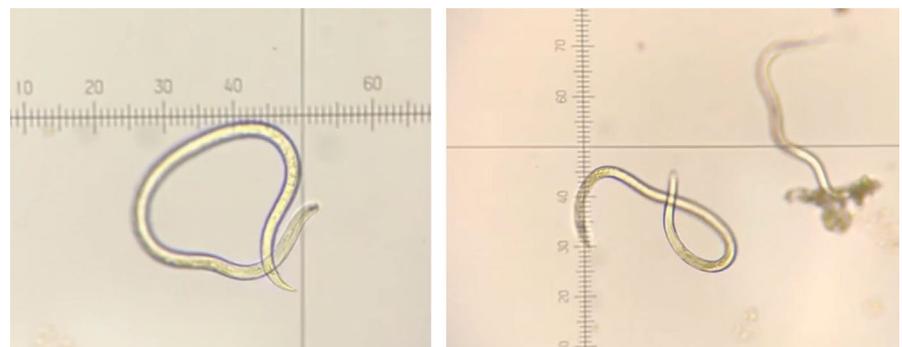


FIGURE 2 Nematode detection in beech tissue samples using molecular methods. PCR was used to determine presence/absence of nematodes in DNA extracted from samples. For visual disease severity, leaf and bud samples were pooled regardless of whether samples were associated with asymptomatic or symptomatic tissue. Total % positive samples for each sample type are shown

FIGURE 3 Nematodes extracted from leaf samples using the modified water soak method. Water was collected in a 15-ml centrifuge tube and centrifuged at 1,252 g for 3 min to collect any potential nematodes. Water was carefully aspirated from the tube until approximately 1 ml remained. Fifty μ L of the remaining water was placed on a concave microscope slide and viewed at 10 \times power



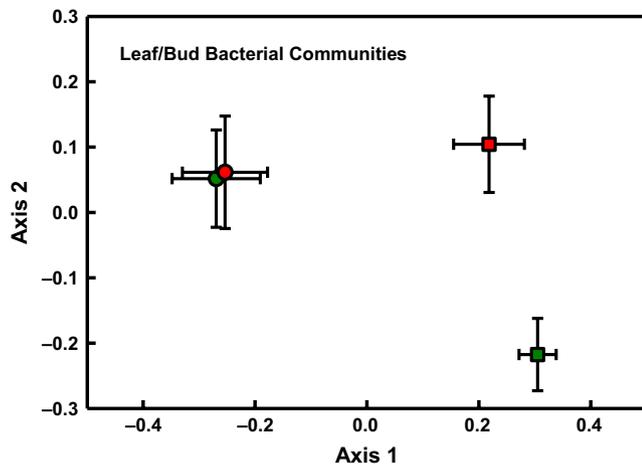


FIGURE 4 NMDS ordination of leaf and bud bacterial communities on trees affected by beech leaf disease. Circles represent buds, and squares represent leaves, while green coloured symbols represent tissue without BLD and red indicates tissue with BLD. Means \pm standard error of the mean are shown for each treatment group. Bacterial communities of buds were not significantly different between samples affected or unaffected by BLD, but bacterial communities on leaves were significantly affected by BLD (see Table 2). Buds and leaves also had different bacterial communities. The 3-dimensional stress for the ordination was 1.3

on leaves (Figure 4). PERMANOVA found significant differences in bacterial communities between leaves and buds, but no effect of BLD despite visual separation of leaf bacterial communities with NMDS (Table 2). One-way PERMANOVA on leaf tissue found a significant effect of BLD on leaf bacterial communities (Table 2). One-way PERMANOVA on bacterial communities from buds found no significant effects ($p = .9896$; data not shown), which confirmed lack of visual separation of bud communities in NMDS (Figure 4).

Sequence analysis was completed for 3 clone libraries representing buds from symptomatic and asymptomatic tissue combined, asymptomatic leaves and symptomatic leaves. Symptomatic and asymptomatic buds were combined since one-way PERMANOVA found no significant differences in those communities. Some taxa were present in all tissue types: taxa in the genera *Geobacter*, *Pedobacter*, *Sphingomonas* and the order Rhizobiales were found in all 3 tissue types (Figure 5, Table S1). Taxa in the Rhizobiales were frequently encountered in our libraries, ranging from 12% to 15% of the clone libraries. Some taxa were found only in leaf libraries: taxa in the Acetobacteraceae, *Beijerinckia*, *Caedimonas* and *Chryseolinea* were found in both leaf libraries but not in buds. There were a number of taxa that were found in only one tissue type. The Bacteroidetes was only in the asymptomatic leaves. Several taxa were found only on leaves symptomatic for BLD; these taxa included the genera *Methylocystis*, *Mucilaginibacter* and *Terimonas*. The genus *Wolbachia* was a large percentage of both the symptomatic leaves and bud library (17%–22%), but this genus was absent in asymptomatic leaves. Bacterial sequences obtained in this study have accession numbers MN628445–MN628553.

3.3 | Molecular analysis of fungal communities

The NMDS ordination produced a 3-dimensional solution with a stress value of 6.8, and there was clear separation in fungal communities between leaves and buds (Figure 6) but little apparent effect of BLD on fungal communities. PERMANOVA confirmed these visual observations; there were significant differences in fungal communities between leaves and buds, but no effect of BLD (Table 3). Sequence analysis was conducted on leaf and bud clone libraries to qualify major taxa within the leaf and bud microbiome. Sixty-four fungal ITS clones were sequenced from leaf samples which represented 17 fungal taxa (Table S2). Fifteen of the taxa were

TABLE 2 Results of two-way PERMANOVA for effect of tissue type (leaf/bud) and disease presence on bacterial community composition (All tissue comparison) and two-way PERMANOVA for effect of disease on leaf bacterial communities

| All tissue comparison | | | | | | |
|-------------------------|----|---------|----------|---------|----------------|--------------|
| Source | df | SS | MS | F | R ² | p |
| Disease | 1 | 0.1449 | 0.14492 | 1.4288 | 0.02692 | .1640 |
| Tissue | 1 | 1.0540 | 1.05395 | 10.3912 | 0.19578 | .0002 |
| Disease \times Tissue | 1 | 0.1274 | 0.12744 | 1.2564 | 0.02367 | .2448 |
| Residuals | 40 | 4.0571 | 0.10143 | | 0.75363 | |
| Total | 43 | 5.3834 | | | 1.0000 | |
| Leaf comparison only | | | | | | |
| Source | df | SS | MS | F | R ² | p |
| Disease | 1 | 0.23722 | 0.237221 | 2.7609 | 0.1213 | .0088 |
| Residuals | 20 | 1.71842 | 0.085921 | | 0.8787 | |
| Total | 21 | 1.95564 | | | 1.0000 | |

Note: Leaf bacterial communities were compared due to separation of sample types as evident by NMDS analysis. Significant differences are in bold. One-way PERMANOVA for bacterial communities on buds was not significant ($p = .9896$).

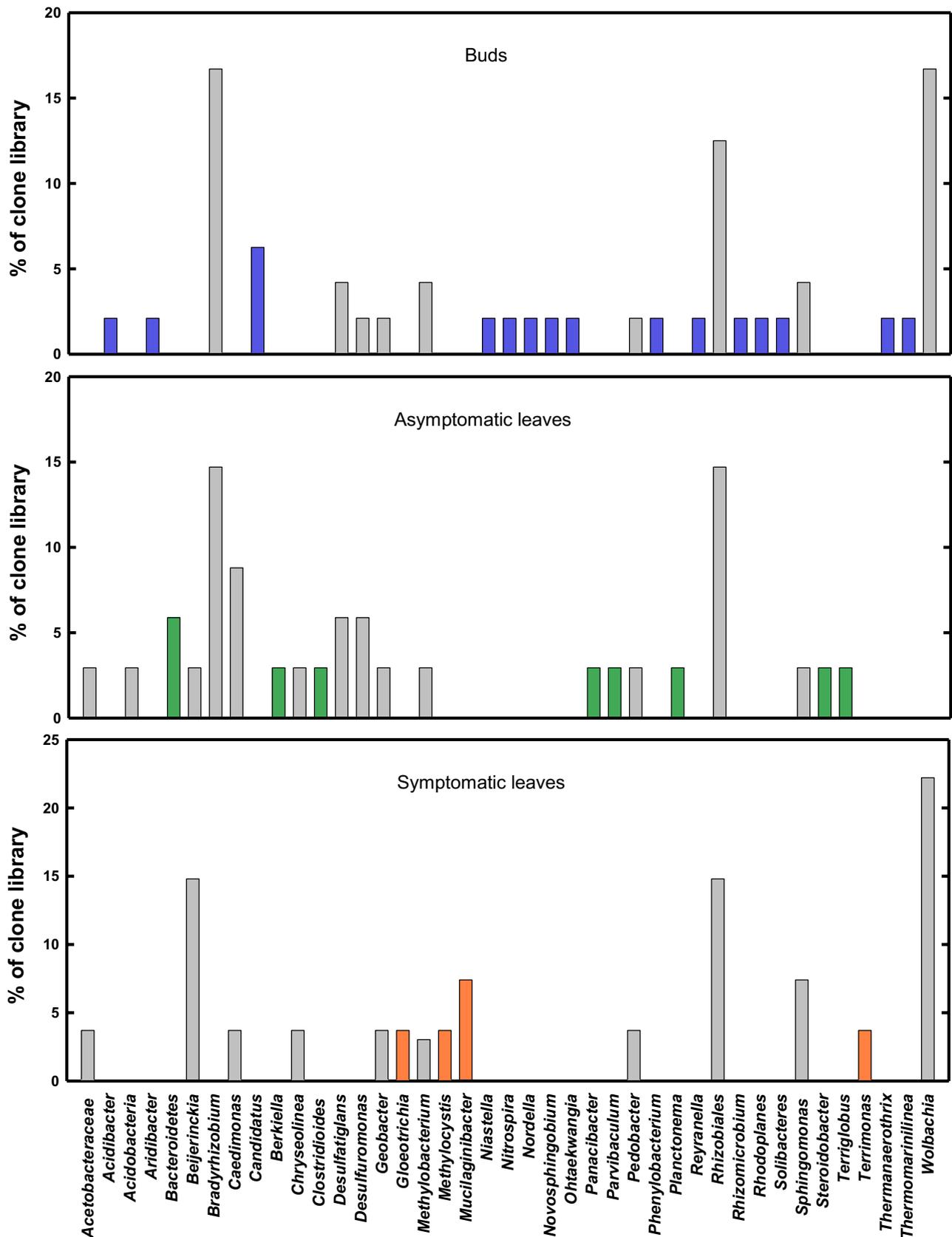


FIGURE 5 Distribution of bacterial taxa between bud tissue, asymptomatic and symptomatic leaf tissue showing dominant taxa within the clone libraries. The % of each clone library dominated by the genera are shown (taxa grouped by genera for ease of visualization-see Table S1 for list of all bacterial affinities). Grey bars represent taxa found in more than one library, blue bars represent taxa found only on buds, green bars represent taxa found only on asymptomatic leaves, and orange bars are taxa only on symptomatic leaves

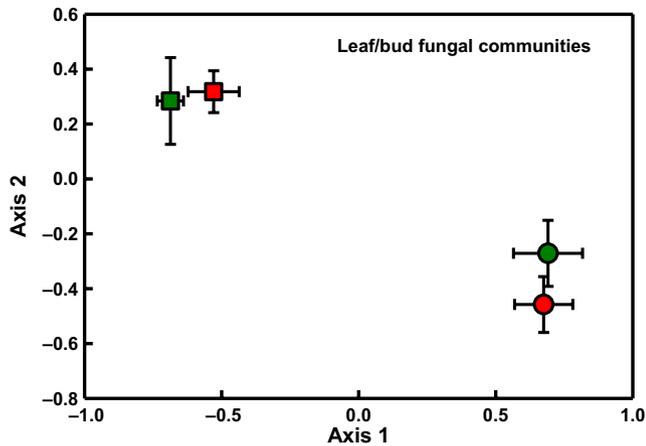


FIGURE 6 NMDS ordination of leaf and bud fungal communities on trees affected by beech leaf disease. Circles represent buds, and squares represent leaves, while green coloured symbols represent tissue without BLD and red indicates tissue with BLD. Fungal communities were significantly different between tissue types (leaf vs. buds), but BLD had no significant effect on fungal communities (see Table 3). The 3-dimensional stress for the ordination was 6.8. Means \pm standard error are shown for each treatment group

Ascomycota while the remaining 2 taxa were Basidiomycota (Table S2). The genus *Ramularia* was the most numerous in the libraries representing 5 species and 42% of recovered clones (Figure 7). *Cladosporium* was the next most numerous genus representing 4 species and 20% of recovered clones. In addition, 118 fungal ITS clones from bud samples were sequenced which represented 25 different fungal taxa (Table S2). Bud fungal communities were dominated by the genus *Taphrina* which consisted of 8 sequence types and 44% of the clone library. Most *Taphrina* sequences had low affinity (90%) to named species within the genus. The second most abundant fungal group on buds was *Cladosporium*, 4 species of which comprised 33% of the clone library. Bud libraries were also dominated by Ascomycota with 20 of the 25 taxa recovered belonging to this group.

Bud and leaf libraries had some overlap in taxa, with 5 of 20 genera shared between the tissue types (Figure 7). Fifteen of the genera appeared in one tissue type or the other but not both. However, the largest differences between bud and leaf

fungal communities focused on 2 taxa: in buds, the genus *Taphrina* dominated (44%) the fungal libraries but was absent from leaf libraries, whereas in leaves, the genus *Ramularia* dominated (42%) the fungal libraries but was absent from bud libraries (Figure 5). Sequence accession numbers for fungi obtained in this work are MN633085-MN633265.

4 | DISCUSSION

Beech leaf disease is an emerging forest disease in northern Ohio and the Great Lakes Region, but the cause of the disease is unknown. Recent work suggested that the anguinid nematode *L. crenatae* subsp. *mccannii* may play a causal role in BLD (Carta et al., 2020), but whether the *L. crenatae* subsp. *mccannii* is solely responsible for the leaf symptoms or whether bacterial and fungal pathogens associated with the nematode play some role is not certain. Our goal was to examine the microbiome of beech trees affected by BLD in one experimental plantation in northern Ohio to determine possible microbial associates of BLD. The findings suggest that (a) fungi are not the cause of BLD or a cause of secondary infections associated with BLD since fungal community composition does not differ between symptomatic and asymptomatic tissues; (b) bacterial communities on leaves but not buds are affected by BLD, and some bacteria could possibly be involved in the disease progression, and (c) the nematode *L. crenatae* subsp. *mccannii* is frequently found on both symptomatic and asymptomatic bud and leaf tissue, although detection was higher in symptomatic tissue, offering further support that *L. crenatae* subsp. *mccannii* is a causal agent of BLD. Although the presence of nematodes on both symptomatic and asymptomatic tissue might also suggest that nematodes are not the direct cause of BLD, recent experimental evidence showed that inoculation of dormant buds with live nematodes results in BLD symptoms (Carta et al., 2020). The data presented here offer further support for the role of this nematode in BLD. Finally, some of our bacterial data suggest a possible transmission route for BLD as the bacterial genus *Wolbachia* was associated with symptomatic leaves and bud tissue (combined symptomatic and asymptomatic buds), but not asymptomatic leaves. This bacterial genus is often associated with arthropods such as mites and ambrosia beetles that could possibly transport eggs of *L. crenatae* subsp. *mccannii* between tree hosts.

TABLE 3 Results of two-way PERMANOVA for effect of tissue type (leaf/bud) and disease presence on fungal community composition (All tissue comparison)

| All tissue comparison | | | | | | |
|-------------------------|----|--------|--------|--------|----------------|--------------|
| Source | df | SS | MS | F | R ² | p |
| Disease | 1 | 0.1897 | 0.1897 | 1.414 | 0.01908 | .2010 |
| Tissue | 1 | 4.6299 | 4.6299 | 34.507 | 0.46564 | .0002 |
| Disease \times Tissue | 1 | 0.1589 | 0.1589 | 1.184 | 0.01598 | .2752 |
| Residuals | 37 | 4.9645 | 0.1342 | | 0.49929 | |
| Total | 40 | 9.9431 | | | 1.0000 | |

Note: Significant differences are in bold.

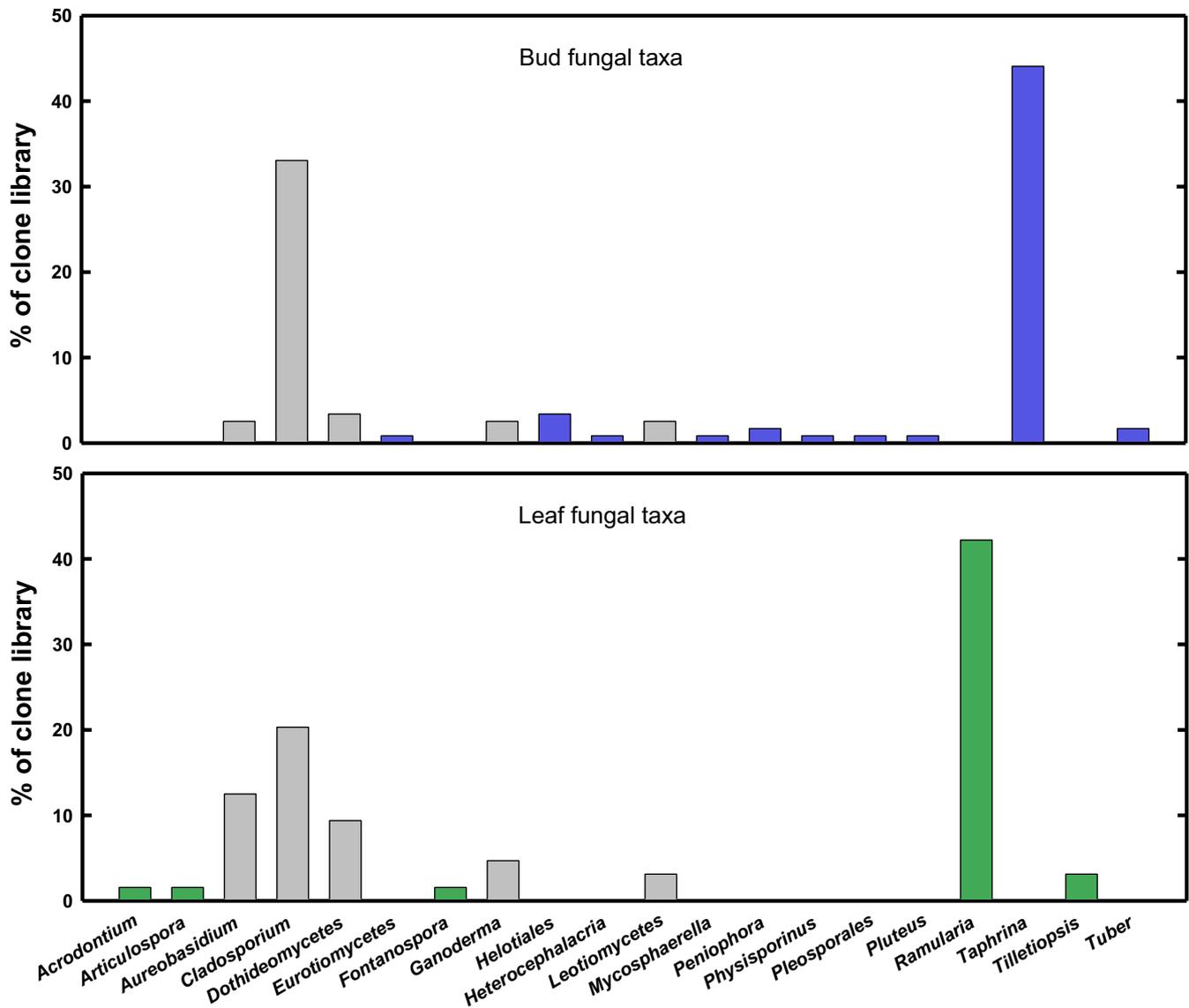


FIGURE 7 Distribution of fungal taxa between bud and leaf tissue showing dominant taxa within the clone libraries. The % of each clone library dominated by the genera are shown (taxa grouped by genera for ease of visualization—see Table S2 for list of all fungal affinities). Grey bars represent taxa found in both bud and leaf fungal communities, while blue bars represent taxa found only on buds and green bars represent taxa found only on leaves

Although no effects of BLD symptomology were observed on fungal communities, we did find some significant differences between leaves and buds although many taxa were shared between tissue types. Many of the taxa observed on beech tissue such as the genus *Aureobasidium*, *Taphrina* and *Mycosphaerella* were some of the most abundant fungal taxa identified on European beech (*Fagus sylvatica*) using next generation sequencing methods (Cordier et al., 2012). Species in the genus *Mycosphaerella* can cause leaf spot disease on Japanese beech (see Kaneko & Kakishima, 2001), but this genus was found only on our bud tissue despite visual confirmation of leaf spot disease at the field site. Leaf tissue was dominated by the genus *Ramularia*, which is an asexual anamorph of *Mycosphaerella*, indicating that this pathogen is an important part of the fungal community on beech leaf and bud tissue, but different stages of the fungal species life cycle may be separated spatially

within the tree (different life stages on bud vs. leaf tissue). *Taphrina* is also a potential pathogen, being the causative agent of leaf curl disease on *Prunus persica* (Cissé et al., 2013), but this genus was also only found on bud tissue. *Taphrina* was the most abundant fungal taxa on European beech leaves (Cordier et al., 2012) but could be commensal on beech. Although we did not find these potential pathogens on leaf tissue, the time of sampling could be a factor. Our samples were collected in autumn when symptoms of BLD are most pronounced, whereas the study by Cordier et al. (2012) collected samples in summer. It is possible that *Taphrina* have disseminated via spores to buds prior to the onset of winter and overwinter in live buds. Nevertheless, the lack of differences between the fungal communities between symptomatic and asymptomatic tissue suggests that fungi do not play a role in BLD symptomology although other fungal pathogens may be present on the leaf tissue.

The evidence for some role of bacterial taxa in BLD appears greater, since significant differences in bacterial communities were observed between symptomatic and asymptomatic leaves. Some bacterial groups were observed across all tissue types examined and may be indicative of the core microbiome of beech leaves. The genera *Beijerinckia*, *Geobacter*, *Methylobacterium*, *Pedobacter*, *Rhizobiales* and *Sphingomonas* were found on all tissue types, and similar groups have been found in the phyllosphere of plants such as rice (e.g., *Methylobacterium*, *Rhizobium*) (Rastogi, Coaker, & Leveau, 2013). However, of particular interest were two bacterial taxa that differed between symptomatic and asymptomatic leaves, the genus *Mucilaginibacter* and *Wolbachia*.

The genus *Mucilaginibacter* is a newly recognized genus, first described by Pankratov, Tindall, Liesack, and Dedysh (2007). The bacteria were isolated from a Sphagnum peat bog in Siberia and showed evidence of degrading pectin and other polysaccharides which might be expected from a saprotrophic bacterium involved in organic matter degradation (Pankratov et al., 2007). Other species within the genus have been isolated from heavy metal contaminated rice paddy soil and also produce exopolysaccharides (Fan, Tang, Nie, Huang, & Wang, 2018). Although this genus has not to our knowledge been found associated with insects or nematodes, the fact that it can degrade pectin makes it a possible symbiotic partner of a nematode that damages leaf tissue. Bacterial pathogens are known to be transferred from nematodes to plant hosts (Starodumova et al., 2017). Several species in the genus *Rathayibacter*, members of the *Actinobacteria*, can be transmitted to plants via seed gall nematodes (Starodumova et al., 2017), and this genus can cause plant disease including ryegrass toxicity (Davis et al., 2018). There is also evidence suggesting that bacterial soft rot of plants caused by the genus *Pectobacterium* could be vectored to plants by soil dwelling nematodes (Nykyri et al., 2013). In addition, entomopathogenic nematodes are known to have symbiotic bacteria that allow the nematodes to kill the insect and use the insect as a food resource (Murfin et al., 2012). Although some nematodes can produce pectinases for cell wall degradation (Ali, Azeem, Li, & Bohlmann, 2017), it is possible that nematode–bacterial partnerships which allow greater production of exopolysaccharides could enhance resource capture and nematode fitness. But this question of whether *L. crenatae* subsp. *mccannii* has a bacterial symbiont or can transfer a bacterial pathogen will require further study.

Of additional interest was the presence of *Wolbachia* in symptomatic leaves but not asymptomatic leaves. *Wolbachia* are common intracellular bacteria of arthropods and filarial nematodes; filarial nematodes can be human pathogens (Werren, Baldo, & Clark, 2008). *Wolbachia* can alter the reproductive biology of host arthropods, are widespread among many insect groups with 70% of insect species possibly colonized by these bacteria (Murfin et al., 2012; Werren et al., 2008). *Wolbachia* are not, to our knowledge, associated with foliar nematodes such as *L. crenatae* subsp. *mccannii*. The presence of these bacteria on symptomatic leaves may be indicative of the presence of an insect vector of *L. crenatae* subsp. *mccannii*, and eggs of nematodes have been found attached to the legs of leaf mites (Carta et al., 2020). However, we found nematodes on all tissue types, and at least 35%

of asymptomatic leaves had positive amplification using nematode ITS primers. It is possible that nematodes can be transported to naïve tissue in rain water or can move to naïve tissue on water film, once established within the tree, without assistance of any insect vector. So, the presence of nematodes without *Wolbachia* could indicate that multiple routes of infection are possible once the nematodes have become established within the tree.

Several bacterial genera were observed on asymptomatic leaves that did not appear in the bud or symptomatic leaf libraries. Some of these taxa, for example *Parvibaculum*, are recently described taxa (Schleheck, Tindall, Rosselló-Mora, & Cook, 2004), and the functional role on the leaf is uncertain. Assessment of bacterial communities in this work is also based on DNA fragment analysis coupled to Sanger sequencing of limited clone libraries. It is highly likely that additional bacterial taxa would be detected if next generation sequencing methods were used. However, this initial approach has identified some bacterial candidates that could be associated with *L. crenatae* subsp. *mccannii* or its potential insect vector. Additional work will need to be conducted to fully understand the role of these bacteria in the colonization of leaves by *L. crenatae* subsp. *mccannii* and the progression of BLD in forest trees. Given some of the novel bacterial taxa found on asymptomatic leaves, the possibility exists that some of these taxa could play a role in the plant's resistance to this invasive nematode species and the development of BLD.

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ORCID

David J. Burke  <https://orcid.org/0000-0003-1774-1617>

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