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Molecular Characterization of Bacterial Community Composition in the Rhizosphere of Invasive Plant Species Amur Honeysuckle (*Lonicera maackii*) in an Urban Wetland Forest

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Abstract

The goal of this research was to determine the effects of the growth of invasive plant Amur Honeysuckle (*Lonicera maackii*) on the rhizosphere bacterial community composition, and diversity in an urban wetland forest ecosystem. Bacterial communities from the rhizosphere of 5 *L. maackii* plants and control bulk soils that did not have any *L. maackii* were investigated at Nina Mason Pulliam EcoLab (NMPE) using a culture-independent pipeline. Bacterial communities were characterized by PCR amplification and cloning 16S rRNA gene fragments following total DNA isolation from the soil samples. Microbial communities associated with both *L. maackii* rhizosphere and control sites showed high bacterial diversity within each site and taxa unique to individual sites were observed. Phylogenetic analyses revealed 80% of 400 16S rDNA clones were classified as *α*-, *β*- and *γ*-*Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Cytophaga-Flexibacter-Bacteroides* (*CFB*) group, and *Verrucomicrobia*. Members of the *Proteobacteria* and *Acidobacterium* represented 66.5% and 14.5% of the clone library, respectively, whereas the remaining bacterial divisions each comprised less than 7% of the clone library. Twenty-five 16S rDNA clones could not be classified into any known bacterial divisions. Statistical analyses showed significant differences in the presence of *L. maackii* on the proportions of 16S rDNA clones affiliated with *Proteobacteria* and *Acidobacterium*, suggesting bacterial community composition and structure does significantly change in the presence of *L. maackii*. However, sequence-based community analysis and the corresponding lack of intact microbial cultures limit understanding of the potential influences of enriched microbial taxa on plant hosts and their roles in ecosystem functioning.

Keywords

Bacterium, Rhizosphere, Forest Soils, 16S rRNA, *Lonicera maackii*

1. Introduction

Soil bacterial communities have significant impact on plant cover as pathogens, decomposers, or beneficial mutualists, influence nutrient cycling and solubilization, as well as have potential to induce production of plant hormones in plants [1]. These impacts can range from highly positive to negative for the plants. Moreover, the impacts of rhizosphere microbes on individual host plants can alter plant communities across the landscape. For example, the relative abundance of plant species in a Canadian meadow was correlated with the extent to which soil microbes are associated with each plant species. This relationship conferred positive impacts on host plant biomass; abundant plant species were more likely to experience positive feedbacks with their rhizosphere-associated microflora, while rare species tended to experience negative feedbacks [2]. Thus, soil microbes, through their positive or negative impacts on individual plants, can be key determinants of plant community diversity and composition. Plants, in turn, have been shown to have significant influences on soil microbial communities [3]-[8]. Plants can have differential effects on microbial community size, composition, and diversity [9] [10]. In particular, root exudates can stimulate growth of bacteria and fungi in the rhizosphere [11] [12] [13] [14]. While there is clear evidence for both positive [15] [16] and negative [17] [18] feedback between plants and soil microbes, most studies to date have failed to show a diverse array of interactions between rhizosphere bacteria and plants. In particular, the potential influences of host plants on non-cultured bacteria, which are believed to represent most of the soil microflora, have received little attention. However, recent advances in sequencing technology offer the opportunity to explore the composition, structure, and diversity of soil microbes associated with distinct host plants in substantially more detail. Compared with culture-based methods, sequence-based analyses provide a means for exploring plant-associated microbial communities that is more representative of their phylogenetic complexity, providing the potential for identifying novel plant-associated taxa while broadening our understanding of the diversity of plant-microbe associations.

Non-native plant species are a worldwide problem with enormous ecological and economic consequences [19] [20]. Though many of these non-natives do not cause harm, many outcompete and crowd native species reducing biodiversity. There is substantial concern that the incidence and severity of these non-native or invasive species have increased in the last few decades as a result of environmental changes at the local and global scale [19]. There is substantial concern over rate of establishments of invasive species in prairie ecosystems in the last few decades because of environmental changes at local (e.g., nitrogen deposition)

and global scales (e.g., climate) [21] [22] [23]. As well, the introduction and rate expansion of these invasive species is of great concern but is often hard to document. The magnitude of these concerns is emphasized by the establishment of major International Soil Reference and Information Centers (ISRIC), and Midwest Invasive Species Information Network (MISIN) including Indiana Invasive Species Council (IISC) efforts to monitor invasive plants. Despite international concerns regarding the devastating impacts of invasive species on native plants, there remains a poor understanding of the factors regulating the establishment and intensity of these plants [24]. This lack of understanding is confounded by poor records of specific species and their extent. Consequently, methods to understand specific factors involved in establishment of specific species are essential [2] [25]. An example of a problematic invasive shrub that is of national concern in the forests of the Eastern United States is Amur honeysuckle, *L. maackii* [26] [27] [28]. It is a species of honeysuckle in the family *Caprifoliaceae* that is native to temperate western Asia, specifically in northern and western China. Despite this, little is known of their relationships with the soil microflora or, specifically, their influences on soil microbial community composition or the extent to which plant-microbe feedbacks influence host establishment or longevity. We believe that a better understanding of the rhizobacteria and soil biological factors associated with the *L. maackii* shrub is required for designing novel strategies for controlling invasive species. Identifying bacterial phyla which are critical for the establishment of *L. maackii* and soil factors that affect their growth is therefore customary in its control.

In this study, we used a molecular phylogenetic approach to characterize the composition and diversity of rhizosphere bacterial communities associated with Amur honeysuckle plant species, *L. maackii* to provide an explicit test of the hypothesis that invasive plant species support distinct and characteristic rhizosphere bacterial communities. We used a spatially stratified sample design to characterize the influences of *L. maackii* on bacterial community composition and diversity in soil. As hypothesized, we report significant differences in relative abundance of bacterial communities associated with *L. maackii* and control soil (away from *L. maackii* canopy) and discuss its implication for the establishment or control in the wetland forest ecosystem.

2. Material and Methods

Urban forest sites, chosen plots and rhizosphere soil sample collections. The Nina Mason Pulliam EcoLab (NMPE) at Marian University, site conditions, and management of invasive species are described elsewhere. Rhizosphere soil (RS) samples were collected from two carefully designated plots of the NMPE Forest in Indianapolis, Indiana (**Figure 1**). The chosen sites (RS 39˚49'5.3"N, 86˚12'9.5"W; CS 39˚49'4.5"N, 86˚12'6.4"W) in the NMPE forest for the control plots (CS) did not have any *L. maackii* shrub whereas test plots (RS) have seedlings as well as some mature *L. maackii* shrubs. The soils on both sites have deep, medium

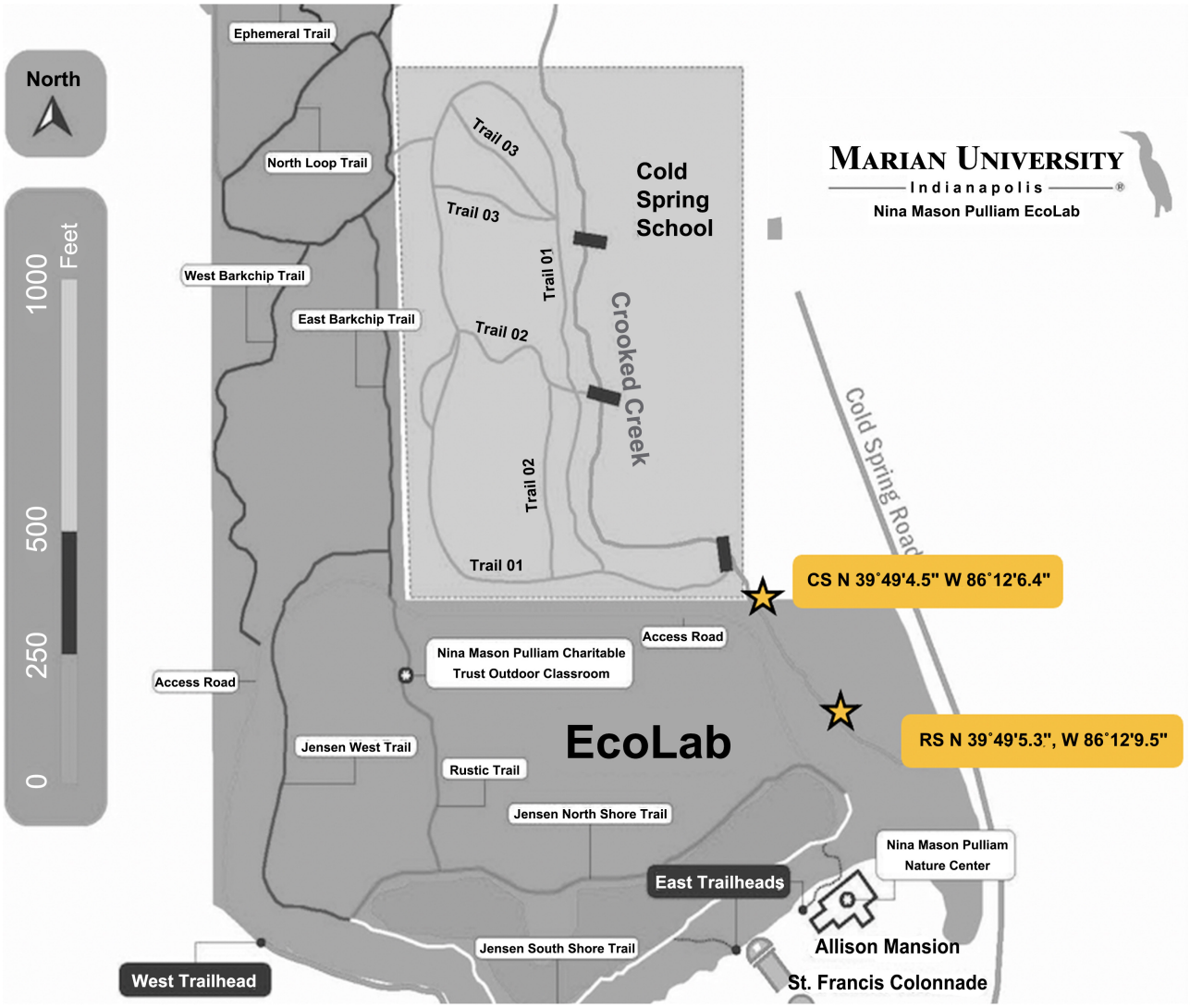

Figure 1. Location of Nina Mason Pulliam EcoLab showing sampling sites with *L. maackii* (RS) and soils without *L. maackii* (CS) in Indianapolis, Indiana, USA.

textured loam soils and trees were 50 - 80 years old (A. Ahmad, pers. communication). The soil characteristics of the control and test plots selected for this study are given in Table 1. Five seedlings (4 - 5 years old) were randomly selected for rhizosphere soil sampling during July 2018 and August 2019 from the test plot. Each seedling was gently lifted from the ground with the intact roots surrounded by abundant soil and the soil was gently shaken from the roots. Random egressed roots plus clinging soil were aseptically cut from the seedling approximately 3 cm from the root plug and placed in a 500 mL sterile bottle. The control plots (CS) were also sampled at the same time. After removing the topsoil, soil cores 12 cm long were collected with a 2 cm soil corer (Lamotte soil corer; Garinger) to a depth of 100 mm. Soil cores were placed individually into plastic bags, transported to the laboratory on ice and stored at −20˚C. Before total DNA extractions, replicate soil samples were pooled to normalize variability.

Table 1. A description of plots with or without *L. maackii* in the Nina Mason Pulliam EcoLab (NMPE) an urban wetland forests site near Indianapolis, Indiana, USA.

Plot	Description	Sample Codes	Field Site Location
Test	Hennepin loam, conditioned by *L. maackii* growth	RS2018.OTU# RS2019.OTU#	NMPE, Indianapolis, IN
Control	Hennepin loam, and *L. maackii* absent	CS2018.OTU# CS2019.OTU#	NMPE, Indianapolis, IN

These samples were maintained at approximately 4˚C and processed within 48 h to isolate DNA (as described below).

Rhizosphere soil was collected by submerging roots plus clinging soil from two seedlings in 250 mL of sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for 20 - 25 min at room temperature with intermittent mixing by hand (one sample). The soil suspension was passed through a sterile mesh screen (1 mm pore size) to remove roots, small rocks, and large pieces of organic matter. An additional 50 mL of TE was added to rinse the screen and the suspension was transferred to a sterile blender chamber and blended for 30 s, at high speed. The liquid portion was decanted to a sterile centrifuge tube and spun at 300 xg at 4˚C for 10 min. The supernatant was centrifuged at 5000 xg at 4˚C for 10 min to pellet the microbial cell fraction. The pellet was washed once in TE, homogenized with a sterile toothpick, and stored at 4˚C until DNA extraction. The FastDNA-Prep™ Kit (Qbiogene, Carlsbad, CA, USA) was used for DNA isolation from 100 mg cell pellet per sample as previously described [1]. Purified DNA samples were stored at −20˚C.

Polymerase chain reaction (PCR) amplification of 16S rRNA gene sequences from rhizosphere soil DNA (0.5 ng) was carried out in 25 µL reactions using universal bacterial primers 16S-336F and 16S-909R [29] [30] [31] as previously described for 16S rDNA clones from surface organic matter and mineral soil samples [32]. A reaction without DNA was prepared as a control. A Mastercycler® X50 Gradient 96 Temperature Cycler (Eppendorf, North America) was used to generate a gradient of four annealing temperatures (52.5˚C, 53.5˚C, 54.5˚C, 55.5˚C) and two reactions were done at each temperature for each soil sample as previously described [11]. The PCR product size was visualized by gel electrophoresis and SYBR™ Green staining. PCR products from different annealing temperatures were pooled for each soil DNA sample and purified with QIAquick PCR purification spin columns (Qiagen, LLC, Maryland, USA). DNA concentrations were quantified using a fluorometer. PCR products were stored at 32˚C. The PCR products amplified with the universal bacterial primers were ligated into the plasmid vector pCR™-2.1-TOPO (3:1 insert to vector ratio) as per TOPO Cloning System technical manual (Invitrogen, Life Technologies, USA). The ligation products were purified and concentrated two-fold using Ultra-free MC Millipore Filters (Millipore, Bedford, MA, USA). An aliquot of the purified ligation product (25 ng) was transformed into the *Escherichia coli* host strain *E. coli* Top10F™ (Thermo Fisher Scientific, USA) as previously described [32].

Serial dilutions were plated as described by the TA Cloning Kit manual, and 50 clones were randomly selected to represent each of 20 composite rhizosphere soil samples. Clones were grown overnight in LB Broth (Thermo Fisher Scientific, USA) with ampicillin (100 μg·mL^{-1}) and stored at −80˚C in 20% glycerol.

Methods for plasmid DNA isolation from 16S rDNA clones and partial sequencing of the 16S rRNA gene inserts in the V3-V5 region (*E. coli* numbering) are described here [33]. The forward primer 336F 5'-GTACTCCTACGGGAGGCAGCA-3'; and reverse primer 909R 5'-CCCCGYCAATTCMTTTRAGT-3'. [31] [34] were employed. PCR clones were sequenced by commercially provided 16S sequencing services at GENEWIZ (Ahmad, unpublished, 2021). Complete information regarding GENEWIZ's 16S rRNA-Sequencing services may be found at this link. Partial 16S rRNA gene sequences were searched using BLAST server (Basic Local Alignment Search Tool, National Center for Biotechnology Information (NCBI): http://www.ncbi.nlm.nih.gov/) to determine the closest matching sequences in the GenBank and to infer possible phylogenetic affiliations. Closely related 16S rRNA gene sequences originating from the same rhizosphere soil sample were aligned to determine similarity using alignment software contained in the CLC Genomics Workbench ver. 21.0.3 (Qiagen, USA). OTU calculations were based on the genetic distance between sequences and were used to estimate the richness and diversity of a sampled community. The partial sequence of a 16S rDNA clone was considered unique, included in phylogenetic analyses, and deposited in the GenBank if it had greater than four nucleotide differences within the region sequenced (approximately 575 nucleotide bases; 99% sequence similarity) compared to other 16S rDNA clones from the same rhizosphere soil sample.

Partial 16S rRNA gene sequences of 16S rDNA clones were designated by a source code to identify the test (RS) and control (CS) plots, sampling year, followed by a semicolon (.), and the clone number (**Table 1**). Phylogenetic analysis included building a series of trees based on variations in 16S clone sequence sets and GenBank sequences representing known and candidate bacterial divisions with 100 bootstraps and edited with programs contained in the CLC Genomics Workbench (Qiagen, USA). All alignment positions were included in the analyses, but insertions and deletions were not included in distance analyses. Final classification of 16S rDNA clones to a phylogenetic division or subdivision, or to an unclassified category, was based on combined results from the phylogenetic group represented by the closest matching sequences in the GenBank and phylogenetic tree analyses [11].

Finally, the resulting matches for each set of sequence data were summarized at various taxonomic levels and filtered to remove non-bacterial lineages. We performed Kolmogorov–Smirnov, goodness-of-fit test, ANOVA (analysis of variance), and Tukey's Honestly Significant Difference (HSD) analyses on taxonomy query results. All statistical analyses were performed using the R-statistical package (version 2.10; http://cran.r-project.org/). Indicator analysis is a composite measure that considers both prevalence and relative abundance [35] [36].

Indicator value analysis is a method to find indicator taxa and assemblages characterizing groups of sites [36]; it combines a taxa's relative abundance with its relative frequency of occurrence in the various groups of sites. Indicator value analysis was performed on taxonomy query results. In these analyses, thresholds of 10 occurrences of a given bacterial taxon per DNA sample (*i.e.*, a requirement that each bacterial taxon must be represented by 10 sequence reads in each DNA sample) and of occurrences of a bacterial taxon in 50% of samples from a field location was applied. Partial 16S rRNA gene sequences from unique clones were deposited in the GenBank database and assigned accession numbers __ to __ for clones classified as *α*-, *β*-, *γ*-, and *δ-Proteobacteri*a, *Actinobacteria*, *Cytophaga-Flexibacter-Bacteroides* (CFB) group, *Acidobacterium*, *Planctomycetes*, *Firmicutes*, and *Verrucomicrobia*; and __ to__ for unclassified 16S rDNA clones.

3. Results and Discussion

DNA yields from the rhizosphere soil samples: Five *L. maackii* seedlings harvested for RS plots showed approximately 75% - 95% root tip mycorrhizal colonization per sample. This indicated similar soil conditions in the two consecutive sampling years, 2019 and 2020. Total DNA yields from the ten rhizosphere soil samples were variable and ranged from 8.1 to 15.3 ng DNA per cell pellet (mean DNA yield = 11.7 ng DNA mg^{-1} cell pellet). DNA template concentrations were standardized for all PCR reactions to 0.5 ng soil DNA per 25 µL reactions to minimize potential sources of variability such as the amount of rhizosphere soil sampled and DNA yields from samples. We followed methods commonly used for sampling rhizosphere soil [37] [38] which included collection of soil clinging to plant roots after plant excavation from soil.

Characterization of 16S rDNA clones: Twenty 16S rDNA clones for 10 each RS and CS soil samples, for a total of 400 clones, were partially sequenced. The length sequenced ranged from 550 - 575 nucleotides. Overall, 27% - 29% of the 16S rDNA clone sequences (108 - 116 clones) shared 99% sequence similarity with another clone from the same soil sample. Identical or nearly identical bacterial 16S rRNA gene fragments have been recovered from non-rhizosphere forest soil samples from the NMPE site (A. Ahmad, data not shown). Retrieval of identical sequences from the same soil sample may indicate species abundant in soils or an artefact due to PCR. Full-length sequence analysis would be required to accurately determine the identical nature of cloned gene fragments.

Phylogenetic analyses revealed that 86.5% of 400 rhizosphere soil 16S rDNA clones were classified as *α*-, *β*-, *γ*-, and *δ-Proteobacteria*, *Actinobacteria*, CFB group, *Acidobacterium*, and *Verrucomicrobia* (**Figure 2**). Members of *Proteobacteria* represented 52.2% of the clone library and *α-Proteobacteria* had the greatest representation followed by *β*-, *γ*-, and *δ-Proteobacteria* except in CS2018, when *β-Proteobacteria* had 20 clones vs 16 *α-Proteobacteria* (**Figure 3**, **Table 2**). *Acidobacterium* comprised 14.25% of the clone library whereas the other bacterial divisions each comprised less than 7% of the clone library. Other

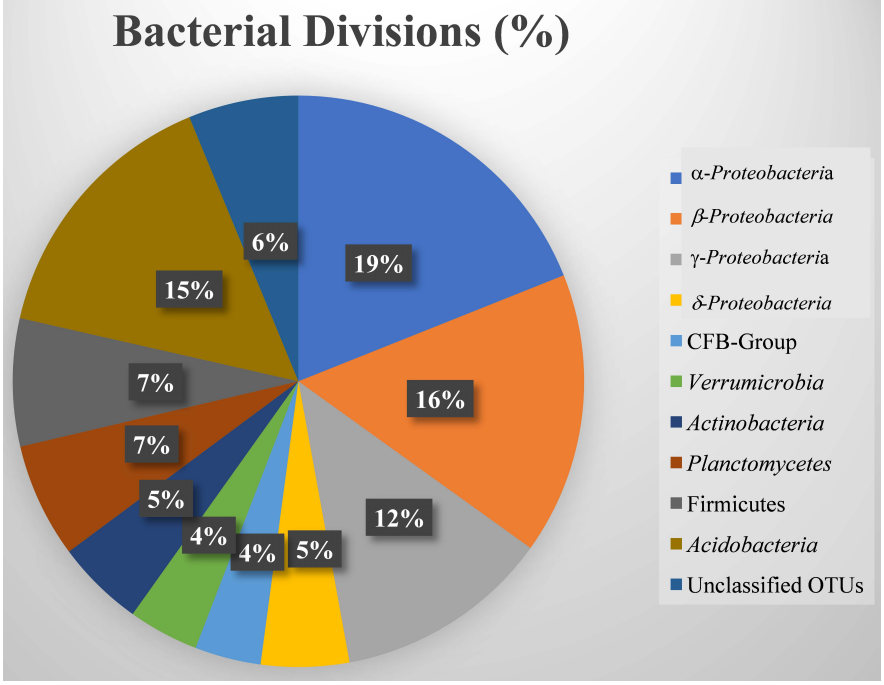

Figure 2. Classification of 16S rDNA clones (n = 400) from rhizosphere (RS) and control soil (CS) samples from NMPE into bacterial phylogenetic groups and showing percentage prevalence of the cloned library of RS soils *α-Proteobacteria*; *β-Proteobacteria*; *γ-Proteobacteria*; *δ-Proteobacteria*; *Actinobacteria*; CFB group; *Acidobacterium*; *Firmicutes*, *Planctomycetes*, *Verrucomicrobia* and unclassified clones.

studies have indicated that *α-Proteobacteria* members were most abundant in 16S rDNA clone libraries from non-rhizosphere forest soil samples [39]. In contrast, *Acidobacterium* members were most abundant in clone libraries from Arizona pinyon pine rhizosphere and bulk soil samples [40]. The relative abundance of *Actinobacteria* for both the RS and CS soils in this study was substantially less compared to clone libraries of mineral soil from forests [39] and rhizosphere soil from grassland plant species [41]. Twenty-five 16S rDNA clones (6.25% of the clone library) could not be classified into known bacterial divisions based on publicly available 16S rRNA gene sequence information and phylogenetic analyses. Many of these clones formed bootstrap-supported clusters with each other and GenBank member sequences were not closely related (results not shown). Unclassified clones with chimeric structures were not detected upon examination of long-distance base pairing in the secondary DNA structure (A. Ahmad, unpublished results).

Phylogenetic analyses revealed that 80% of 16S rDNA clone sequences from both CS and RS soils formed bootstrap-supported clusters that contained GenBank member sequences originating from diverse soil sources, prairie and aquatic environments [23] [38] [42]. The remaining clones either were represented in eight bootstrap-supported clusters which excluded GenBank member sequences (266 clones which represented *Proteobacteria* and *Acidobacterium*) (**Table 2**). These 16S rDNA clone sequences may represent members of novel lineages

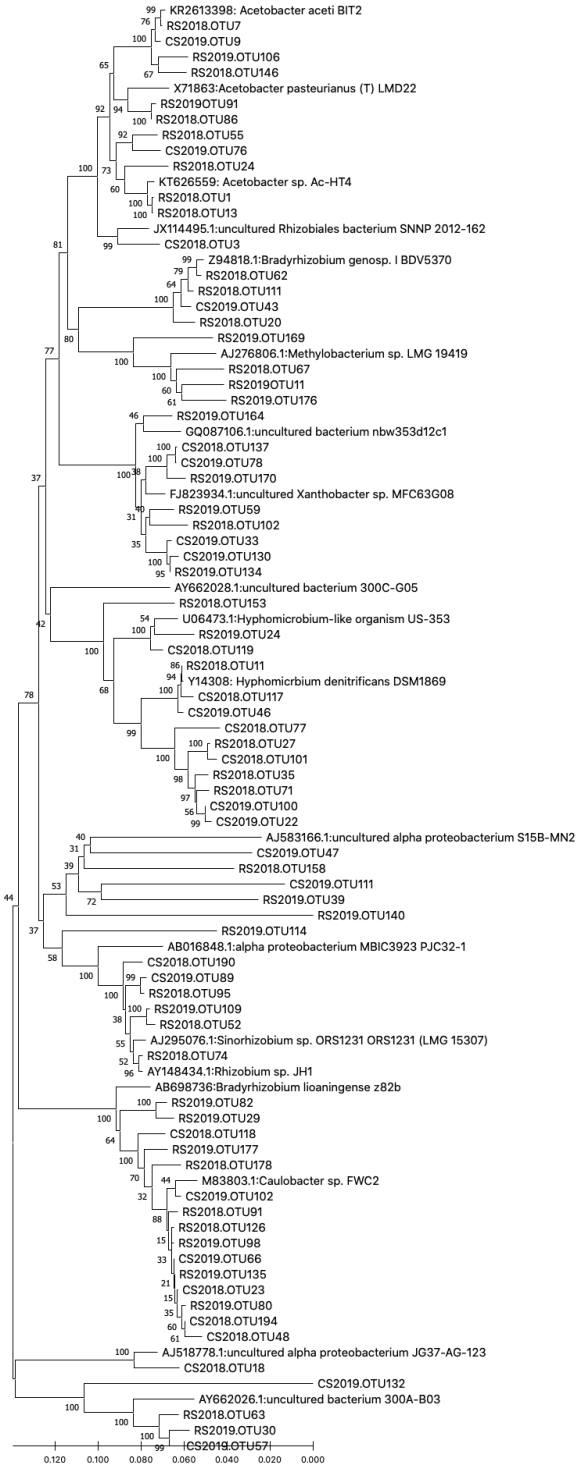

Figure 3. Phylogenetic tree of *α-Proteobacteria* partial 16S rRNA gene sequences representing 43 rhizosphere 16S rDNA clones and 33 control soils from the NMPE forest soils and 17 reference sequences from the RDP database representing various groups are shown. The number of rhizosphere 16S rDNA clones represented in each cluster is: cluster 1: 41 clones; cluster 2:29 clones; cluster 3: 29 clones; cluster 4: 10 clones; cluster 5: two clones; cluster 6: 13 clones; cluster 7: seven clones; and cluster 8: no clones, the cluster is represented by two GenBank member sequences. Two clones, C47.23PG and N42.38PG, were not affiliated with the eight proposed subdivisions of *Acidobacterium*.

Table 2. Summary of the 16S rDNA clone affiliation in phylogenetic tree clusters based on the most closely related GenBank member sequences included in trees[a] and present in clusters.

Phylogenetic tree cluster affiliation	Number of clusters
α-Proteobacteria	
Rhizobiaceae group	3
Acetobacteriaceae	3
Caulobacter group	1
Hyphomicrobium	*2*
No known cultivated relative (GenBank sequence(s) excluded from clusters)	6
Number of single sequences not in clusters:	8
β-Proteobacteria	
Burkholderia group	5
Comamonadaceae	2
Spirallaceae	*1*
Nitrosomonadaceae	*2*
Rhodocyclus group	1
No known cultivated relative (GenBank sequence(s) excluded from clusters)	5
Number of single sequences not in clusters:	3
γ-Proteobacteria	
Enterobacteriaceae	2
Pseudomonadaceae	1
Xanthomonas group	2
No known cultivated relative (GenBank sequence(s) excluded from clusters)	2
Number of single sequences not in clusters:	3
δ-Proteobacteria	
Desulfuromonas group	2
Myxobacteria	1
No known cultivated relative (GenBank environmental sequence(s) in clusters)	2
Number of single sequences not in clusters:	5
CFB group	
*Flavobacteriacea*e and *Flexibacter* group	2
Flavobacteriaceae and *Cytophagaceae*	2
Acidobacterium	5
Actinobacteria	1
Verrucomicrobia	3

within *Proteobacteria* and *Acidobacterium* that have not been previously reported. A summary of the cluster affiliation from all phylogenetic trees is pre-

sented in Table 2 and the highlights for *Proteobacteria* and *Acidobacterium* are discussed below. Detailed phylogenetic trees were not included in this paper due to their size and complexity (A. Ahmad, unpublished).

Proteobacteria. Two hundred and nine (52%) 16S rDNA clone sequences were affiliated with *α*-, *β*-, *γ*-, and *δ*-*Proteobacteri*a and were related to 16 bacterial families and groups representing cultivated bacterial genera (Table 2). In contrast, 3.5% of the *Proteobacteri*a 16S rDNA clone sequences were present in phylogenetic tree clusters containing only uncultivated GenBank representatives. The largest cluster of related *Proteobacteria* 16S rRNA gene sequences consisted of 33 clones affiliated with *Burkholderia*, also represented as cluster 1 in the *β*-*Proteobacteria* tree (data not shown), most probably has an important role in NMPE rhizosphere ecology. *Burkholderia* species are nutritionally versatile, common residents of rhizosphere soil, and beneficial attributes of some members include nitrogen fixation, plant growth promotion and biological disease control [43]. Only eleven of the 33 16S rDNA clone sequences shared 99% sequence similarity with other clones originating from the RS soil sample suggesting high diversity in the *Burkholderia* group sequences (Figure 4). At the same time, a notable feature of this cluster is that RS sequences were identical or nearly identical to partial 16S rRNA gene sequences from CS sites indicating some conserved nature of the 16S rRNA gene in the recovered *Burkholderia* group sequences from NMPE sites.

Although *Pseudomonas* is also recognized as a common rhizosphere colonist [44], 19 of the 200 rhizosphere 16S rDNA clone sequences formed a bootstrap-supported cluster with GenBank member *Pseudomonas* sequences (data not shown). Cloned 16S rRNA genes affiliated with *Pseudomona*s were infrequently recovered from grassland rhizosphere soil samples [45]. *Pseudomona*s were readily cultivated from mineral soil samples though a significantly greater proportion of *Pseudomonas* 16S rRNA gene sequences were cloned from CS soil samples compared to RS. Eleven clones affiliated with *Pseudomonas* were obtained from CS at the NMPE sites. Further research is warranted to examine rhizosphere-associated *Pseudomonas* abundance and diversity from NMPE sites. Twenty-one clones of *Rhizobiaceae* group sequences were recovered from RS rhizosphere soil and six from CS samples at the two NMPE sites (data not shown). Though the vegetation present on NMPE plots does not include leguminous plants (A. Ahmad, personal communication), it is also possible that *Rhizobium* is a native member of non-legume rhizospheres.

Acidobacteria: Fifty seven 16S rDNA clone sequences were affiliated with seven of the eight monophyletic *Acidobacterium* subdivisions proposed by Hugenholtz and co-workers [46] indicating broad diversity and potential ecological significance of this bacterial division in NMPE rhizospheres (Table 2). Seventy-four percent of the clone sequences were affiliated with *Acidobacterium* subdivisions 1, 2 and 3. The sequences did not cluster with GenBank member Holophaga and Geothrix 16S rRNA gene sequences which belong to *Acidobacterium* subdivision 8 (Data not shown). Full-length sequence analysis is needed to further

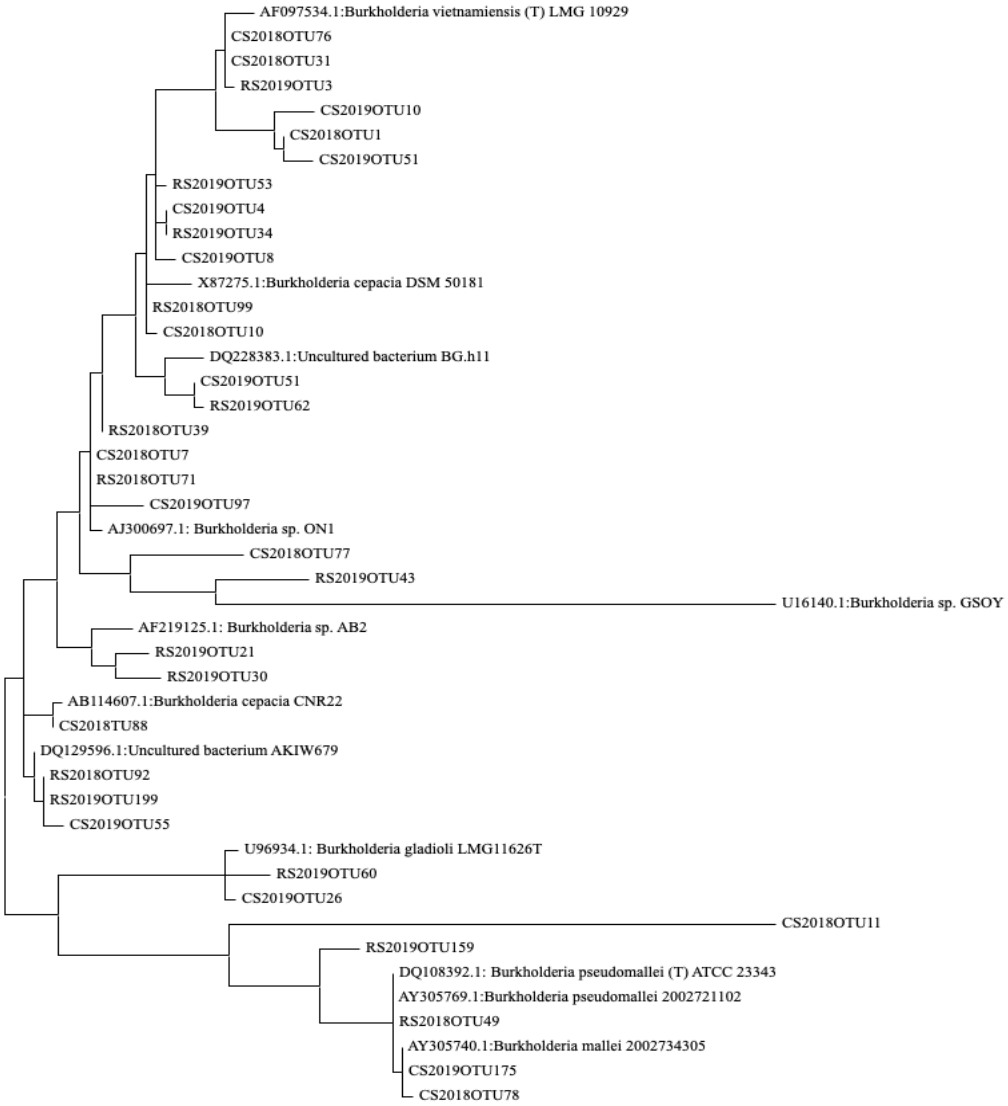

Figure 4. Bootstrap-supported cluster of *Burkholderia* group partial 16S rRNA gene sequences extracted from a phylogenetic tree containing *Proteobacteria*16S rDNA clone sequences from NMPE forests16S rDNA clones. Partial 16S rRNA gene sequences from the Genbank are designated by the accession numbers.

explore the affiliation of *Acidobacterium* clones. Although *Acidobacterium* has few cultivated members, DNA sequences from uncultivated representatives have been frequently recovered from soil [46] [47] including forest environments [39] [48].

Comparison of bacterial community profiles of CS and RS of NMPE sites. Few different trends in community profiles were apparent among 16S rDNA clones from the RS and CS plots in the NMPE sites. The relative abundance of *Proteobacteria* was greatest for CS plot, followed by *Acidobacterium* (**Table 3**). Statistical analyses revealed that within the *Proteobacteria*, the trend from highest to lowest relative abundance was *α*-, *β*-, *γ*-, and *δ*-*Proteobacteri*a respectively, for both the CS and RS plot sites and showed significant NMPE site effects. All likelihood

Table 3. Classification of 16S rDNA clones from NMPE plot sites into bacterial phylogenetic groups.

Phylogenetic groups	Number of 16S rDNA clones				
	RS-2018	CS-2018	RS2019	CS-2019	Total
Proteobacteria, total	46	57	52	54	209
α-Proteobacteria	23	16	20	17	76
β-Proteobacteria	11	20	16	17	64
γ-Proteobacteria	9	13	12	15	49
δ-Proteobacteria	3	7	4	5	19
Uncultivated *Proteobacteria*	4	3	2	3	12
Acidobacteria	12	16	13	16	57
Actinobacteria	5	5	7	3	20
CFB group	5	2	3	5	15
Firmicutes	6	8	7	5	26
Planctomycetes	3	8	4	7	22
Verrucomicrobia	5	3	4	3	15
Unclassified, total	5	2	10	8	25
Total	100	100	100	100	400

ratio *P* values exceeded 0.2 when analyses were completed for *Proteobacteria* at the division level and for individual analyses of *α*-, *β*-, and *γ-Proteobacteria.* For *Acidobacterium*, *P* value (testing for plot effects) was approximately 0.06 and the magnitudes of differences in the number of *Acidobacterium* clones among sites were not dramatic. The numbers of 16S rDNA clones belonging to other bacterial groups were too low for statistical analyses. Felske and Akkermans [49] also found more similarities than differences in community profiles when they examined temperature gradient gel electrophoresis fingerprints from 160 soil samples representing three grassland field sites.

Examination of the source of 16S rRNA gene sequences within individual bootstrap-supported clusters from phylogenetic trees provided a better comparison of the bacterial community profile from different NMPE soil sites. This approach may have more biological relevance as opposed to a general analysis of the relative abundance of 16S rDNA clones belonging to individual bacterial divisions or subdivisions. Clusters were selected that contained four or more members (a minimum of 1% of the clone library) and that were supported by bootstrap values > 80 to allow for comparisons of related 16S rRNA gene fragments. Only 17 of the 41 clusters each contained one or more 16S rRNA gene fragments originating from the two NMPE sites. Therefore, it was less common for phylogenetic tree clusters to contain related 16S rRNA gene fragments representing 16S rDNA clones from both RS and CS plot sites. Only four clusters had more than six clones, and three of these clusters (including the *Burkholderia* group

cluster, **Figure 4**) each contained one or more 16S rRNA gene fragments originating from the RS plot sites. The above results indicate that the rhizosphere environment generated by *L. maackii* may have exerted a stronger selective pressure in determining the rhizosphere bacterial community than soil conditions prevailing in CS soils. Historically, it has been thought that rhizosphere microbial populations are directly or indirectly related to root exudates [50] indicating the importance of the host plant. Numerous studies under field and controlled conditions have reported the importance of the host plant and/or soil factors in influencing the composition of rhizosphere bacterial communities [51] [52] [53]. PCR-based methods have proved valuable in generating cultivation-independent microbial diversity data. Yet, it is recognized that PCR artefacts may cause biases [54] and the relative proportions of different bacterial groups represented in clone libraries may not reflect the relative proportions present in template DNA samples [55]. The analysis of multiple soil samples representing host plant or soil types strengthens the conclusions which can be drawn from bacterial diversity studies.

4. Conclusion

Management practices such as controlled burns, tree harvesting, site preparation and subsequent planting of native seedlings can result in changes in the soil environment. In this study, we addressed two main questions about NMPE soils: 1) What the bacterial community profile is in the *L. maackii* rhizosphere soils? 2) Does this profile differ from the control soils devoid of *L. maackii*? The 16S rDNA clone library from NMPE LM rhizosphere soils represented seven known bacterial divisions. There were significant site-effects on the relative abundance of 16S rDNA clones belonging to *Proteobacteria* or *Acidobacterium* which together comprised 66.5% of the clone library. Phylogenetic analyses indicated that 16S rRNA gene fragments from CS and RS corresponding to different NMPE sites only somewhat group together in bootstrap-supported clusters. These results suggest that the LM rhizosphere is a niche that supports extremely diverse microbial communities which though shares similar but not identical profiles in the control soils. Torsvik and coworkers [56] stress that information about bacterial communities and their diversity is required to explore questions regarding the impact of environmental factors on ecosystem function. However, Staddon and co-workers [57] note that information is lacking about the variability of soil microbial communities in forest ecosystems. This study established a library of diverse 16S rRNA gene fragments from NMPE rhizosphere soil which can be used to construct specific DNA primers and probes to target bacterial groups of interest. Microbial indicators could prove valuable for assessments of soil quality [58] relating to ecological forest management [57]. Future studies on NMPE sites can be strengthened by using combinations of molecular methods to profile and fingerprint whole rhizosphere bacterial communities. Furthermore, a unique opportunity exists for future investigations into invasive tree species addressing the

relationship between soil bacterial communities and forest ecosystem functions through integration with other forest sites across climatic gradients in North America.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., *et al.* (2001) Bulk and Rhizosphere Soil Bacterial Communities Studied by Denaturing Gradient Gel Electrophoresis: Plant-Dependent Enrichment and Seasonal Shifts Revealed. *Applied and Environmental Microbiology*, **67**, 4742-4751. https://doi.org/10.1128/AEM.67.10.4742-4751.2001

[2] Klironomos, J.N. (2002) Feedback with Soil Biota Contributes to Plant Rarity and Invasiveness in Communities. *Nature*, **417**, 67-70. https://doi.org/10.1038/417067a

[3] Bever, J.D. (2002) Negative Feedback within a Mutualism: Host-Specific Growth of Mycorrhizal Fungi Reduces Plant Benefit. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **269**, 2595-2601. https://doi.org/10.1098/rspb.2002.2162

[4] Miethling, R., Wieland, G., Backhaus, H. and Tebbe, C.C. (2000) Variation of Microbial Rhizosphere Communities in Response to Crop Species, Soil Origin, and Inoculation with *Sinorhizobium meliloti* L33. *Microbial Ecology*, **40**, 43-56. https://doi.org/10.1007/s002480000021

[5] Lemanceau, P., Corberand, T., Gardan, L., Latour, X., Laguerre, G., Boeufgras, J., *et al.* (1995) Effect of Two Plant Species, Flax (*Linum usitatissinum* L.) and Tomato (*Lycopersicon esculentum* Mill.), on the Diversity of Soilborne Populations of Fluorescent Pseudomonads. *Applied and Environmental Microbiology*, **61**, 1004-1012. https://doi.org/10.1128/aem.61.3.1004-1012.1995

[6] Hart, S.C. and Classen, A.T. (2003) Potential for Assessing Long-Term Dynamics in Soil Nitrogen Availability from Variations in delta15N of tree rings. *Isotopes in Environmental and Health Studies*, **39**, 15-28. https://doi.org/10.1080/1025601031000102206

[7] Sanders, I.R., Streitwolf-Engel, R., van der Heijden, M.G.A., Boller, T. and Wiemken, A. (1998) Increased Allocation to External Hyphae of Arbuscular Mycorrhizal Fungi under CO_2 Enrichment. *Oecologia*, **117**, 496-503. https://doi.org/10.1007/s004420050685

[8] Callaway, R.M., Thelen, G.C., Rodriguez, A. and Holben, W.E. (2004) Soil Biota and Exotic Plant Invasion. *Nature*, **427**, 731-733. https://doi.org/10.1038/nature02322

[9] Shively, J.M., Samuel English, R., Baker, S.H. and Cannon, G.C. (2001) Carbon Cycling: The Prokaryotic Contribution. *Current Opinion in Microbiology*, **4**, 301-306. https://doi.org/10.1016/S1369-5274(00)00207-1

[10] Zakharova, O.S., Zenova, G.M. and Zviagintsev, D.G. (2003) Selektivnye priemy vydeleniia iz pochvy aktinomitsetov roda Actinomadura [Selective Isolation of Actinomycetes of the Genus Actinomadura from Soil]. *Mikrobiologiia*, **72**, 126-130.

[11] Bever, J.D., Platt, T.G. and Morton, E.R. (2012) Microbial Population and Community Dynamics on Plant Roots and Their Feedbacks on Plant Communities. *Annual*

Review of Microbiology, **66**, 265-283.
https://doi.org/10.1146/annurev-micro-092611-150107

[12] Cline, L.C. and Zak, D.R. (2015) Soil Microbial Communities Are Shaped by Plant-Driven Changes in Resource Availability during Secondary Succession. *Ecology*, **96**, 3374-3385. https://doi.org/10.1890/15-0184.1

[13] Rudrappa, T. and Bais, H.P. (2008) Rhizospheric Pseudomonads: Friends or Foes? *Plant Signaling & Behavior*, **3**, 1132-1133. https://doi.org/10.4161/psb.3.12.7093

[14] Gibbons, S.M., Lekberg, Y., Mummey, D.L., Sangwan, N., Ramsey, P.W. and Gilbert, J.A. (2017) Invasive Plants Rapidly Reshape Soil Properties in a Grassland Ecosystem. *mSystems*, **2**, e00178-16. https://doi.org/10.1128/mSystems.00178-16

[15] Carvalho, R.F., Carvalho, S.D. and Duque, P. (2010) The Plant-Specific SR45 Protein Negatively Regulates Glucose and ABA Signaling during Early Seedling Development in Arabidopsis. *Plant Physiology*, **154**, 772-783.
https://doi.org/10.1104/pp.110.155523

[16] Zhang, Q., Yang, R., Tang, J., Yang, H., Hu, S. and Chen, X. (2010) Positive Feedback between Mycorrhizal Fungi and Plants Influences Plant Invasion Success and Resistance to Invasion. *PLoS ONE*, **5**, e12380.
https://doi.org/10.1371/journal.pone.0012380

[17] Oliveira, D.F., Pereira, A.C., Figueiredo, H.C.P., Carvalho, D.A., Silva, G., Nunes, A.S., *et al.* (2007) Antibacterial Activity of Plant Extracts from Brazilian Southeast Region. *Fitoterapia*, **78**, 142-145. https://doi.org/10.1016/j.fitote.2006.09.027

[18] Nijjer, S., Rogers, W.E. and Siemann, E. (2007) Negative Plant-Soil Feedbacks May Limit Persistence of an Invasive Tree due to Rapid Accumulation of Soil Pathogens. *Proceedings of the Royal Society B: Biological Sciences*, **274**, 2621-2627.
https://doi.org/10.1098/rspb.2007.0804

[19] Pimentel, D., Zuniga, R. and Morrison, D. (2005) Update on the Environmental and Economic Costs Associated with Alien-Invasive Species in the United States. *Ecological economics*, **52**, 273-288. https://doi.org/10.1016/j.ecolecon.2004.10.002

[20] Lazzaro, L., Bolpagni, R., Buffa, G., Gentili, R., Lonati, M., Stinca, A., *et al.* (2020) Impact of Invasive Alien Plants on Native Plant Communities and Natura 2000 Habitats: State of the Art, Gap Analysis and Perspectives in Italy. *Journal of Environmental Management*, **274**, Article ID: 111140.
https://doi.org/10.1016/j.jenvman.2020.111140

[21] Dukes, J.S., Chiariello, N.R., Loarie, S.R. and Field, C.B. (2011) Strong Response of an Invasive Plant Species (*Centaurea solstitialis* L.) to Global Environmental Changes. *Ecological Applications*, **21**, 1887-1894. https://doi.org/10.1890/11-0111.1

[22] Early, R., Bradley, B.A., Dukes, J.S., Lawler, J.J., Olden, J.D., Blumenthal, D.M., *et al.* (2016) Global Threats from Invasive Alien Species in the Twenty-First Century and National Response Capacities. *Nature Communications*, **7**, Article ID: 12485.
https://doi.org/10.1038/ncomms12485

[23] Schuster, M.J. and Dukes, J.S. (2017) Rainfall Variability Counteracts N Addition by Promoting Invasive *Lonicera maackii* and Extending Phenology in Prairie. *Ecological Applications*, **27**, 1555-1563. https://doi.org/10.1002/eap.1547

[24] Pyšek, P. and Richardson, D.M. (2010) Invasive Species, Environmental Change and Management, and Health. *Annual Review of Environment and Resources*, **35**, 25-55.
https://doi.org/10.1146/annurev-environ-033009-095548

[25] Kueffer, C. (2010) Transdisciplinary Research Is Needed to Predict Plant Invasions in an Era of Global Change. *Trends in Ecology Evolution*, **25**, 619-620.

https://doi.org/10.1016/j.tree.2010.08.001

[26] Christopher, C.C. and Cameron, G.N. (2012) Effects of Invasive Amur Honeysuckle (*Lonicera maackii*) and White-Tailed Deer (*Odocoileus virginianus*) on Litter-Dwelling Arthropod Communities. *The American Midland Naturalist*, **167**, 256-272. https://doi.org/10.1674/0003-0031-167.2.256

[27] Christopher, C.C., Matter, S.F. and Cameron, G.N. (2014) Individual and Interactive Effects of Amur Honeysuckle (*Lonicera maackii*) and White-Tailed Deer (*Odocoileus virginianus*) on Herbs in a Deciduous Forest in the Eastern United States. *Biological Invasions*, **16**, 2247-2261. https://doi.org/10.1007/s10530-014-0661-x

[28] McNeish, R.E. and McEwan, R.W. (2016) A Review on the Invasion Ecology of Amur Honeysuckle (*Lonicera maackii*, Caprifoliaceae): A Case Study of Ecological Impacts at Multiple Scales. *The Journal of the Torrey Botanical Society*, **143**, 367-385. https://doi.org/10.3159/TORREY-D-15-00049.1

[29] Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., *et al.* (2013) Evaluation of General 16S Ribosomal RNA Gene PCR Primers for Classical and Next-Generation Sequencing-Based Diversity Studies. *Nucleic Acids Research*, **41**, e1. https://doi.org/10.1093/nar/gks808

[30] Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Titus Brown, C., *et al.* (2014) Ribosomal Database Project: Data and Tools for High throughput rRNA Analysis. *Nucleic Acids Research*, **42**, D633-D642. https://doi.org/10.1093/nar/gkt1244

[31] Tuan, N.N., Chang, Y.-C., Yu, C.-P. and Huang, S.-L. (2014) Multiple Approaches to Characterize the Microbial Community in a Thermophilic Anaerobic Digester Running on Swine Manure: A Case Study. *Microbiological Research*, **169**, 717-724. https://doi.org/10.1016/j.micres.2014.02.003

[32] Ahmad, A., Barry, J.P. and Nelson, D.C. (1999) Phylogenetic Affinity of a Wide, Vacuolate, Nitrate-Accumulating *Beggiatoa* sp. from Monterey Canyon, California, with *Thioploca* spp. *Applied and Environmental Microbiology*, **65**, 270-277. https://doi.org/10.1128/AEM.65.1.270-277.1999

[33] Wang, F., Men, X., Zhang, G., Liang, K., Xin, Y., Wang, J., *et al.* (2018) Assessment of 16S rRNA Gene Primers for Studying Bacterial Community Structure and Function of Aging Flue-Cured Tobaccos. *AMB Express*, **8**, Article No.182. https://doi.org/10.1186/s13568-018-0713-1

[34] Munyaka, P.M., Eissa, N., Bernstein, C.N., Khafipour, E. and Ghia, J.-E. (2015) Antepartum Antibiotic Treatment Increases Offspring Susceptibility to Experimental Colitis: A Role of the Gut Microbiota. *PLoS ONE*, **10**, e0142536. https://doi.org/10.1371/journal.pone.0142536

[35] Dufrêne, M. and Legendre, P. (1997) Pecies Assemblages and Indicator Species: The Need for a Flexible Assymetrical Approach. *Ecological Monographs*, **67**, 345-366. https://doi.org/10.1890/0012-9615(1997)067[0345:SAAIST]2.0.CO;2

[36] Karlin, S. and Altschul, S.F. (1990) Methods for Assessing the Statistical Significance of Molecular Sequence Features by Using General Scoring Schemes. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 2264-2268.

[37] Barillot, C.D.C., Claude-Olivier, S., Tarnaud, E. and Cochet, N. (2013) A Standardized Method for the Sampling of Rhizosphere and Rhizoplan Soil Bacteria Associated to a Herbaceous Root System. *Annals of Microbiology*, **63**, 471-476. https://doi.org/10.1007/s13213-012-0491-y

[38] McPherson, M.R., Wang, P., Marsh, E.L., Mitchell, R.B. and Schachtman, D.P. (2018) Isolation and Analysis of Microbial Communities in Soil, Rhizosphere, and

Roots in Perennial Grass Experiments. *Journal of Visualized Experiments*, **137**, Article ID: 57932. https://doi.org/10.3791/57932

[39] Axelrood, P.E., Wang, P., Marsh, E.L., Mitchell, R.B. and Schachtman, D.P. (2002) Molecular Characterization of Bacterial Diversity from British Columbia Forest Soils Subjected to Disturbance. *Canadian Journal of Microbiology*, **48**, 655-674. https://doi.org/10.1139/w02-059

[40] Dunbar, J., Takala, S., Barns, S.M., Davis, J.A. and Kuske, C.R. (1999) Levels of Bacterial Community Diversity in Four Arid Soils Compared by Cultivation and 16S rRNA Gene Cloning. *Applied and Environmental Microbiology*, **65**, 1662-1669. https://doi.org/10.1128/AEM.65.4.1662-1669.1999

[41] McCaig, A.E., Glover, L.A. and Prosser, J.I. (1999) Molecular Analysis of Bacterial Community Structure and Diversity in Unimproved and Improved Upland Grass Pastures. *Applied and Environmental Microbiology*, **65**, 1721-1730. https://doi.org/10.1128/AEM.65.4.1721-1730.1999

[42] McNeish, R.E., Benbow, M.E. and McEwan, R.W. (2012) Riparian Forest Invasion by a Terrestrial Shrub (*Lonicera maackii*) Impacts Aquatic Biota and Organic Matter Processing in Headwater Streams. *Biological Invasions*, **14**, 1881-1893. https://doi.org/10.1007/s10530-012-0199-8

[43] Parke, J.L. and Gurian-Sherman, D. (2001) Diversity of the *Burkholderia cepacia* Complex and Implications for Risk Assessment of Biological Control Strains. *Annual Review of Phytopathology*, **39**, 225-258. https://doi.org/10.1146/annurev.phyto.39.1.225

[44] Qessaoui, R., Bouharroud, R., Furze, J.N., El Aalaoui, M., Akroud, H., Amarraque, A., *et al.* (2019) Applications of New Rhizobacteria *Pseudomonas* Isolates in Agroecology via Fundamental Processes Complementing Plant Growth. *Scientific Reports*, **9**, Article No. 12832. https://doi.org/10.1038/s41598-019-49216-8

[45] Vieira, S., Sikorski, J., Dietz, S., Herz, K., Schrumpf, M., Bruelheide, H., *et al.* (2020) Drivers of the Composition of Active Rhizosphere Bacterial Communities in Temperate Grasslands. *The ISME Journal*, **14**, 463-475. https://doi.org/10.1038/s41396-019-0543-4

[46] Hugenholtz, P., Goebel, B.M. and Pace, N.R. (1998) Impact of Culture-Independent Studies on the Emerging Phylogenetic View of Bacterial Diversity. *Journal of Bacteriology*, **180**, 4765-4774. https://doi.org/10.1128/JB.180.18.4765-4774.1998

[47] Barns, S.M., Takala, S.L. and Kuske, C.R. (1999) Wide Distribution and Diversity of Members of the Bacterial Kingdom *Acidobacterium* in the Environment. *Applied and Environmental Microbiology*, **65**, 1731-1737. https://doi.org/10.1128/AEM.65.4.1731-1737.1999

[48] Kuske, C.R., Barns, S.M. and Busch, J.D. (1997) Diverse Uncultivated Bacterial Groups from Soils of the Arid Southwestern United States That Are Present in Many Geographic Regions. *Applied and Environmental Microbiology*, **63**, 3614-3621. https://doi.org/10.1128/aem.63.9.3614-3621.1997

[49] Felske, A. and Akkermans, A.D.L. (1998) Spatial Homogeneity of Abundant Bacterial 16S rRNA Molecules in Grassland Soils. *Microbial Ecology*, **36**, 31-36. https://doi.org/10.1007/s002489900090

[50] de B. Richter, D., Oh, N.-H., Fimmen, R. and Jackson, J. (2007) CHAPTER 8—The Rhizosphere and Soil Formation. In: Cardon, Z.G. and Whitbeck, J.L., Eds., *The Rhizosphere*, Academic Press, Burlington, 179-200, IN1-IN2. https://doi.org/10.1016/B978-012088775-0/50010-0

[51] Koo, B.J., Adriano, D.C., Bolan, N.S. and Barton, C.D. (2005) Root Exudates and

Microorganisms. In: Hillel, D., Ed., *Encyclopedia of Soils in the Environment*, Elsevier, Oxford, 421-428. https://doi.org/10.1016/B0-12-348530-4/00461-6

[52] Marschner, P. (2012) Chapter 15—Rhizosphere Biology. In: Marschner, P., Ed., *Marschner's Mineral Nutrition of Higher Plants*, 3rd Edition, Academic Press, San Diego, 369-388. https://doi.org/10.1016/B978-0-12-384905-2.00015-7

[53] Mendes, R., Garbeva, P. and Raaijmakers, J.M. (2013) The Rhizosphere Microbiome: Significance of Plant Beneficial, Plant Pathogenic, and Human Pathogenic Microorganisms. *FEMS Microbiology Reviews*, **37**, 634-663. https://doi.org/10.1111/1574-6976.12028

[54] Schloter, M., Nannipieri, P., Sørensen, S.J. and van Elsas, J.D. (2018) Microbial Indicators for Soil Quality. *Biology and Fertility of Soils*, **54**, 1-10. https://doi.org/10.1007/s00374-017-1248-3

[55] Amann, R. (2000) Who Is Out There? Microbial Aspects of Biodiversity. *Systematic and Applied Microbiology*, **23**, 1-8. https://doi.org/10.1016/S0723-2020(00)80039-9

[56] Torsvik, V., Sørheim, R. and Goksøyr, J. (1996) Total Bacterial Diversity in Soil and Sediment Communities—A Review. *Journal of Industrial Microbiology*, **17**, 170-178. https://doi.org/10.1007/BF01574690

[57] Staddon, W.J., Duchesne, L.C. and Trevors, J.T. (1999) The Role of Microbial Indicators of Soil Quality in Ecological Forest Management. *The Forestry Chronicle*, **75**, 81-86. https://doi.org/10.5558/tfc75081-1

[58] Bünemann, E.K., Bongiorno, G., Bai, Z., Creamer, R.E., De Deyn, G., de Goede, R.G.M., *et al.* (2018) Soil Quality—A Critical Review. *Soil Biology and Biochemistry*, **120**, 105-125. https://doi.org/10.1016/j.soilbio.2018.01.030