BACTERIAL COMMUNITY STRUCTURE IN SOILS OF THE OLDEST AGRONOMIC EXPERIMENT FIELDS IN THE UNITED STATES, THE MORROW PLOTS, AND OF THE ORIGINAL TALLGRASS PRAIRIE

by

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ABSTRACT

Diversity of soil microbial communities and their influence on plants growth are widely studied to develop and improve sustainable agricultural practices. Soil-microbe interactions are very complex to interpret given that it involves other biotic and abiotic environmental factors, and the traditional culture-based methods followed by physico-chemical analysis are either extremely laborious or not robust enough to investigate the broad picture and intricate details of these complex interactions. The use of molecular technologies like *q*PCR has resolved some of the shortcomings of culture-based methods, but still exhibited biases in both qualitative and quantitative analyses of microbial communities. New molecular techniques that focus on high throughput DNA sequencing techniques such as 454 pyrosequencing and the MiSeq sequencing platforms revolutionized the field of microbial diversity studies. These techniques are widely used in projects such as the Earth Microbiome Project (EMP), a collective attempt to establish microbial fingerprints in different environments of the planet. Additional applications include studies on long-term effects of crop rotation and different fertilization regimen on bacterial community structure. We tried to build on these studies and assess microbial community structure in the Morrow Plots, the oldest agronomic experimental fields in the United States, and adjacent tallgrass prairie with emphasis on members of the genus *Frankia*. The Morrow Plots were established in 1876 on tallgrass prairie soils to evaluate the effects of different cropping systems and soil treatments on crop yields, and include the oldest continuous corn plots in the world. Illumina-based 16S rRNA V3 gene

amplicon sequencing retrieved a total of 26.47 M effective sequences obtained from 44 samples, i.e. 12 soils with different vegetation and fertilization regimen, and 3 to 6 replicates per soil, with 313,695 to 906,328 reads per sample. At a sequencing depth of 300,000 sequences for each sample, Acidobacteria, Actinobacteria, Proteobacteria and Verrumicrobia were the most abundant bacterial phyla present across all soil samples accounting for 74±4% of the reads. Crop rotation increased diversity of the bacterial community, which was also affected by the fertilization regimen. Reads representing frankiae accounted for 0.1 to 1.0% of all reads, with generally higher percentages in fertilized soils. Reads represented frankiae of clusters 1a, 2, 3, and 4, but also a group of frankiae that could not reliably be assigned to a cultured relative. The results provide evidence of long-term establishment of *Frankia* populations in agricultural soils under different management conditions.

I. INTRODUCTION

Soils represent highly heterogeneous environments with a high diversity of microhabitats that are occupied by large numbers of microbes at a tremendous diversity [1-4]. Bacterial numbers alone can exceed more than 10^9 cells g^{-1} soil [5, 6], with estimated diversities of up to 10^6species g^{-1} soil [4]. Although many soils contain significant amounts of organic material, these carbon (C) resources are generally not easily available, and thus soils are often C limited environments for microbes [7, 8]. Easily available C resources can be provided by plants that can overcome C limitation through rhizodeposition, the release of C compounds such as sugars, organic acids, and amino acids from roots into the soil [9-12]. In contrast to the generally oligotrophic conditions in bulk soil, rhizodeposition stimulates abundance and microbial activities in the rhizosphere [10]. The plant species, the developmental stage, the plant growth substrate, and stress factors affect the composition and the amount of root exudates [13-15]. These can also be influenced by rhizosphere microbes themselves [16, 17].

Additional C or nitrogen (N) resources can be generated by rhizosphere priming effects stimulating leaf litter decomposition in natural habitats, or mineralization of manure and other organic fertilizers in agricultural environments [18-20]. In agriculture, additional mineral fertilization can provide nutrients not only to enhance plant growth performance, but also for microbes effectively accelerating decomposition of plant material and thus releasing additional C and N resources [21]. Thus, availability and composition of nutrients for microbes in the rhizosphere are highly dynamic [22]. As a consequence of rhizosphere and priming effects, plants are involved in shaping microbial community structure in soil [23-25]. Plant effects on microbial community structure are

potentially magnified by additional modifications of the environment such as e.g. crop rotation, different management practices or different fertilization regimen, and could result in the differentiation and stabilization of specific communities following consistent and long-term application of these conditions [26].

The study of microbial communities in soils is affected by the complexity of the environment characterized by highly variable soil-plant-microbe interactions in microhabitats, but also by the limitations of methodology for analyzing physico-chemical conditions, interactions or transformations on size levels important for microbes as well as for studying the composition and environmentally controlled changes in the structure of microbial communities. Analyses of both physico-chemical conditions and microbial community structure still require destructive sampling and samples up to 10^4 times the volume of the target organisms (i.e. 1 g soil corresponding to a volume of about 1 cm^3 compared to a soil bacterium with a volume of 1μm^3). Arrays of microelectrodes are available for some physico-chemical analyses of environmental samples at the microscale [27-29], while new molecular techniques that focus on high throughput DNA sequencing such as 454 pyrosequencing and the MiSeq sequencing platforms revolutionized the field of microbial diversity studies [30-33].

Ribosomal RNA genes are generally used as standard markers to study phylogenetic diversity, and are widely applied in high-throughput DNA sequencing projects such as the Earth Microbiome Project (EMP), a collective attempt to establish microbial fingerprints in different environments of the planet [34].Next generation sequencing (NGS)-based analyses of soil microbial communities using rRNA gene fragments as target generally focus on phylogenetic levels that encompass diversity

between phyla and families, though occasionally analyses include assessments on the genus level [35, 36]. Analyses on lower taxonomic levels such as species or subspecies levels are usually not attempted, since they are often impacted by the limited phylogenetic resolution of comparative sequence analyses of 16S rRNA gene fragments, the limited number of reads available at this level, as well as by the lack sufficient sequences for distinct species or subspecies in the databases.

Elaborate databases of 16S rRNA genes or gene fragments have only been created for a few bacterial genera, and species can be distinguished based on 16S rRNA gene sequence information. An example is members of the genus *Frankia* that consists of diverse group of filamentous, gram-positive bacteria that are capable of fixing atmospheric nitrogen (N_2) [37-39]. These soil actinomycetes are mostly detected as symbionts forming root nodules with members of eight plant families representing about 25 genera of woody, dicotyledonous, perennial angiosperms, collectively called "actinorhizal plants" [38]. These *Frankia* symbionts fix atmospheric N_2 and provide reduced nitrogen compounds to the plant that in exchange provides carbon sources to the symbiont. This symbiotic interaction enables the actinorhizal plants to flourish even in nutrient poor soils and in niches that have limited nitrogen availability [37].

Members of the genus *Frankia* can be assigned to four major clusters based on the N_2-fixing capability and host plant specificity. The first three clusters comprise nitrogen-fixing members, while the fourth cluster represents non-nitrogen fixing species [40].

(i) Cluster 1: *Frankia alni* group and related species

This includes the nitrogen-fixing microsymbionts of *Alnus, Comptonia and Morella* (often referred to as *Alnus* host infection group) (*Frankia alni*) and the *Casuarina*-infective strains (*Frankia casuarinae*) [41]

(ii) Cluster 2: Microsymbionts of *Dryas*, *Coriaria*, *Ceanothus*, *Cerocarpus*, and *Datisca* sp.

This group represents *Frankia* strains with only one strain isolated so far (often referred to as *Dryas* host infection group) [42] (*Frankia coriariae*) [43]

(iii) Cluster 3: *Elaeagnus*-infective strains

This includes the nitrogen-fixing microsymbionts of *Elaeagnus, Shepherdia, Hippophae* and *Morella* (often referred to as *Elaeagnus* host infection group) (*Frankia elaeagni*) [41]

(iv) Cluster 4: "Atypical" strains (Non-nitrogen fixing)

This group represents *Frankia* strains generally not capable of fixing nitrogen. They have been isolated from many host plants, including *Alnus*, *Coriaria*, *Datisca* and *Purshia* (*Frankia inefficax*) [44]

Previous studies in our lab have generated large databases of *nif*H and 23S rRNA gene fragments from pure cultures as well as from uncultured endophytes in root nodules of diverse host plant species and locations [45-50]. These databases were used to develop SybrGreen- and Taqman-based *q*PCR methods that enabled us to detect and quantify *Frankia* without the need to culture them and also to study and quantify *Frankia* populations in soil [49, 51-55]. Sequences for 16S rRNA gene fragments are available in

the databases since they are commonly used as basis for phylogenetic identification and characterization of isolates and uncultured endophytes. However, their use in structural analyses of the genus *Frankia* in natural environments has been limited so far to one study [56].

II. OBJECTIVE OF THIS STUDY

The goal of our study was to assess long-term effects of crop rotation and different fertilization regimen on bacterial community structure in the oldest agronomic experimental fields in the United States, the Morrow Plots, and from the original tallgrass prairie.

The Morrow Plots are not only the oldest agronomic research fields in United States, but also include the oldest continuous corn plots in the world[57]. They were established in 1876 to evaluate the effects of different cropping systems and soil treatments on crop yield [58, 59]. Major soil management steps were performed in 1904 and in 1955, with the start of MLP (Barnyard manure-M, Limestone-L and ground rock Phosphorus_P) and NPK (nitrogen-N, processed phosphorus-P, and potassium-K) fertilization regimen, respectively, to the sub-plots to check whether addition of chemical fertilizer can improve soil productivity [59].

Illumina high-throughput sequencing of amplified 16S rRNA gene fragments has been used in the past for comparative analyses of bacterial taxonomic diversity among the land-use types of the Morrow Plots following the Earth Microbiome Project (EMP) protocol [60]. This protocol was also used in a second study analyzing members of the genus *Frankia*, specifically cluster 2 frankiae, in soils as a function of presence/absence of host plants [56].

The basic goals addressed in this study were to expand previous studies on bacterial taxonomic diversity among the land-use types of the Morrow Plots to the original prairie soils, and to more specific analyses of *Frankia* cluster distribution in Morrow Plot soils and their original prairie soils. Specifically, we tried to

a) Assess the bacterial diversity of soils sampled from different plots with different long-term managements (i.e. crop rotation and fertilization) from the oldest agronomic experimental fields in the United States, the Morrow Plots, and of the original tallgrass prairie, and
b) Assess the diversity of members of the genus *Frankia*, a generally nitrogen-fixing actinomycete commonly found in symbiosis with specific woody plants (i.e. actinorhizal plants such as *Alnus*, *Casuarina*, *Elaeagnus*, etc) in managed systems kept without host plants for a long time.

The analyses were performed using the EMP protocol for Illumina next generation sequencing of bacterial communities with 16S rRNA gene fragments as target for amplification and sequence analyses. Sequence data retrieved were subsequently analyzed with an open-source computational pipeline, QIIME (Quantitative Insights Into Microbial Ecology) [61].

III. MATERIALS AND METHODS

Soil samples

Composite samples from the upper 20 cm of soil were obtained September 15, 2014, from 3 sites: Loda Cemetery Prairie Nature Preserve, about 50 km north of Champaign (Illinois, USA) (40.5284721; -88.0717537), Meadow Brook Park (40.0789008; -83.7852567) and the Morrow plots, located near the center of the University of Illinois' Urbana campus (40.6331249; -89.3985283).

Loda Cemetery Prairie sites represented either original mesic black soil tallgrass prairie that had never been plowed or used for pasture, and soil cultivated continuously with corn, while Meadow Brook Park represented restored tallgrass prairie dominated by *Sorghastrumnutans* (L.) (Indiangrass) and *Andropogongerardii* Vitman (Big bluestem) on black prairie soil. The Morrow Plots represent the oldest agronomic experiment fields in the United States, established in 1876 on black soil tallgrass prairie. Nine sites were sampled, with different crop rotation and fertilization regimen (Table 1). All sites were classified as AquicArguidolls, that developed on Flanagan silt loam soil (6.4 - 9.0% sand, 67.1 - 66.8% silt, and 24.2 - 26.5% clay), under prairie vegetation with poor natural drainage and high available-moisture-holding capacity.

Selected physico-chemical characteristics of all soils and treatments (i.e. % organic material, pH, estimated nitrogen release, cation exchange capacity and macronutrient concentrations -P, K, Mg, Ca, NO_3^-, and NH_4^+-) were analyzed by Agricultural Soil Management, Inc (Urbana-Champaign, IL) (Table 2, 3).

Table 1: Soil samples used in this study

Location	Vegetation	Fertilization
Loda Cemetery Prairie		
(40.5284721; -88.0717537)	Native tallgrass prairie	none
	Corn	none
Meadow Brook Park		
(40.0789008; -83.7852567)	Restored Prairie	none
Morrow Plots		
(40.6331249; -89.3985283)	Corn-corn	none
		MLP
		NPK
	Corn-soy	none
		MLP
		NPK
	Corn-oat-alfafa	none
		MLP
		NPK

(MLP- Barnyard manure, limestone and phosphate; NPK-Nitrogen, phosphate and potassium)

Table 2: Basic physico-chemical conditions in prairie and Morrow Plot soils

Location Vegetation, Fertilization[1]	Organic Material (%)	pH	ENR[3] (ppm)	CAC[4] (meq/100 g)
Loda Cemetery Prairie				
Native Prairie	4.6	6.6	46.3	23.9
Corn	3.7	6.0	36.6	20.9
Meadow Brook Park				
Restored Prairie	3.5	6.1	35.3	22.0
Morrow Plots				
Corn-Corn, no fertilization	1.7	6.0	16.9	15.2
Corn-Corn, MLP fertilization	1.9	6.6	19.1	17.0
Corn-Corn, NPK fertilization	2.1 (0.1)	5.6 (0.1)	20.9 (0.9)	15.9 (0.3)
Corn-Soy, no fertilization	2.0 (0.1)	5.9 (0.2)	19.6 (0.7)	16.5 (0.1)
Corn-Soy, MLP fertilization	2.4	6.3	23.8	19.0
Corn-Soy, NPK fertilization	2.0	5.9	20.1	16.4
Corn-Oat-Alfalfa, no fertilization	2.9	5.7	28.6	19.3
Corn-Oat-Alfalfa, MLP fertilization	2.6 (0.1)	6.0 (0.1)	25.4 (0.8)	18.2 (1.3)
Corn-Oat-Alfalfa, NPK fertilization	2.3	5.7	22.7	17.7

[1]Vegetation, Fertilization: Loda Cemetry Prairie (native prairie dominated by *Sorghastrum nutans* (L.) (Indiangrass) and *Andropogon gerardii* Vitman (Big bluestem) on black prairie soil; or cultivated continuously with corn); Meadow Brook Park (restored prairie dominated by *Sorghastrum nutans* (L.) (Indiangrass) and *Andropogon gerardii* Vitman (Big bluestem) on black prairie soil); and Morrow Plots (crop rotation on black prairie soil, with additional fertilization: MLP (manure+lime+P treatment implemented 1904) or NPK (lime+N+P+K treatment implemented 1955); [2]ENR, Estimated Nitrogen Release, released over the season;[3]CAC, Cation Exchange Capacity;[4]NS, no samples

Table 3: Macronutrient concentrations in prairie and Morrow Plot soils

Location	Macronutrient concentration (ppm)					
Vegetation, Fertilization[1]	P	K	Mg	Ca	NO_3^-	NH_4^+
Loda Cemetery Prairie						
Native Prairie	9	186	724	2997	11.4	6.1
Corn	17	194	646	2357	NS[5]	NS
Meadow Brook Park						
Restored Prairie	27	174	627	2796	4.4	3.9
Morrow Plots						
Corn-Corn, no fertilization	16	105	315	1894	4.6	2.5
Corn-Corn, MLP fertilization	30	163	360	2235	6.1	2.5
Corn-Corn, NPK fertilization	71 (9)	236 (30)	278 (9)	1943 (54)	4.7 (1.2)	3.8 (0.9)
Corn-Soy, no fertilization	11 (2)	86 (4)	347 (6)	1986 (47)	6.1 (0.7)	3.2 (0.4)
Corn-Soy, MLP fertilization	21	114	373	2639	10.9	3.7
Corn-Soy, NPK fertilization	57	177	320	2021	5.0	2.5
Corn-Oat-Alfalfa, no fertilization	12	142	386	2267	12.4	3.3
Corn-Oat-Alfalfa, MLP fertilization	36 (8)	226 (11)	395 (8)	2298 (26)	32.8 (8.7)	2.3 (0.4)
Corn-Oat-Alfalfa, NPK fertilization	72	170	388	2002	22.2	3.3

DNA extraction

DNA was extracted from soils using the SurePrep^{TM} Soil DNA Isolation Kit (Fisher Scientific, Houston, TX) with small modifications as described before [62]. Extractions of all samples were done in triplicate, and DNA concentrations measured with a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, USA). Concentrations ranged from 8 to 30 ng μl^{-1}, corresponding to soil DNA concentrations between 3.2 and 12.0 µg (g soil)$^{-1}$.

Illumina sequencing

For Illumina sequencing, 16S rRNA gene fragments were amplified using primer 515f ($^{5'}$GTG CCA GCM GCC GCG GTA A) and barcoded primer 806r ($^{5'}$GGA CTA CHV GGG TWT CTAAT), which target the V3-V4 region. Both primers included linker sequences following the instructions from the Earth Microbiome Project (www.earthmicrobiome.org/emp-standard-protocols/16s/) [63]. PCR was carried out in a 100 µl volume with 1 x *Taq* Buffer, 1.5 mM $MgCl_2$, 0.2 mMdNTPs, 0.2 µM primers, 2.5 µg μl^{-1} BSA, 1U *Taq* polymerase (GenScript, Inc., Piscataway, NJ) and 1 µl DNA extract. PCR conditions followed those of the Earth Microbiome project with an initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, with a final 72°C extension for 10 min. PCR products were cleaned using the UltraClean® 15 DNA Purification Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) and then checked and quantified on a 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA) using the Agilent DNA 7500 Kit. Samples were analyzed at the Genomic Sequencing and Analysis Facility at the University of Texas (Austin, TX), on the

Illumina MiSeq v3 with paired end 2 x 300 bp reads using the respective sequencing and index sequence primers (www.earthmicrobiome.org/emp-standard-protocols/16s/).

Data analyses

The sequences were analyzed using the QIIME software (Quantitative Insights Into Microbial Ecology, version 1.9.1) [61] on a LINUX platform available on the High Performance Computing (HPC)-STAR-Cluster at Texas State University, San Marcos. The 16S rRNA V4 Data was mapped to Reference Sequence following the steps mentioned in the standard EMP protocol for mapping Databases (http://www.earthmicrobiome.org/emp-standard-protocols/16s-taxonomic-assignments/).

Quality filtering and demultiplexing

Fastq files containing the raw reads were received from the sequencing facility with barcodes, primers and adapter/linker sequences removed from them. Quality filtering and demultiplexing of sequences were done using "split_libraries_fastq.py". The sequences were truncated at a point where the quality/phred scores of three consecutive bases fell below 20. The resulting truncated sequences were about 75% of the length of the original sequence. These filtered sequences were screened for ambiguous bases (N) and those containing Ns were discarded.

OTU picking

The resulting files containing the demultiplexed and filtered sequences were subjected to open reference operational taxonomic unit (OTU) picking using "pick_open_reference_otus.py", with sequencesof more than 97% similarity selected from the most recent 16S rRNA geneGreengenes database (16s) - gg_otus-13_8-relaese/rep_set/97_otus.fasta (http://qiime.org/home_static/dataFiles.html,

http://greengenes. secondgenome.com/downloads)[64]. Similar sequences were clustered into OTU's using UCLUST [65] and alignments of representative OTU's were done using PYNAST [61]. The OTU picking step was run in parallel mode by passing the parameters "–a" and "–O" which indicates the number of parallel jobs to be done. The resulting BIOM table without singleton OTU's and sequences failed to align by PYNAST removed was used to further downstream diversity analysis process.

Diversity analyses

Core diversity analyses were performed to study alpha and beta diversity and to generate summarized taxa plots at different hierarchies. Initially, the resulting taxonomy table generated by QIIME was analyzed at the phylum level to establish the diversity of bacteria in all soil samples. For both alpha and beta diversity analysis, the samples were rarified at a depth of 300,000sequences per sample, and a phylogenetic tree generated through the previous OTU picking step was used as reference tree. The samples were categorized based on the soil type. To study alpha diversity, three metrics that includedPD_whole tree (Faith's Phylogenetic diversity index), Chao1 (species richness estimator index) and observed_OTUs (number of distinct OTU's) counts data were generated in conjunction with alpha rarefaction plots. Statistical analyses of data from all three metrics were carried out using R and ANOVA followed by Tukeys's Post Hoc analysis. Values were represented as mean ± standard error. To compare bacterial communities across different soil types, both quantitative (weighted unifrac) and qualitative (unweighted unifrac) [66] phylogenetic beta diversity metrics were employed and distance matrices were generated. The distance matrices were visualized through distance box plots. Statistical analyses of the box plots were carried out by applying

parametric student's two-sample t-tests followed by Bonferroni correction, comparing every pair of boxplots to determine if they are significantly different from each other. As a part of core diversity analysis, Principal Coordinate Analysis (PCoA) plots were also generated using the EMPeror software [67]to estimate beta-diversity.

Screening and identification of *Frankia* sp.

To study the *Frankia* populations in prairie and Morrow Plot soils, Illumina sequencing data were analyzed using QIIME and Geneious 9.1.7 (Biomatters, New Zealand).In order to construct a reference sequence/mapping reference file, 16S rRNAgene sequences of various representative *Frankia* strains representing the four currently delineated clusters 1 to 4 were retrieved from the NCBI database. About 92 16S rRNA gene sequences of a length ~1500 bp each were truncated and only the V3-V4 region of each of the sequences were arranged in tab delimited format in a fasta file with a unique identifier (i.e. the GenBank accession number). A phylogenetic tree was constructed from these sequences by the neighbor-joining method using the Geneious software.

Fasta files containing quality filtered and demultiplexed sequences were passed as input to the QIIME script "parallel_pick_otus_blast.py"*(parallel_pick_otus_blast.py -i $PWD/seqs.fna -r $PWD/refseqs.fna –s 0.97 -o $PWD/blast_otus/)* and sequences thatwere similar to reference *Frankia* sequences with a similarity threshold of 97% and higher were extracted using the BLAST program. The output files were analyzed in Geneious to generate and visualize multiple alignments of the extracted *Frankia* sequences against the reference sequences (i.e. representative *Frankia* sp., Fig 1) to identify the *Frankia* sequences present in these soils.

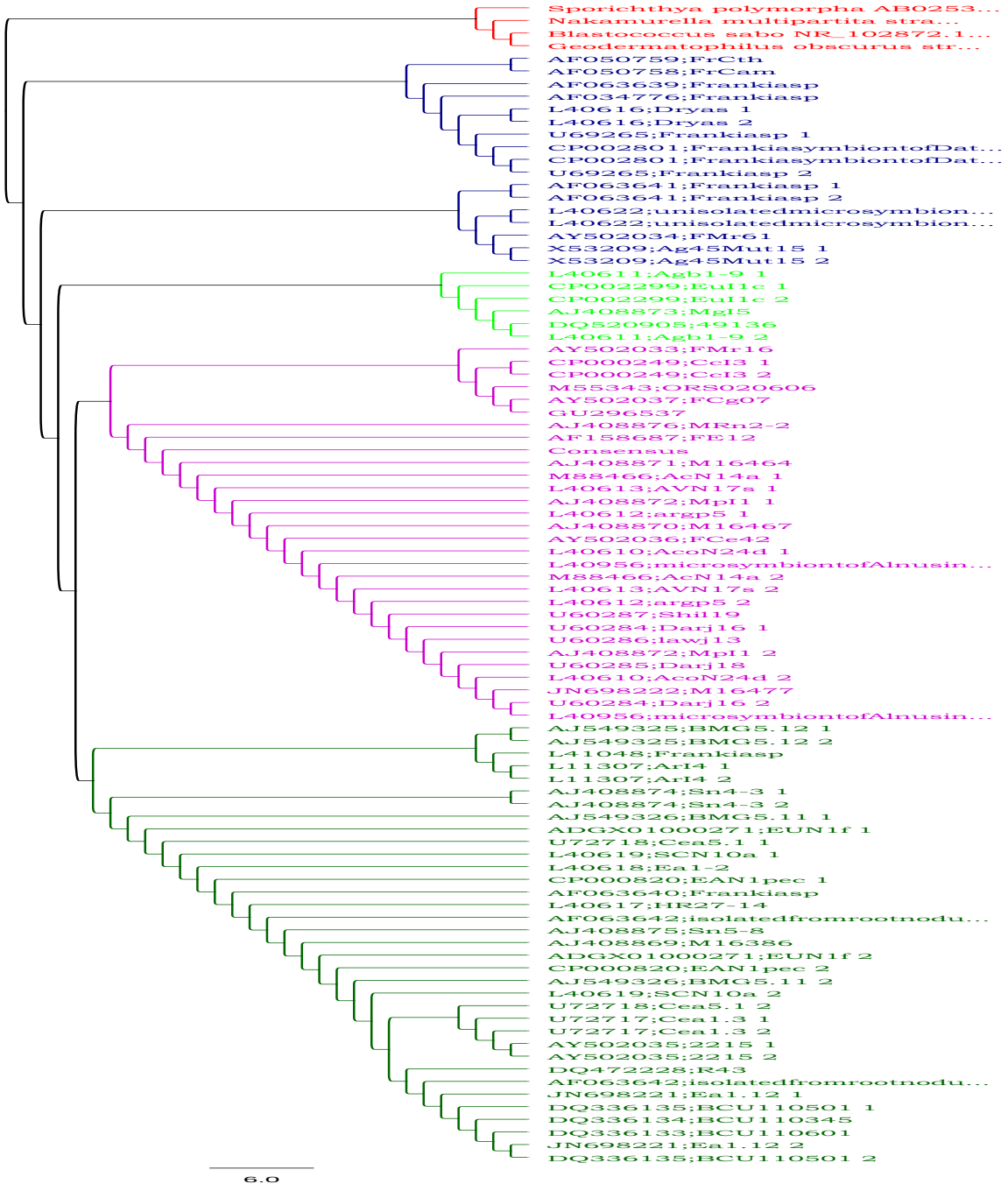

Figure 1: Classification of genus *Frankia*- representative reference sequence tree (Neighbor joining method)

IV. RESULTS

Quality filtering

After demultiplexing and quality filtering, a total of 26,465,154 sequences were obtained from the 44 samples (12 soil types) with sequences/sample ranging from 313,695 to 906,328, with an average of 601,481 (±128,660) sequences/sample.

Bacterial diversity in soil samples

The core diversity analyses described the major bacterial communities present in the soil samples at various taxonomic levels. Acidobacteria, Actinobacteria, Proteobacteria and Verrumicrobia were the most abundant bacterial phyla (10%-35%) present across all soil samples, followed by Bacteroidetes, Chloroflexi, Gemmatimonadetes, Nitrospirae, Plancomycetes (0.5% - 5%) and among the Archaea, Crenarchaeota was the major phylum observed (Fig.2).

The major bacterial phyla were further analyzed at the order level (Fig.3). The phylum Acidobacteria was mainly comprised of the bacteria belonging to the order Acidobacteria-6 and Chloracidobacteria. The majority of the observed Actinobacteria belonged to one of the four orders Acidimicrobiia, Actinobacteria, MB-A2-108 and Thermoleophilia. Proteobacteria were mainly represented by the orders Alpha-, Beta-, Gamma- and Delta-proteobacteria. Most of the Verrumicrobia belonged to the orders Pedosphaerae and Spartobacteria (Fig. 3).

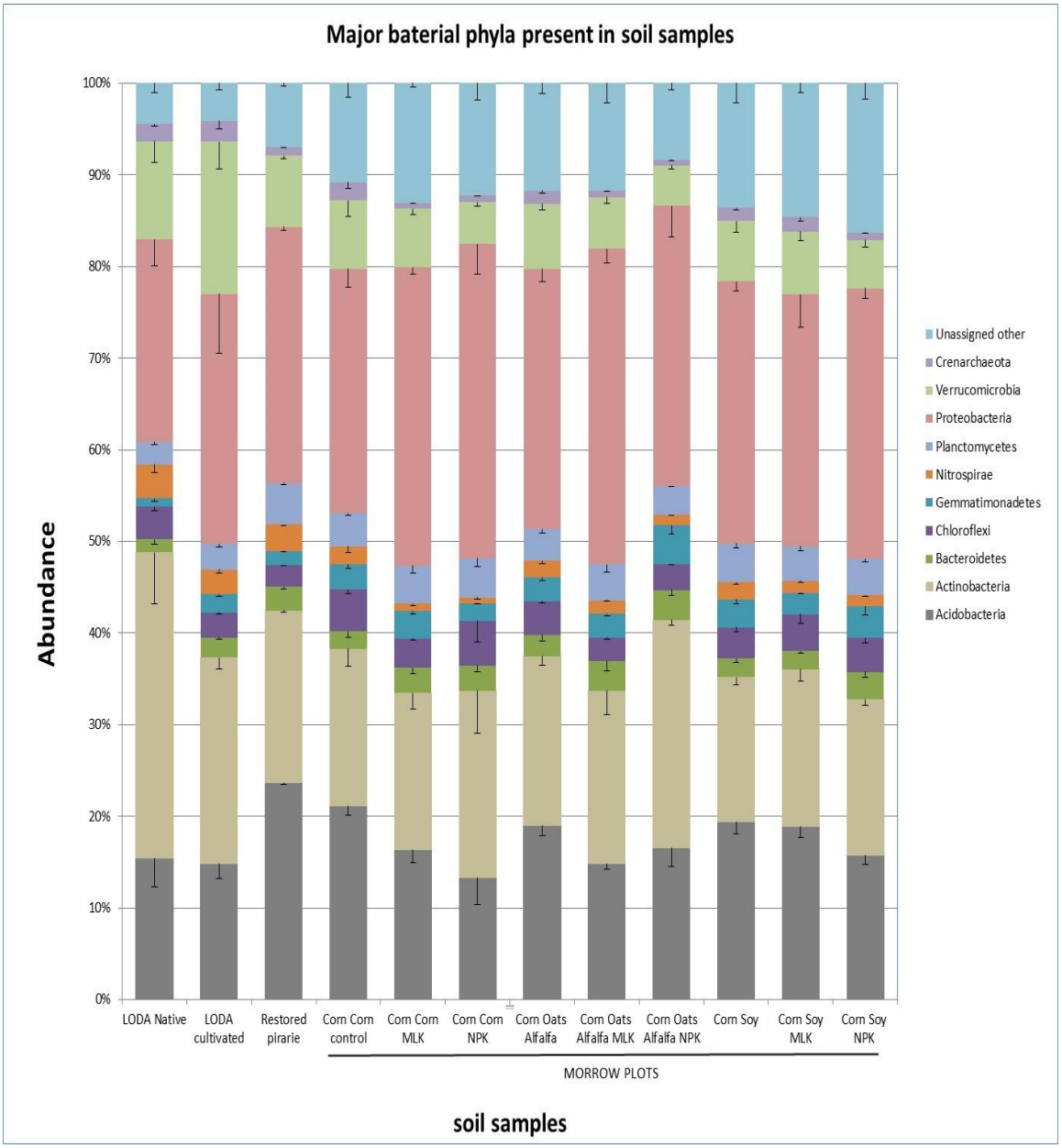

Figure 2: Major bacterial phyla present in soil samples

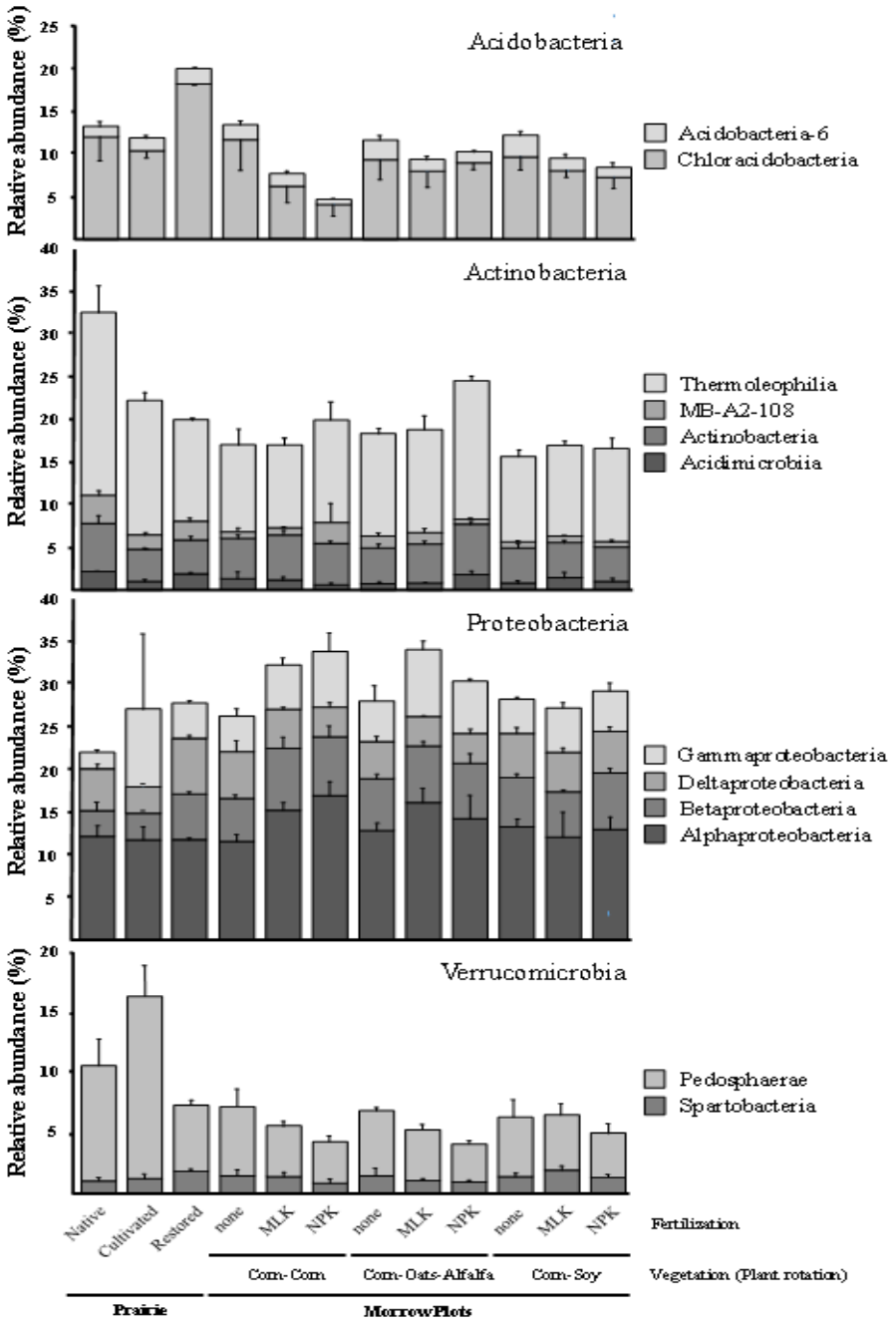

Figure 3: Orders of the major bacterial phyla present in soil samples

Alpha diversity

Alpha diversity analyses revealed that soil types exhibited significantly different phylogenetic diversity ($p<0.001$) as demonstrated by the alpha diversity PD metric. In general, the Morrow Plots cultivated with corn-oat-alfalfa or with corn-soy exhibited higher phylogenetic diversity than the other soil types (native prairie, restored prairie and Morrow Plot soils cultivated with corn-corn and applied with MLP/NPK fertilization). In addition, phylogenetic diversity in the Morrow Plot soils with corn-oat-alfalfa and corn-soy applied with MLK fertilization was higher than those of the Morrow Plot soils with corn-corn and NPK fertilization. There was no significant difference in the phylogenetic diversity of the native prairie and cultivated soils and between the LODA and Meadow Brook prairie restored soils (Fig 4 and Table 4).

With respect to species richness, there was no significant difference among the soil types. However, the soil types differed significantly ($P<0.001$) with respect to the observed OTUs, i.e. the number of distinct OTUs. Morrow Plot soil cultivated with corn-soy had more distinct OTUs than native prairie soil ($p<0.01$), or Morrow Plot soils with corn-corn ($p<0.01$), corn-corn with MLP ($p<0.05$) and corn-corn with NPK ($p<0.01$). Morrow Plot soils with corn-oat-alfalfa and the same ones applied with MLP fertilization had a higher number of distinct OTUs than the Morrow Plot soil with corn-corn and NPK fertilization ($p<0.05$) (Fig 4 and Table 4).

Beta diversity

The distance matrices box plots and **s**tatistical analysis of the unweighted and weighted distance matrices revealed that the "all within soil type" distances differed significantly from those of the "all between soil type" ($p<0.001$). In other words, no

significant differences were found among the samples belonging to same soil type that were similar to each other in all the 12 soil types. Soil samples from different sites and with different crop cultivation / fertilization regimen differed significantly from each other (as revealed by weighted and unweighted UNIFRAC methods, Fig 5, 6 and 7).

Weighted UNIFRAC analyses showed slightly different results as the unweighted analyses. Results were taken into account based on statistical analysis of the weighted distance matrix. Based on crop cultivation, the Morrow Plot soils with corn-soy cultivation differed significantly from the Morrow Plot soils with corn-oat-alfalfa cultivation ($p<0.001$) and the ones with corn-corn cultivation ($p<0.001$). Morrow Plot soils with corn-oat-alfalfa cultivation differed significantly from that of with corn-corn cultivation ($p<0.05$). The microbial composition of Morrow Plot soils with corn-corn cultivation and NPK fertilization differed significantly from that of all the three prairie soils ($p<0.001$), and the Morrow Plot soils with corn-corn cultivation and MLP fertilization differed significantly from the native ($p<0.001$) and restored prairie soils ($p<0.01$). With respect to the fertilization regimen, the Morrow Plot soils cultivated with corn-corn and NPK fertilization ($p<0.001$) and corn-soy plots and MLP ($p<0.05$) had different microbial communities from that of their respective control/ones with no fertilization. Such differences, however, were not observed for corn-oat-alfalfa plots.

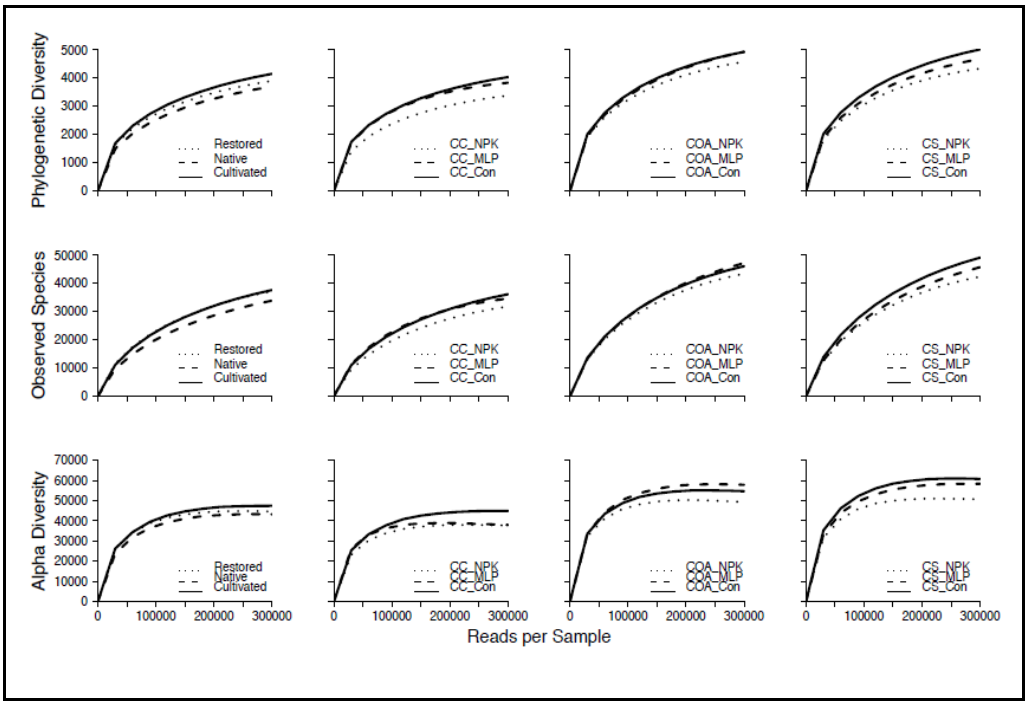

Figure 4: Alpha diversity-rarefaction analyses

Table 4: Diversity measures for Prairie and Morrow plot samples (X±SE)

Site[1] Vegetation/crop rotation Fertilization	Phylogenetic diversity	Species richness (Chao1)	Number of distinct OTUs
Prairie			
Native	3703 (451)	43273 (7167)	33888 (6290)
Cultivated	4152 (248)	47486 (3271)	37646 (2302)
Restored	3899 (58)	44653 (1032)	37178 (795)
Morrow Plots			
Corn-Corn			
None	4036 (448)	44937 (6989)	36157 (4710)
MLK	3837 (249)	37977 (5671)	34626 (3202)
NPK	3381 (213)	37706 (6201)	31847 (2781)
Corn-Oat-Alfalfa			
None	4931 (342)	54759 (9342)	46097 (4096)
MLK	4931 (204)	57951 (5704)	47207 (2453)
NPK	4580 (187)	49366 (2628)	43442 (2165)
Corn-Soy			
None	5020 (340)	60844 (7086)	49163 (3985)
MLK	4687 (487)	58453 (15671)	45759 (6628)
NPK	4339 (526)	50797 (16072)	42374 (7054)

[1]n=3 per treatment, except for untreated (none) Morrow Plot samples (n=6), with 300,000 reads analyzed for each sample

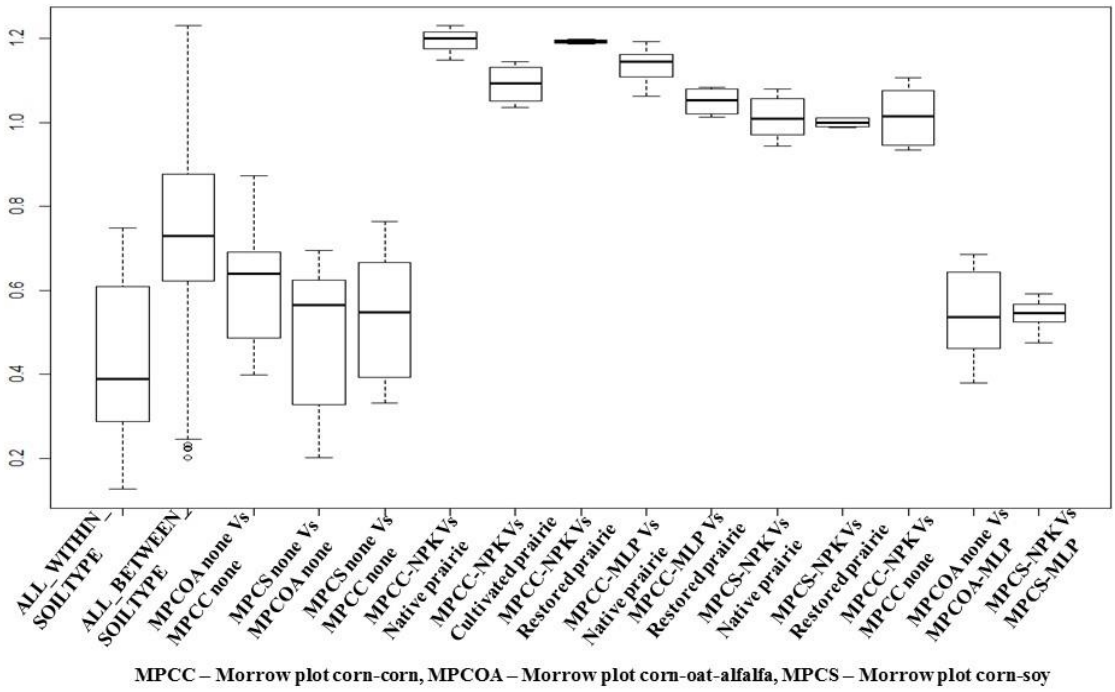

Figure 5: Beta-diversity weighted UNIFRAC

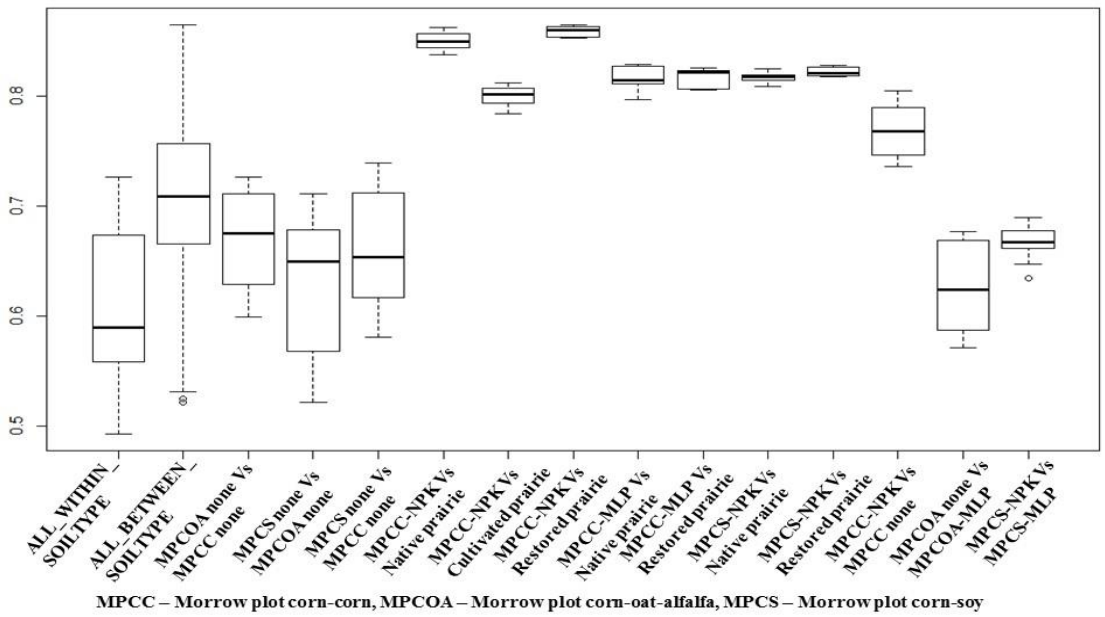

Figure 6: Beta-diversity-unweighted UNIFRAC

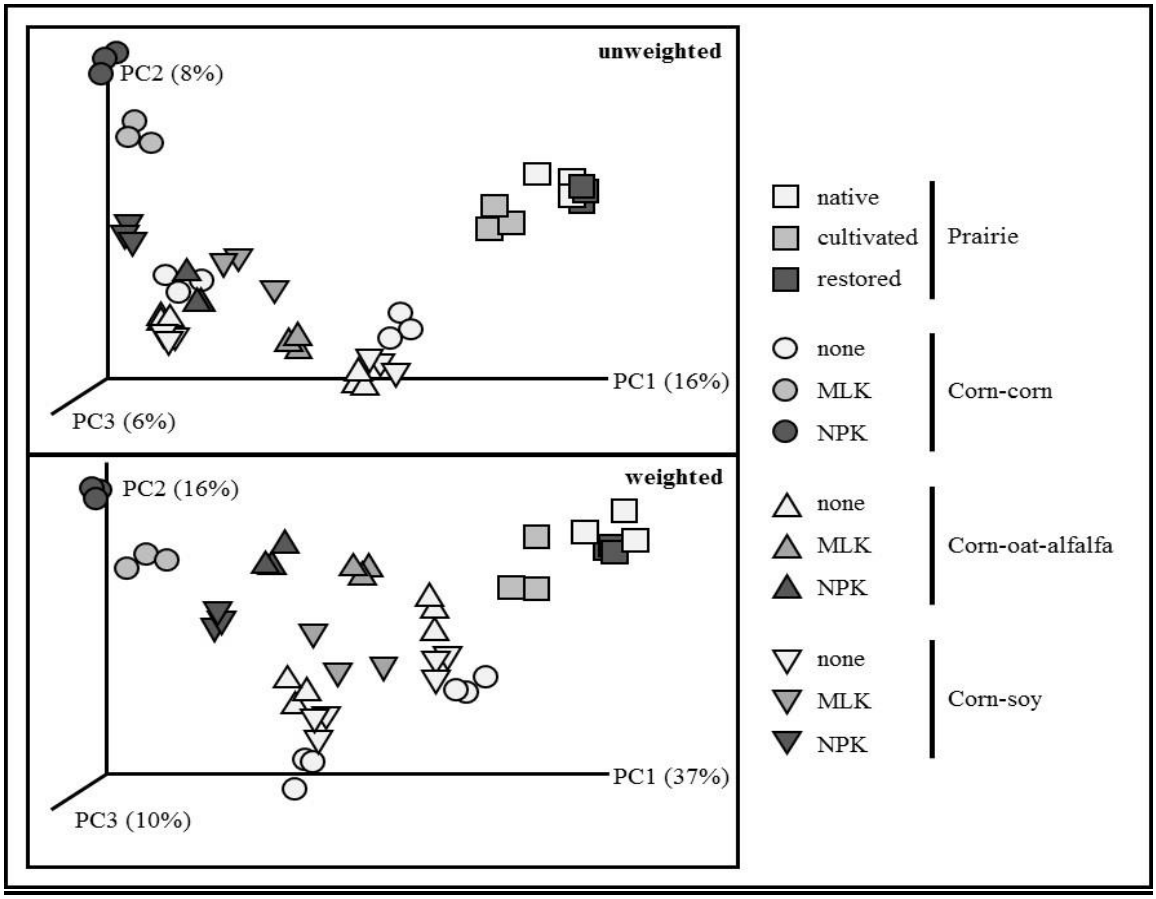

Figure 7: Beta-diversity-PCoA plots

Genus *Frankia*

Sequences representing *Frankia* of all four clusters were retrieved from native and restored prairie soils, while those cultivated from prairie and Morrow Plots were generally less diverse with usually cluster 2 frankiae absent. All soils were dominated by cluster 1 and 3 frankiae, with cluster 1 being most prominent in native and restored prairie soils, while cluster 3 was generally dominant in cultivated soils followed by cluster 1 and cluster 4.

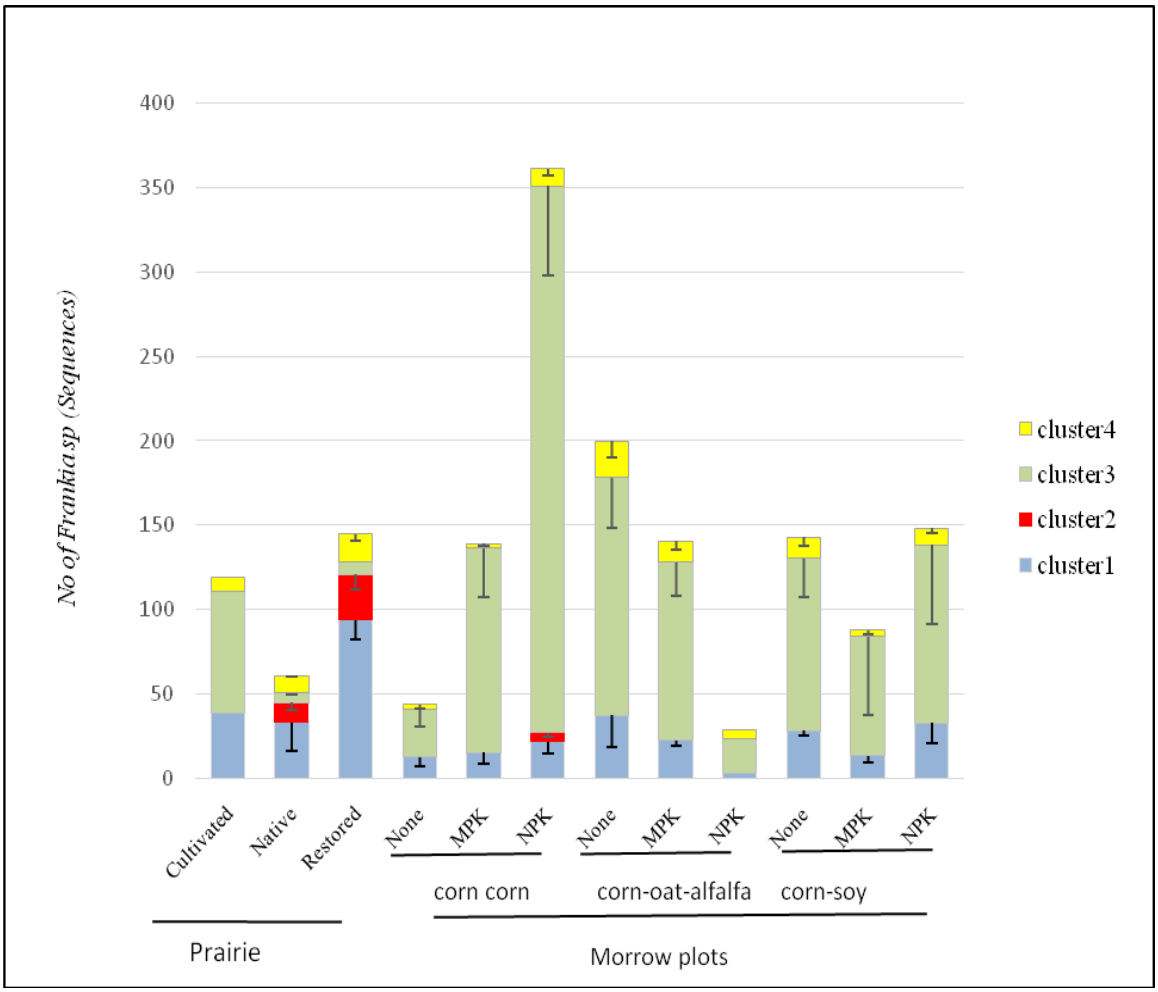

Figure 8: Genus *Frankia* identified in morrow plots and prairie soils.

V. DISCUSSION

Soil microbes play an intimate role in biogeochemical cycling, and thus influence the basic chemical and physical properties of soils and consequently affect soil fertility and plant growth [68]. Gaining insights into soil bacterial diversity and functioning was therefore postulated to be a prerequisite to develop strategies to design sustainable agricultural practices [69]. Agricultural practices like crop rotation and fertilization regimens influence soil microorganisms by affecting the quantity and quality of plant residues entering the soil, and changes in nutrient inputs. Changes in residues also alter the composition of soil fauna and flora, including both pests and beneficial organisms [70]. Many studies have proven that crop rotation and the use of organic and inorganic fertilizers play a key role in increasing plant growth and enhancing crop yield by stimulating soil biological activity and concomitantly inhibiting the growth of pathogenic microbes [71].

Usually, fertilization needs a long time and repeated applications to produce any significant changes to the physico-chemical characteristics and structure of soils. Consequently, responses of microbial community to these changes in soil might be slow. Therefore, analyzing the long-term effects of crop rotations and fertilization regimen on microbial communities will provide more accurate information about their effects on soil characteristics and microbial communities. The results of these analyses could then serve as baseline data to improve sustainable farming practices [72]. The current study explored the bacterial community structure of oldest agronomic experimental fields in the USA, the Morrow Plots, located on campus of the University of Illinois in Urbana-Champaign and of the original tallgrass prairie soils. The Morrow Plot soils are

established with common agricultural treatments such as crop rotation and fertilization regimes [73]. Bacterial community structure of these soils have recently been analyzed employing modern next generation sequencing tools such 454 pyrosequencing analysis [74] and more recently Illumina sequencing [60]. High throughput next generation sequencing tools have revamped the scientific area of molecular microbial ecology in their approaches towards microbial diversity analyses. The 16S ribosomal RNA gene amplicon analysis using the Illumina sequencing platforms is the current standard for cultivation-independent investigations of microbial diversity [75] as it can be used as a tool to contrast the results from previously used methods to study microbial diversity and overcome the biases caused by these methods.

Our Illumina sequencing results showed that all soils were dominated by bacteria belonging to phyla Proteobacteria (in particular Alpha-proteobacteria) followed by Acidobacteria, Actinobacteria, and Verrumicrobia, respectively. Using the phylum level for comparison, these results were in line with both studies conducted on Morrow Plot soils previously by either pyrosequencing analyses [76] or Illumina sequencing [60]. Many Proteobacteria are meant to represent copiotrophic organisms and have shown to increase in numbers after fertilization with barnyard manure rich in organic material [77]. However, we retrieved contrasting results as no significant increase was observed for Proteobacteria in fertilized plots, except for corn-corn and corn-oats-alfalfa crop rotations treated with manure and compared to native prairie soils, despite having lower organic material contents than prairie soils. This may be attributed to the fact that crop rotation and rhizosphere effects might have affected population development of Proteobacteria. Microbes present in soil rely on combined nitrogen resources present in organic matter.

Before nitrogen resources can be taken up, however, organically bound nitrogen has usually to be transformed into inorganic forms to be available for microbes [78]. Application of nitrogen resources through fertilizers as in our NPK-amended plots thus might increase readily available N resources and consequently support growth of those bacteria that prefer copiotrophic conditions and higher N availability.

In contrast to Proteobacteria, Acidobacteria are generally assumed to be slow-growing oligotrophs [79, 80]. Numbers of Acidobacteria declined in the plots treated with N fertilizer, though only in corn-corn rotation plots and not in plots with other crop rotations. NPK treatments generally resulted in a shift towards potentially copiotrophic phyla (Actinobacteria and Proteobacteria) though shifts were not statistically significant, while populations of potentially oligotrophs (Acidobacteria and Verrumicrobia) were declining in numbers, similar to previous reports [81]. This may be due to priming effects of NPK amendment increasing organic matter decomposition [82] and thus encouraging growth of copiotrophs [83] that might outcompete oligotrophic microbes for growth.

Crop rotation is common practice in agriculture and viewed as a potential step leading towards more sustainability [84]. Studies indicate that crop rotation influences microbial diversity of the soil by affecting physico-chemical characteristics of the soil. Excretion of root exudates and accumulation of plant litter from previous cropping seasons attribute to the chemical changes caused by crop rotation and serve as nourishment for the upcoming crops and enhances microbial activity as it provides organic matter for their cells to thrive [85]. Organic matter accumulation also affects physical changes of soil structures such as aggregate composition and sizes as well as pore size distributions that result in better water retention, temperature stabilization, etc

and thus creates better growth conditions for the proliferation and survival of microbes [86].

Alpha diversity metric analysis showed that the Morrow Plots with crop rotation (both corn-soy and corn-oat-alfalfa) exhibited greater phylogenetic diversity than all other soils (Morrow Plot corn-corn, prairie soils from other locations) and thereby increased number OTUs clearly demonstrating the influence of crop rotation on bacterial community richness.

A main challenge faced by the agriculture industry is to achieve higher and higher crop yields to accommodate the ongoing increase in global food demand [76]. Fertilization regimen mainly augment the levels of organic matter and N contents in the soils [87] to increase nutrient availability to plants [88]. However, these treatments not only affect plant growth performance, but also often cause shifts in microbial community structure of those soils, and, as a consequence, might affect the function of soil microorganisms [89].

Beta diversity analysis revealed that in addition to the crop rotation, fertilization regimen also influence the bacterial communities present in these soils. Many studies in the past focused on changes in total soil organic carbon (SOC) caused by crop rotation, but not much information is available on the positive correlation between higher microbial diversity (thereby increased plant growth) and increased N content in soil due to crop rotation [90]. This was mainly due to the limitations of methods to study and analyze such a vast microbial diversity present in soil and the requirement to culture microbes *in vitro*. Current metagenomic approaches like high throughput next generation sequencing studies can possibly overcome these drawbacks [74].

Most microbial diversity studies carried out using the EMP protocol analyze major phyla present but usually not particular genera or even species. The genus *Frankia* that belongs to the phylum Actinobacteria was mostly studied in the context of its symbiotic association with actinorhizal plants and the potential of exploiting this symbiotic interaction in agroforestry and land reclamation [91, 92]. Most of the studies for *Frankia* sp.have been carried out in soils with their respective host plants, but not in crop lands without host plants. Thus, it is not surprising that no reports exist on the prevalence of *Frankia* sp. in soils from the Morrow Plots.

*q*PCR based tools have recently been developed for the specific detection and quantification of frankiae and applied for the analyses and quantification of *Frankia* sp. both in root nodules and in soils [51, 52]. *Frankia* sp.were studiedin soils from the Morrow Plots and the original prairie as part of another study, with results indicating that native, cultivated and restored prairie soils were diverse in terms of *Frankia* populations as all four *Frankia* clusters were detected [49]. Illumina sequencing results were in line with the *q*PCR-based analyses and reported the presence of all four clusters in these soils. However, in soils from the Morrow Plots both methods showed contrasting findings. *q*PCR suggested that the Morrow Plots soils were mostly populated equally by cluster 3 and 4, while the Illumina sequencing data reported that the Morrow Plot soils were dominated by cluster 3 followed by cluster 1 and 4, respectively. The dominance of cluster 3 members can be explained by the fact that they occur even in soils with no host plants [93] whereas the cluster 2 *Frankia* will be mostly present only in soils containing their host plants [56] which explains their absence in cultivated prairie and Morrow Plot soils.

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