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A 16S & ITS Soil Microbiome Analysis of Native & Old-World Bluestem Invaded Soils of Kansas Grasslands

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A 16S & ITS SOIL MICROBIOME ANALYSIS OF NATIVE & OLD-WORLD BLUESTEM INVADED SOILS OF KANSAS GRASSLANDS

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ABSTRACT

Invasive species are becoming an increasing problem throughout the world. Their effect on local ecosystems is detrimental and widespread, harming productive efforts such as biomass accumulation, forcing native species (plant, animals, microbes) out, and ultimately, reducing biodiversity. Investigations of how invasive plants spread are widely studied; however, there has been little to no examination of how native plants influence the microbiome in the surrounding soil. In this study, I performed a fungal and bacterial metagenomic analysis of the soil and root microbiomes of both native grasslands and grasslands invaded by Old-World Bluestem Grasses to help determine if they influence the soil microbiome to assist in their invasion. Old-World Bluestem Grasses are grasses in the *Bothriochloa* genus, namely *Bothriochloa ischaemum* and *Bothriochloa bladhii*. Utilizing the QIIME2 software platform, I performed a variety of comparative diversity analyses between the two types of grasslands sampled across Kansas. The fungal portion of the microbiome seemed mostly unaffected by species of grass and their native vs. invasive status; however, location played a much large role in which fungi were present and their abundance. The bacterial component was also mostly unaffected by any variable; these being species, nativity, and location. This result may have been strongly influenced by both poor read count and the number of operational taxonomic units that could be assembled. In conclusion, location was what affected the fungal microbiome with nativity showing a measurable but insignificant effect; the bacterial portion was inconclusive due to the poor read counts and operational taxonomic unit assembly.

INTRODUCTION

Old-World Bluestem (OWB)

The spread of Old-World Bluestem (OWB) has become an increasing concern for ecologists and land managers. Hard to control, tolerant of harsh conditions such as drought and poor soils, and resistant to most herbicides OWB have been shown to be extremely competitive, frequently out competing native grass species by displacing them from their original habitats (Ruffner, 2012). With origins from Eastern Europe, Australia, and part of Asia, OWB are well adapted to overgrazing and drought. They also produce many more seeds compared to natives making them very capable competitors (Harmoney, 2016). There has also been evidence of OWB, specifically *Bothriochloa ischaemum*, having allelopathic capabilities discouraging any native plants seeds from germinating (Greer et al., 2014). These competitive qualities produce a monoculture reducing biodiversity as well as bird and arthropod populations (Hickman et al. 2006). In addition, the loss in biodiversity increases the chances of new and established zoonotic diseases spreading among humans (Keesing & Ostfeld, 2021) and reduces the overall productivity of the ecosystem for humans (Diaz, 2006).

Old-World Bluestems refer to a group of mostly *Bothriochloa* grasses. They were originally introduced in the early 20th century to prevent soil erosion along newly constructed highways, stabilize marginal farmland, and as forage throughout the southern Great Plains (Harmoney, 2016). OWB's ability to resist drought and to quickly establish a population through seed bank development made them uniquely capable of both preventing erosion and providing livestock sustenance during harsh seasons (Wied et al., 2020) The specific grasses within the OWB complex that are commonly found and causing problems in Kansas now are *Bothriocloa ichaemum* (Yellow Bluestem), and *Bothriochloa bladhii*, (Caucasian Bluestem). OWBs have

since escaped their original areas of introduction, outcompeting native grasses like *Andropogon gerardii* and *Schizachyrium scoparium* throughout the Great Plains (Ruffner, 2012).

Early detection of OWB grasses is paramount to successful mitigation and control of spread before the use of containment methods such as herbicides and mechanical removal become uneconomical (USDA, 2018). If detected early enough, OWB mitigation can be successful, but this requires careful timing and repeated applications of established conventional methods of control, such as a combination of burning and herbicide application (USDA, 2018). However, due to OWBs rapid and prolific seed development, resistance to drought, heavy grazing, and fire, control is difficult to achieve without proper equipment and knowledge of OWBs (USDA, 2018). Biological controls, like targeted livestock grazing or insects, are generally ineffective; and because livestock prefer native grasses to OWBs, which rapidly lose their palatability as they mature, there are currently no other available methods of targeted removal (USDA, 2018). Physical controls, such as mowing, tilling, and fire, are indiscriminate and time and energy intensive requiring application during seed head formation to remain effective (USDA, 2018). However, research into effective fire applications is ongoing (Charlotte et al. 2021). Chemical controls remain limited to specific herbicides like glyphosate and imazapyr due to their effectiveness, especially when compared to other herbicides, and are the primary candidates for research into effective applications (Ruffner, 2012). With the continued spread of OWB across the Great Plains, investigations into the ecology and behavior of these grasses are becoming more common place. Investigations into control measures using the aforementioned herbicides and specific grazing patterns provide short-term relief for native species to reestablish a population (Harmoney, 2016). However, longer term control methods

have been elusive with only some success in using carefully controlled and timed burning of OWB invaded grasslands (Charlotte et al. 2021).

These warm-season perennial grasses, once established into a monoculture, have long lasting impacts on the biotic and abiotic aspects of the ecological community and require costly interventions to properly manage the infested grasslands. Alterations to the food chain and biogeochemical cycles are only some of the problems that develop with OWB establishment (USDA 2018). OWB have also been found to have allelopathic effects preventing the germination and growth of various native species including *Andropogon gerardii* and *Schizachyrium scoparium* (Greer 2014).

Microbiomes and Bioinformatics

The microbiome is the entire microbial ecology of a site or host. These biomes have shown to be an enormously important aspect of life affecting human mood and digestion (Gomaa, 2020), successful plant immune responses (Bakker et al. 2020), and soil processes (Fierer, 2020). Its widespread effects and pervasive presence throughout the environment warrant study into how the microbiome affects its environment, those within that environment, and its host. Continuing advancements in gene marker development, gene sequencing, and genetic analysis have allowed the identification of numerous microbiome compositions resulting in a growing knowledge base of what each microbial species is capable of within its environment (Elijovich et al. 2020).

With these new genetic analysis tools, large and complex genomes can be sequenced in short amounts of time. Thanks to these techniques, it is estimated that up to 600 giga base pairs (GBp) can be sequenced within 10 days (Caporaso et al., 2012). Moving from a non-

parallelization, low throughput, short read sequencing method to a high parallelization, high throughput, long read method has both decreased the cost and time needed to produce high quality sequence data (Heather & Chain, 2016). Combined with the continued development of software dedicated to the analysis of these sequences, the development of these new sequencing methods has created a large toolset for bioinformaticians to tackle problems and produce solutions (Escobar-Zepeda et al., 2015). One of these newer sequencing concepts is called shotgun metagenomics, a technique that lets researchers sequence the entire genome of an environment's microbiome that allows for taxonomic classification to species level and identification of novel genetic sequences giving us an insight into what each microbe is doing within its environment (Escobar-Zepeda et al., 2015).

While whole genome metagenomics provides an incredible amount of data toward the study of microbiomes, it is expensive and computationally taxing. Therefore, other methods have been developed to identify individual genera or species within microbiomes in a less computationally intensive and simpler process. The general idea behind these developments has allowed for the identification of specific nucleotide sequences that are conserved enough to be pervasive throughout a taxonomic kingdom while remaining unique enough to identify the genera. These specific sequences, 16S ribosomal RNA (rRNA) sequences for bacteria as an example, are a highly conserved region of the bacterial genome with just enough variation between genera to allow for identification of bacteria to that level; the internal transcriber spacer, or ITS, segment has the same characteristics as the 16S except found in fungi (Matsumoto & Sugano, 2013; Ruiheng et al., 2018). These characteristics allow researchers to identify the taxonomic composition of microbiomes and facilitate experimentation or comparisons between

microbiomes to observe or predict how the effects of changes to the composition may have on the host or environment (Köberl et al., 2020).

The sensitivity of these microbiomes to changes in their surroundings can influence host health. For example, a study by Elijovich et al.,(2020), showed that implantation of some halophilic bacteria, taken from a mouse that was suffering from salt sensitive hypertension, into a healthy rat resulted in that healthy rat developing salt influenced hypertensive characteristics. This finding can be extended to other systems such as in soil, water, and plant environments. Examination of microbial compositions and of any changes that occur within its environment, such as a shift in pH, could give insight to how the host or surrounding environments respond. This might allow for preemptive measures to be taken before damage to either host or environment can occur.

If discussing soil microbiomes these preemptive measures, such as liming to raise pH, would need to address soil characteristic changes before they became irreversible or financially prohibitive to either prevent or correct. With how complex soil microbiomes are, and their reactive nature to changes in the soil, management practices need to be quick and definitive to correct those shifting characteristics (Köberl et al., 2020). These practices also need to identify how reactive different microbiomes are, both in terms of its diversity and abundance, to different soil characteristics (Islam et al., 2020).

Each type of microbe has different role to play in the environment, ranging from nitrogen fixation to carbon cycling (Jacoby et al., 2017). Both the presence and concentration of different microbes can indicate a specific process being undertaken within the soil. Some bacteria have a unique ability to reduce atmospheric nitrogen into fixed nitrogen compounds, such as ammonia;

while fungi are widely responsible for carbon recycling with both microbes assisting in nutrient uptake of plants through a close-knit interaction (Jacoby et al., 2017 & Tomer et al., 2021).

Each species of plant has slightly different biological needs, so each requires slightly different microbiome compositions to assist its propagation. Close association between plant species and microbiome composition show correlation between the species and its accompanying microbiome. For example, studies showed that microbiomes shift from plant species to plant species and even between the wild and domesticated varieties of the same species (Bulgarelli et al. 2015; Pérez-Jaramillo et al. 2017).

Research Question/Objective

Current procedures for the control and management of OWB revolves around surveying for new growth and the early application of herbicides which are being actively researched for the most efficient and timely application for greater effectiveness (Harmoney, 2016) as well as their effects on the microbiome (Liao et al. 2021). In this paper, we examine the potential application of metagenomic surveys to test for common characteristics between OWB species microbiomes as well as verifying any differences between OWB and native species to use as a starting point for the application of preemptive control measures before OWB can establish.

This study has the potential to answer several questions. First, and the question which prompted this study, is whether or not species significantly influences the soil microbial community. Second, does nativity affect the soil microbiome. We can investigate any differences between native and invasive species, specifically OWB, and if these can be utilized to predict where OWB may propagate in the future. Lastly, does longitude and its various gradients, specifically the precipitation gradient, change the soil microbiome.

MATERIALS AND METHODS

Description of Field Sites

The targeted plants for this study included two native and two invasive grasses. The native species are Big Bluestem *(Andropogon gerardii)*, and Little Bluestem (*Schizachyrium scoparium)*. The invasive grasses are Caucasian Bluestem (*Bothriochloa bladhii*), and Yellow Bluestem (*Bothriochloa ischaemum*). These native grasses were chosen because they are common throughout Kansas and the United States, while the invasive grasses were chosen due to a growing concern that their spread from original plantings are now outcompeting native grasses and forming monocultures and disrupting ecological processes.

Four sample locations were chosen near the cities of Hays, Oakley, Manhattan, and Lawrence, along the natural precipitation gradient. Smoky Valley Ranch is managed by The Nature Conservancy, a non-profit corporation and is the driest of the three. Three of these sites are managed by state universities: the Relict Area and Sheep Pasture are managed by Fort Hays State University (FHSU) near Hays, KS, Konza Prairie Biological Station is managed by Kansas State University (KSU) near Manhattan, KS, and the University of Kansas Field Station is managed by Kansas University (KU) near Lawrence, KS, which is the site receiving the most precipitation.

Smoky Valley Ranch is a short grass 19,000-acre prairie with several mixed-grass areas and is actively managed for native grasses. It has several cattle herds and wild bison throughout the area with pronghorns commonly passing through the land. Sampling of *Bothriochloa ischaemum* occurred near the edge of the property next to a neighboring agriculture field where corn was being grown. It is the most western located of the sampled areas and is located at 38.889847, -101.019231.

The Relict Area is a 35-acre mixed grass prairie that only had fire controls in place for a century. Plant diversity in the Relict Area is high providing ample sample opportunities for native grasses collection. The Sheep Pasture, part of a 3,825-acre agricultural area, is the responsibility of FHSU Agriculture Department and is a near monoculture of invasive *Bothriochloa* species. During the time of collection, the pasture was being actively grazed by sheep. Both locations are located near latitude 38.859124 longitude -99.377305.

The 8,600-acre Konza Prairie is part of the National Science Foundation's Long-Term Ecological Research Site. It's actively managed to encourage native grass propagation and invasive control. Sampling of invasive *Bothriochloa* only occurred near active research plots; however, we were allowed to sample the researchers' control plots. Most sampling efforts occurred near the edge of these research areas' control plots, with some being located next to fertilized plots. This area is near the coordinate's latitude 39.108763, longitude -96.609431.

The University of Kansas Field Station is the easternmost located at latitude 39.045800, longitude -95.201324. The area has various types of land covers ranging from hard-wood forests to tall-grass prairie. The management targeted Caucasian bluestem (*Bothriochloa ischaemum*), for active removal. Some Caucasian bluestem were growing along walkways throughout the prairie.

These non-FHSU locations are found along a longitudinal gradient. This gradient can introduce various physical differences and possibly differences in microbiome composition between the sample sites such as differences in precipitation, changes soil type, and types of ecosystems with the differences ranging from semi-arid prairie with average precipitation at 455mm in the most western to the development of continental forests in the east with precipitation averaging 1016 mm in the most eastern (USDA, 2013). Figures 1 and 2 show the

differences in precipitation and soil type distribution across the state of Kansas (USDA, 2013 & Beck et al., 2018). From least to most yearly average precipitation is Smoky Valley at 519mm, the FHSU sites at 595mm, Konza Prairie at 905mm, and the KU Field Station at 1013mm.

Sample Collection

Soil collection utilized a soil corer to collect sample cores approximately six inches (152mm) deep and three (76mm) inches in diameter. The corer was centered around the targeted plant stalk to collect as much of the root system as possible. Little bluestem is a native bunchgrass that lacks a distinct stalk to core so instead we sampled the center bunch. Soil was placed in plastic freezer bags and homogenized before storing on dry ice until transportation to a -70 C freezer for long term storage. A total of 189 samples were collected with some locations lacking the targeted species which are summed in Table 1.

DNA Extraction, Amplification, and Sequencing

To extract genetic material from both fungi and bacteria, a Qiagen PowerSoil Extraction Kit (Hilden, Germany) was used following the manufacturer's recommended protocol. DNA was quantified using a Qubit. DNA was amplified by Polymerase Chain Reaction (PCR) until each sample reached a concentration of 20ng/µl. The samples were sent to the Kansas State University Genome Sequencing Core for sequencing on MiSeq Paired-End 300 Illumina using the Earth Microbiome Project 16S and ITS Illumina Amplicon Protocols. The 16S targeted region was V4 with primers 515F-806R, FWD: GTGYCAGCMGCCGCGGTAA and REV: GGACTACNVGGGTWTCTAAT with a thermocycler setting of 94℃ for 3 minutes, 94℃ for 45 seconds 35x, 50℃ for 60 seconds 35x, 72℃ for 90 seconds 35x, 72℃ for 10 minutes, and hold at 4℃. ITS utilized the ITS1f-ITS2 primers, FWD: AATGATACGGCGACCACCGAGATCTACAC GG CTTGGTCATTTAGAGGAAGTAA

REV: CAAGCAGAAGACGGCATACGAGAT NNNNNNNNNN CG GCTGCGTTCTTCATCGATGC, and used different thermocycler settings. These settings are 94℃ for one minute, 94℃ for 30 seconds 35x, 52℃ for 30 seconds 35x, 68℃ for 30 seconds 35x, 68℃ for 10 minutes and hold at 4℃. The samples were sent to the Kansas State University Genome Sequencing Core for sequencing on MiSeq Paired-End 300 Illumina using the Earth Microbiome Project 16S and ITS Illumina Amplicon Protocols.

Data Analysis

Bioinformatic analyses were performed for the ITS and 16S gene regions separately, utilizing the Kansas State University's Computer Science Departments Supercomputer called 'Beocat,' and the QIIME2 software package version 2019.7 (Bolyen et al. 2019). The analysis steps are summarized in Firgure 3. The resulting sequences were put through vsearch, via q2-vsearch, to join the pairs of complementary sequences together using default values (Torbjørn, 2016). The sequences were then quality filtered using the default plugin values, minimum quality value of 4 and quality window of 3, utilizing PHRED scoring to remove ambiguous nucleotide sequences and primer sequences by using the quality-filter plugin, q2-quality-filter developed by Nicholas et al. (2013). PHRED scores are log based scoring values, ranging from 10 to 60; anything higher than 30 has a base call accuracy of 99.9% (Ewing et al. 1998), any sequences below this threshold were removed. These sequences were further filtered using Deblur via q2-deblur (Amnon et al. 2017) to remove nucleotide sequences too short to be the targeted 16S or ITS gene, these being a trim length of 250bp and 300bp respectively. The value 250bp for 16S is slightly shorter than the targeted sequence. This was to include as many assemblies as possible due to how feature sparce the 16S sequences were with the median number of features in the sequences equaling two.

After these quality filters were applied, the sequences were pushed through the vsearch plugin again utilizing its de-novo assembly capabilities to assemble OTUs at 97% matching identity. This percentage was a common value for de-novo assembly and allows distinguishing microbes to genus level. However, this technique has recently been shown to require adjustment based on the metric being measured, such as Mathews' Correlation Coefficient (Edgar, 2018). These representative sequences produced by vsearch were then aligned using mafft via the q2-alignment plugin (Katoh & Standley, 2013) and then constructed with fasttree2 (Morgan et al. 2010) using the q2-phylogeny plugin.

Alpha diversity matrices were produced using the assembled OTU frequencies to measure various metrics of in-sample diversity. The metrics measured were Faith's Phylogenetic Diversity (Faith 1992), Pielous Evenness (Perilous, 1966), Shannon's entropy (Shannon, 1948) and observed operational taxonomic unit (OTU) vectors (DeSantis et al. 2006) all of which use Kruskal-Wallis (Kruskal & Wallis, 1952) all group and pairwise analysis using the q2-diversity plugin. The measured beta diversity matrices, which quantify various between sample measurements include weighted UniFrac (Chang et al. 2011), unweighted UniFrac (Lozupone & Knight, 2005), Jaccard Similarity Index (Jaccard, 1908), and Bray-Curtis dissimilarity (Sørensen, 1948) using the q2-diversity plugin at a sampling depth of 2 for the 16S sequences and 9079 for the ITS sequences. The sampling depth normalizes the data allowing for analysis without outliers causing undue influence. The 16S sampling depth was chosen to keep as many OTUs possible in the analysis to make up for feature sparsity, thus, no samples were removed. The ITS sampling depth was near the median of the of feature counts of the samples; this depth retained as many samples as possible within the analysis while still removing the disproportionally low-featured

samples. PERMANOVA analysis was also performed measuring Bray-Curtis and Unweighted Unifrac metrics with a sample size of 93 and 999 permutations.

All of these tests use the assembled OTUs and compares either within or between samples to determine significance like how one would compare larger scale ecologies. For the alpha metrics, Faith Phylogenetic Diversity measures the diversity of a sample utilizing a previously assembled phylogenetic tree which utilizes the assembles OTUs. Pielous Evenness measures the evenness within a sample, or how numerically similar the count of all OTUs within a sample are to each other. Shannon's Index describes the number of species and their abundance using a natural log function. Lastly, the adonis test utilizes the 'vegan' package in R to run a permutation-based statistical test analogous to a MANOVA test; specifically it tests for the effect size of each group using vegan while remaining in the QIIME environment.

For beta metrics, Unifrac can be used in a qualitative or quantitative form described as weighted or unweighted, weighted considers abundance whereas unweighted does not. Unifrac utilizes a phylogenetic tree to measure the fraction of unique branch lengths to determine phylogenetic diversity between samples. Bray-Curtis Dissimilarity measures the fraction of over abundant counts between samples, this quantifies the composition of the samples and describes their dissimilarity on a 0 to 1 scale. Jaccard's Similarity Index measures the fraction of unique between samples regardless of abundance.

To finish this analysis, ordination plots using the q2-emporer plugin (Yoshiki et al. 2013) were developed from the various beta analysis. These plots form a three-dimensional principal coordinates analysis (PCoA) plot to assist in visualizing samples that are most similar to each other. Lastly, an Adonis test (Marti, 2013) was performed using the q2-diversity plugin to determine if samples community characteristics were significantly different from one another

and to calculate an effect size. This test utilizes the R package 'vegan' (Oksanen et al. 2018) to test for significant differences and is analogous to a MANOVA test while remaining within the QIIME environment. Assembled sequences were taxonomically identified using BLAST+ (Camacho et al. 2009). This process is summarized in the flowchart provided by the QIIME2 development team in Figure 3.

RESULTS

Sequence Metrics

A total of 126 16S samples were sequenced and assembled 22 features at a total feature frequency of 1198. In this study, sample PHRED scores were found to be greater than 23 in the 9^{th} percentile with the median having a quality score of 38. The median demultiplexed sequence length was 334 nucleotides (nts) with 10000 sequences sampled. A total of 126 samples were processed with a median demultiplexed sequence count of 118, a mean of 118, with a total sequence count of 19679. Frequency per sample summary has a median of 8 features per sample, the mean was 9 per sample. To normalize the samples, 33 were removed during rarifying at a sample depth of 2 to allow for even sampling while retaining as many samples as possible; this left 93 samples for analysis. This rarefaction was done for all subsequent diversity analyses of the sample's species, location, and nativity status. This left a total of 93 samples which of 37 invasive and 56 native samples: 7 from the KU field station, 3 from Konza Prairie, 22 from the FHSU Relict Area, 24 from the FHSU Sheep Pasture, and 37 from Smoky Valley. These 93 samples also represented 33 from *Andropogon gerdii,* 12 from *Bothriochloa bladhii,* 25 from *Bothriochloa ischaemum,* and 23 from *Schizachyrium scoparium*. These are summarized in Table 2.

A total of 166 ITS samples were processed with a median demultiplexed sequence count at 16,632 and a mean of 18,685 with total sequence count at 3,101,719. Sequence length summary with 10000 sequences randomly subsampled at position 301 has 2% being lower than 303nts and a median of 345nts; PHRED quality scores from 25th percentile and are above 41. Total number of features assembled was 1,780 with a total frequency of 1,093,891. Frequency per sample summary has a median frequency of 5,623 and a mean of 6,589. Frequency per feature summary has a median of 69 and a mean of 614. These sequences were rarified at a sample depth of 3952 removing 48 samples. This left 118 samples which were made up of 38 native and 80 invasive samples: 18 from the KU Field Station, 35 from Konza Prairie, 12 from the FHSU Relict Area, 15 from the FHSU Sheep Pasture, and 38 from the Smoky Valley Ranch. These 118 also represented 40 from *Andropogon gerdii*, 18 from *Bothriochloa bladhii*, 20 from *Bothriochloa ischaemum*, and 40 from *Schizachyrium scoparium*. These are summarized in Table 2.

Adonis Results

The 16S adonis results show each tested variable being significant at $p < 0.05$. There was an error in the calculation for species resulting in a missing degree of freedom, regardless of this error the R^2 values reported have location causing 41% of the change while nativity causes 1.9% and species causes 2.9%. The ITS adonis results mimic the trends of the 16S results with all tested variables being significant and location having the most effect in changes seen at 19%. Nativity and species have R^2 values of 2.2% and 1.9% respectively.

Alpha Diversity

Bacterial Communities

Pielou's Evenness, Faith's Phylogenetic Diversity, Observed OTU Vectors, and Shannon Index were measured and the results can be found in Figures 4 to 6 respectively. For 16S

comparing nativity status evenness (Figures 4a-4d), we see non-significance with a p-value > 0.05 and very narrow variation within the invasive samples. Faith's Phylogenetic Diversity between the two also shows no significance; however, the invasive phylogenetic diversity has a lower median and more variation, but less diversity of species between species within samples. Examining observed OTU's shows no significance as well with more variation in the invasive samples but similar medians between the native and invasive samples. Lastly, we see more variation in Shannon's Index in the invasive samples with the same medians between variables.

Looking at species (Figures 5a-5d), we see evenness medians being equal across all species soil communities with *Schizachyrium scoparium* showing the most variation and *Bothriochloa bladhii* showing little variation in abundance. Evenness metrics for both groupwise and all pairwise comparisons are not significant. Faiths Phylogenetic Diversity shows little change in medians in the soil communities between sampled species. *Bothriochloa bladhii* and *Schizachyrium scoparium* have less variation than the other two species indicating similar levels of phylogenetic diversity within samples. Observed OTU's show no change in community variation with only *Bothriochloa ischaemum* having a higher median. Neither pairwise nor groupwise comparisons were significant; there is no change in amount of OTU's present between species. For Shannon's Index, we see similar variations and medians. Both groupwise and pairwise comparisons are not significant.

For location (Figures 6a-6d), evenness shows very different values from the previous variables; medians are similar aside from Konza Prairie whose median is below the other locations. Level of community variation is highly variable with the KU Field Station showing the most variation, the Relict Area a moderate amount of variation, and the last three having little to no variation. Despite this variation, both groupwise and pairwise comparisons are not

significantly different. Faiths Phylogenetic Diversity shows KU Field Station and Konza Prairie having the lowest median, the FHSU Relict Area having the highest, and the Sheep Pasture and Smoky Valley Ranch being below, but close, to the Relict Area's median. There is less variation compared to the evenness results with Smoky Valley Ranch having the widest range and KU Field Station having the smallest. Groupwise and several pairwise comparisons show significance with a p-value < 0.05. The only comparisons that are not significant are the Sheep Pasture versus Smoky Valley, and the Relict Area versus the Sheep Pasture.

Fungal Communities

The same alpha diversity tests ran on the 16S samples were performed on the ITS samples whose results can be found in Figures 7a-7d. Looking at evenness in nativity, invasive samples showed a reduction in variation and a higher median. Despite little variation, p-values remain insignificant. Faith's Phylogenetic Diversity is, again, consistent with the 16S sequences; however, the variation of the invasive samples is narrower than the native showing more consistency between samples. The p-value for this comparison is > 0.05, so there was not a statistically significant difference. For observed OTU's invasive and native medians are the same, with invasive having less variation. Regardless, these differences are observable but not significant with the p-value > 0.05. Shannon's index shows the reverse of the 16S sequences. Invasive samples have a higher median and lower variation than native species, once again though it is observable but not significant with a p-value > 0.05.

Looking at species (Figures 8a-8d) we see evenness medians and variation being similar as well; apart from *Bothriochloa ischaemum* which has a less variation. p-values remain > 0.05 with a borderline significant pairwise comparison between *Andropogon gerdii* and *Bothriochloa ischaemum.* These results contrast with the 16S sequences, showing more variance between

samples with *B. ischaemum* having an observable difference in variation with the rest being roughly equivalent. Looking at Faith's Phylogenetic Distance, we see a narrow range of medians with *B. ischaemum* and *B. bladhii* having the lowest and *Schizachyrium scoparium* having the highest. Range of variation is narrow with *S. scoparium* having the most, *B. ischaemum* the lowest, and equivalent ranges for the last two. p-values, both group and pairwise, remain insignificant at $p > 0.05$. Observed OTU's show *B. ischaemum* having the lowest variation and highest median with *S. scoparium* having the widest range in variability and lowest median. All p-values are insignificant at $p > 0.05$.

Examining location, (Figures 9a-9d), evenness measurements show KU and Konza having the widest variation. The Sheep Pasture, Smoky Valley Ranch, and the Relict Area all have similar medians, with the Sheep Pasture having the lowest variation of the locations. A few pairwise comparisons and the group comparison have a p-value < 0.05. The KU site versus the Smoky Valley sequences shows a significant difference, and Konza Prairie versus the Sheep Pasture and Smoky Valley site show differences; the Relict Area remains similar to both KU and Konza. These results indicate a larger variation in the more eastern locations than the western locations. Faith's Phylogenetic Distance follows the same pattern as evenness with KU and Konza having the larger variation, however their medians reversed with Konza having the highest median and KU having the lowest. The Sheep Pasture contains the lowest variation and lowest median with Smoky Valley and the Relict Area having similar ranges and medians. Groupwise and several pairwise comparisons are significant with p-value < 0.05. KU is significantly different from Konza, the Relict Area, and Smoky Valley; Konza Prairie is significantly different from the Sheep Pasture while being borderline significant with Smoky Valley. The Relict area is significantly different from the Sheep Pasture, and lastly the Sheep

Pasture is different from Smoky Valley. These results show a distinct difference between eastern and western sites with the exception of the Relict Area. Observed OTU's mimics the Faith's Phylogenetic Distance results except in the Relict Area where variation increases and swaps its place in the order of medians with Smoky Valley. Shannon entropy shows more variation in the KU and Konza samples than the others with the Sheep pasture having the narrowest. The Relict Area has the highest median value with Smoky Valley and the Sheep Pasture being close behind. Konza has the lowest median with KU being just above. Significant differences mimic the Observed OTU results with east and west being distinct.

Beta Diversity

Bacterial Communities

The 16S sequences in both the Bray-Curtis PERMANOVA tests was insignificant with a p-value > 0.05 when testing between nativity status and the Unifrac test being significant with a pairwise p-value < 0.05; these can be seen in Figures 10a-10d. The Unifrac pairwise PERMANOVA indicates a significant difference in the calculated distance of the branch lengths in the generated phylogenetic tree where descendants appeared in one or the other group. The Adonis test tests how much each variable effects the samples. We see location (df=4) having an R^2 value of 0.419 with a p-value <0.05, indicating that about 42% of the variation we see is explained by location. Nativity (df=1) has an R^2 of 0.020 with a p-value < 0.05, indicating only 2% of the variation seen is explained by whether a sample was native or from an invasive species. And species (df=2) is inadmissible due to an error during calculation resulting in a missing degree of freedom. It reported an R^2 of 0.030 and a p-value < 0.05 indicating that only 3% of variation is explained by the 3 species included in the calculation.

Looking at the emperor plots and starting with nativity (Figures 11a-14d), these figures correspond with the Bray-Curtis, Unweighted UniFrac, Weighted Unifrac, and Jaccard nativity

plots respectively. Species (Figures 15a-18d) show few patterns. Within the Bray-Curtis plots (Figures 12a-12d) we only see *Andropogon gerdii* weakly grouping on the end of Axis 1 but still spread throughout the plot; all other samples show no patterns. Unweighted UniFrac plots for species (Figures 13a-13d) shows no pattern. Weighted Unifrac (Figures 14a-14d) shows only a small group of *Bothriochloa ischaemum* with the rest spread about the plot. The species Jaccard Distance plots (Figures 18a-18d) also show no patterns.

Looking at the location plots (Figures 19a-22d) several patterns are revealed. The Bray-Curtis plots (Figures 19a-19d) reveal the KU field station occupying its own section of the plot with the other locations spreading along a single axis, specifically Smoky Valley and the Sheep Pasture. The Unweighted UniFrac (Figures 20a-20d) shows the KU field station on its own within the plot with Relict Area forming two clusters along the same plane as the larger Smoky Valley cluster along the same axis. Weighted Unifrac location plots (Figures 21a-21d) show the KU field station occupying its own section of the plot again. Meanwhile, the Relict area and Smoky Valley Ranch are spread along similar axis in a narrow field of occupancy. There is a clear horseshoe effect in this metric. Jaccard Distance location plots (Figures 22a-22d) shows Smoky Valley Ranch clustering together in three rough groups. The KU field station samples are occupying their own section in the plot, the other locations are mixed.

Fungi Communities

The ITS sequences for both the Bray-Curtis and Unweighted Unifrac PERMANOVA tests (Figures 23a-23d) show p-values < 0.05. The Bray-Curtis group significance plots show a higher median and less variance in the native samples in the ‘Distance to Invasive’ plot and similar medians with less variance in the ‘Distance to Native’ plot indicating that the native samples are more dissimilar than the invasive samples. The Unweighted Unifrac plot ‘Distances

to Invasive' show native samples having a higher median and less variance than the invasive with the 'Distances to Native' having similar values between native and invasive.

Examining the emperor nativity plots, the Bray-Curtis graphs (Figures 24a-24d) shows no pattern. The Unweighted Unifrac plots (Figures 25a-25d) show four distinct groups with two of these having a distinct separation between native and invasive samples. The Weighted UniFrac plots (Figures 26a-26d) show no grouping based on nativity. The Jaccard Distance plots (Figures 27a-27d) show four very distinct groupings with three of the four containing a clear separation between native and invasive samples.

Looking at species, the Bray-Curtis emperor plots (Figures 28a-28d) shows no pattern. The Unweighted Unifrac plots (Figures 29a-29d) shows some weak clustering of samples from the same species, namely *B. bladhii* and *A. gerdii*. The Weighted Unifrac plots (Figures 30a-30d) show some weak grouping of *Bothriochloa bladhii*. The Jaccard Distance plots (Figures 31a-31d) show two of the three invasive groupings are made up of *B. bladhii* while the last is made up of *B. ichaemum.*

Lastly, examining location, the Bray-Curtis plots (Figures 32a-32d) shows 4 distinct groups separated based on the four counties each set of samples was collected from; this pattern is repeated in the Unweighted UniFrac plots (Figures 33a-33d) with the FHSU sites mixing and the other sites having distinct clusters. These distinct groupings are not as strong in the Weighted UniFrac plots (Figures 34a-34d) but are still clearly separate. The Jaccard Distance plots (Figures 35a-35d) show the strongest distinctions between each sampling sites minus the FHSU sites.

Species Present

Bacterial Communities

The 16S features were put through BLAST to identify any common genera. All but one of the sequences were listed as part of Family Gemmatimonadaceae, that one being Class Ktedonobacteria. One of the sequences from the Gemmatimonadaceae was identified to be in the *Gemmatimonas* genus, a commonly found bacteria in arid or low moisture soils (DeBruyn et al. 2011). This species of *Gemmatimonas* was found in all locations alongside all species being found in 35 samples at a frequency of 111; however, they were more common at Smoky Valley Ranch than other locations. This OTU was found only in *Bothriochloa ischaemum* and *Andropogon gerdii* samples.

Fungal communities

ITS results were much more complicated. Over 1700 OTU's were assembled, and both the most widespread and abundant, as well as some of the rarest, were run through BLAST to identify and characterize a portion of what is occurring in the soil and roots. Many of those BLASTed are from the Basidiomycota and Ascomycota phyla with a few from Phylum Mortierellomycota. Several of the more widespread and abundant OTU's are from Phylum Ascomycota. One of these was sequenced in 133 of our samples, with 55,300 occurrences spread among those samples. It was narrowed to the Order Onygenales, the Family and Genus was unspecified.

Another of the more common Phyla to appear is the Phylum Basidiomycota, however, individual OTU's were not widespread but were in high abundance within the few samples they appeared in. Two examples were identified as *Clavaria fumosa* and *Clavaria fragilis*. These OTU's were found in only 4 samples but had a frequency of 25,265 and 11,217 respectively.

The third most common OTU was from Phylum Mortierellomycota which specified to species within the Genus *Mortierella*; specifically, the species *Mortierella alpina*, *Mortierella amoeboidea*, and *Mortierella elongate* were identified being present in 161, 127, and 72 samples respectively. These species are within an exceptionally diverse Family with a wide variety of functions, allowing them to perform many ecological roles.

Some specific OTUs that were only found in samples of certain variables include *Clavaria tenuipes* and a species within the *Cladophialophora* genus. The *Clavaria* genus is a known fungus within soil, shown to collect trace elements or assist in neutralizing oxidative chemicals. The species of fungus, *Clavaria tenuipes,* was found only in native *Andropogon gerdii* species samples being found in 24 samples with a frequency count of 7854. The species within the Genus *Cladophialophora* is a previously seen but unclassified species. The assembled OTU for the *Cladophialophora* species was found in 41 samples with a frequency of 1564 and was only found in invasive *Bothriochloa ischaemum* samples.

DISCUSSION

16S Sequences

Alpha Metrics

The nativity and species results for the 16S alpha metrics show no trend or pattern regarding the make-up of the community. The location seems to influence community an observable, but not a statistically significant amount. These measurements may be inconclusive due to the poor OTU assembly caused by the low read counts and OTU quantities observed in the OTU count plots in Figures 3c, 4c, and 5c. Nativities Shannon Index metric is the only metric with a significant Kruskal-Wallace groupwise comparison indicating a difference between invasive and natives' community abundance and evenness. The metrics for location has evenness being the only metric without any significance in groupwise comparisons. Together, these

indicate location having an effect when comparing between sampling locations separated by a significant distance despite the poor OTU counts.

PERMANOVA

The 16S beta metrics break from the alpha metrics lack of statistical significance. The PERMANOVA results, both Bray-Curtis and Unweighted Unifrac, show an observable but insignificant difference between distance to invasive and distance to native samples with the distance to native samples having less variation and a higher median. This indicates more dissimilarity and more phylogenetically unique individual species between samples. Distances to native show the same medians between native and invasive in both Bray-Curtis and Unweighted Unifrac metrics; however, the Bray-Curtis distance to native shows less variation in the native samples. This all seems to indicate that invasive samples are exerting selective pressure to reduce diversity within the community, whether these are similar species between samples or just similar amounts of diversity with different species is yet to be determined.

Emperor Plots

The 16S emperor plots show few trends. Nativity only reveals one grouping within the Unweighted Unifrac graphs. This grouping is made up of native *A. gerdii*. Something about this group seems to have similar levels of phylogenetic diversity despite being sampled from different areas. The rest of the metrics when looking at species shows no patterns. The Weighted Unifrac graphs show a very prominent horseshoe phenomenon indicating the secondary gradient is not well represented and any results that could have been interpreted may not be valid. As for location, no pattern was revealed.

Bacterial Species Present

The BLASTed 16S sequences show little variety with the majority being from the Family *Gemmatimonadaceae* with one exception from Family *Ktedonobacteria*. The majority of these

sequences were not able to be identified beyond Family. One of the sequences that was able to be identified beyond Family was a sequence belonging to the Genus *Gemmatimonas,* a common Genus found in drier soils. This OTU does not have a preference regarding location but was restricted to two species, the *Bothriochloa ischaemum* and *Andropogon gerdii*. Without being able to identify beyond Family and with the extremely low variety of OTUs no patterns could be discerned from these samples beyond establishing that there are preferences of OTUs within certain variables.

16S Adonis

The adonis results measure effect size, or how much of the difference between samples is caused by the tested variable. While each variables effect is significant only location has a reasonable effect on the measured changes. This is likely due to the longitudinal gradient present in the Midwest. However, the poor OTU assembly and quality has probably unduly exaggerated the measured differences increasing the effect sizes. The missing degree of freedom from species may also be misconstruing the results to a minor degree.

ITS Sequences

Alpha Nativity

The ITS nativity alpha metrics show a much more decisive and clearer result than the 16S samples. No metric was significant however there are observable differences between native and invasive species. The invasive evenness measure has less variability than native species which seems to indicate an attempt to keep a similar population of different species was present within the soil. We also see invasive species having a lower median in phylogenetic diversity values as well as OTU counts; this makes sense considering the more species present could increase the amount of diversity and since the invasive samples have fewer OTUs we would see less diversity. In contrast the native samples don’t seem to exert selective pressure on its microbiome.

Lastly the Shannon Index values show invasive species having less variation in abundance and evenness confirming the OTU count metric and Pielous evenness metric. Together, this indicates that the invasive species are applying some selective pressures to the microbiome.

Alpha Species

The ITS species metrics did not contain any significant values; however, these results reveal more information of what's happening within the invasive and native categories. Looking at evenness we see similar medians and variation between *A. gerdii*, *B. bladhii*, and *S. scoparium*. The *B. ischaemum* samples showed far less variation with a higher median indicating a pressure to keep the community populations at least similar. Phylogenetic diversity values mirror the nativity values with *A. gerdii* and *S. scoparium*, the native species, having more variation and a higher median indicating more diversity with the invasive species, the Bothriochloa species, having less. The OTU counts for each species is mixed with *A. gerdii* and *B. ischaemum* having higher medians and less variation while the other two are reversed. Lastly the Shannon Index shows similar values to the evenness measurements confirming that *B. ischaemum* is pressuring the microbiome community to at least have similar population numbers and richness.

Alpha Location

The ITS location metrics reveal consistently significant results in contrast to the previous variables. Evenness shows a clear contrast between more eastern and western locales with the KU Field Station and Konza Prairie having the most variation and lower medians than the FHSU sites and Smoky Valley Ranch showing a clear trend of increased variety in species populations in eastern locations. Phylogenetic diversity is more varied without a clear distinction but still shows more variety within the eastern locations than the western locations, medians are mixed showing no trends in total diversity. The OTU counts follow the same pattern as evenness with

eastern locations having more variation and lower medians than the western locations. Lastly, Shannon Index confirms the evenness metrics while showing abundance has little to know effect on the established pattern.

PERMANOVA

The ITS beta diversity forms a much clearer picture of how each variable effects the community when compared to the 16S community beta metrics. The PERMANOVA results examining nativity shows significant differences between native and invasive species level of dissimilarity and phylogenetic diversity. Both the Bray-Curtis and Unweighted Unifracs' distance to native plots show no differences between native and invasive, however the distance to invasive plots show more variation and lower medians in the invasive samples. This tells us that these samples are showing less diversity, they are more similar and aren't as phylogenetically diverse when not accounting for quantity of individual OTUs.

Nativity Emperor Plots

The ITS sequences emperor plots for nativity shows some distinction between native and invasive. The Bray-Curtis graph and the Weighted Unifrac graph shows no clustering, however, we do see some patterns in the Unweighted Unifrac and Jaccard Distance. Both graphs show clustering of invasive samples within four larger clusters; there seems to be some differing influences on the microbiome between invasive and native species.

Species Emperor Plots

The ITS emperor plots for species show more detail within the different clusters that formed when examining nativity. The Bray-Curtis plot shows some strong grouping of *B. ischaemum* and *B. bladhii* showing the two species influencing their respective communities into having similar amount of dissimilarity. The other two species do show single pockets of weakly clustering samples that is worth noting but is not on the same level at the *Bothrochloa* species.

The Unweighted Unifrac plots shows each species forming lose clusters within the four larger groups of samples. Specifically, *B. bladhii* and *B. ischaemum* formed distinct subgroups within the larger formations. Examining the Weighted Unifrac, we see those patterns shown in the Unweighted graphs disappearing resulting in no distinct pattern; this shows that there are more unique species that have a very low population and when these are excluded the phylogenetic diversity of these communities loses similarity. Finally, Jaccard Distance shows each species forming its own cluster within the four larger structures. Each species forms a community with a similar amount of unique OTUs within the total OTU assemblage.

Location Emperor Plots

Looking at location we see what the four larger cluster found in the Unweighted Unifrac and the Jaccard Distance graphs are formed from, location. The Bray-Curtis plots show the four clusters occupying their own area of the graph based entirely on their longitude. We see the FHSU sites clumped together while the other three sites separate with some minor mixing between locations that are closer together. The Unweighted Unifrac graph shows the four larger clusters entirely based on longitude with no mixing beyond the FHSU sites. There are three outliers from the Konza Prairie samples, but these remain near the KU Field Site cluster and not the very distance FHSU or Smoky Valley locations. The Weighted Unifrac plots lose the distinct clustering in the previous graph to form a much larger single mass that remains sectioned based on location. The minor mixing between locales remains between those that are closer together. Lastly, Jaccards Distance repeat the distinct four clusters seen in the Unweighted Unifrac and forms even denser clusters. To sum up, nativity seems to have a minor effect on community make up and is overshadowed by the influence location has, this is likely related to the various gradients found along the longitude of the Midwest which include precipitation and soils.

ITS Adonis

The ITS results mirror and are more conservative than the 16S results. Each tested variable remains significant and location the largest effect size. The gap between the locations R^2 value, nativities value, and species value has shrunk considerably and is likely more representative of the differences between the variables thanks to the higher OTU assembly rate. Regardless of the possible reasons behind the differences between the ITS and 16S results, the reasoning behind location being the cause of the most change remains the same in the ITS samples as the 16S, the longitudinal gradient is likely the cause of the differences.

Species Present

The BLASTed ITS sequences show much more variety than its 16S counterpart. A small part of the assembled OTUs were investigated for being either omnipresent in among the samples or for only appearing in samples of a specific tested variable. These specific OTUs were from a variety of phyla and several were able to be identified to the species level. A common OTU found was *Mortierella alpina* which was almost omnipresent throughout the samples with no clear pattern in the samples where it was absent. Two identified species that were specific to certain species of grass are *Clavaria tenuipes* which was found exclusively in *Andropogon gerdii* samples and an unclassified species from the Genus *Cladophialophora* which was found only in *Bothriochloa ischaemum* samples. These specific examples add to the pool of evidence that grass species do influence the microbiome for their own purposes; it shows that there are species that are omnipresent throughout the soil.

CONCLUSION

The spread of Old-World Bluestem (OWB) grasses throughout the Midwest is having a drastic and negative effect on prairie biodiversity increasing the cost for controlling and preventing its spread. Recent studies have shown OWB not only drive native grasses out of

habitats but also actively prevent the reestablishment of those native grasses, complicating remediation attempts. This prevention of native grass reestablishment may be related to the microbiome and how interconnected plant functions and soil characteristics are to this microbial community. Determining how microbiomes are influenced by its host species could provide insight into how invasive species propagate and push native grasses out of their habitat.

The metagenomic analysis shows that location has the largest effect on the fungal microbial community with nativity being overshadowed but still measurable and significant between samples. The bacterial community results are inconclusive due to the poor OTU assembly, however, because of the consistency it is likely that location has the largest effect on the bacterial community differences like the fungal results. The identification of species of microbes that only appear in samples of specific grass species increases the pool of evidence that grass species are influencing the microbiome for their own ends.

The development or application of techniques from other fields of study is needed to rescue the Midwest prairies. The continued encroachment of OWB continues to reduce biodiversity and push native species out of their habitats. Further experiments into the differences between each plant species root & soil microbiome is prudent, along with examining how various physical characteristics that change along the longitudinal gradient affect these microbiomes to identify the best and most economical way to preserve and restore our natural prairies.

FIGURES

	Relict	Sheep	Konza	KU	Smoky
B. bladhii	Absent	Present	Present	Present	Absent
B. ischeamum	Absent	Present	Absent	Absent	Present
S. scoparium	Present	Absent	Present	Present	Present
A. gerardi	Present	Absent	Present	Present	Present

Table 1: The presence of targeted grass species in each location

	Native	Invasive	KU Field	Konza	Relict	Sheep	Smoky	A. gerdii	S. scoparium	B. bladhii	B. ischaemum
16S	56	37	7	3	22	24	37	33	23	12	25
ITS	38	80	18	35	12	15	38	40	40	18	20

Table 2: Summation of the number of samples that represent each variable

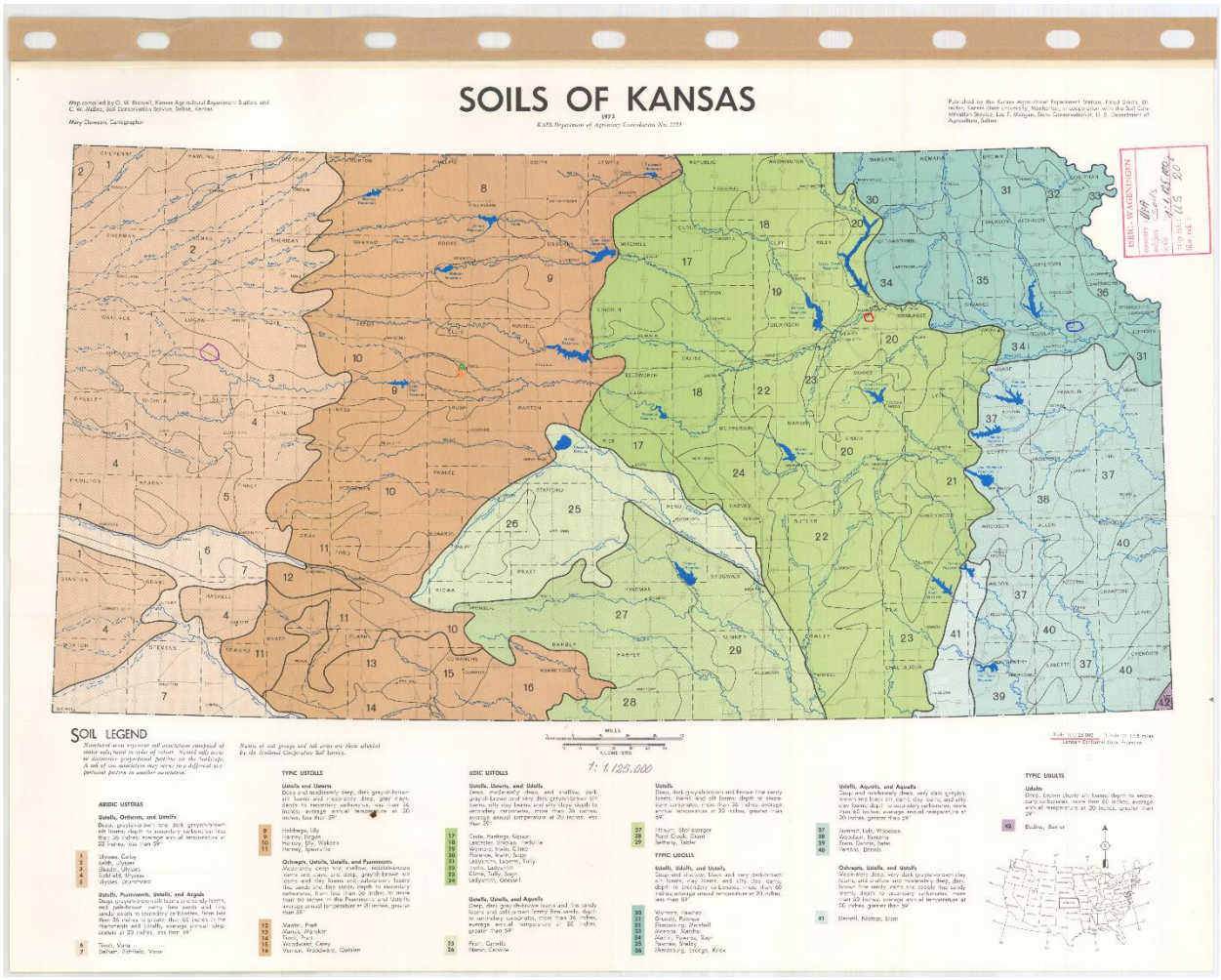

Figure 1

This map shows the stratification of soil types from east to west. Each location can be found circled with its respective color found in the emperor plots. We see each location, with the exception of the FHSU sites, being located in an area with different soil mixtures. (O.W. Bidwell et al. 1973)

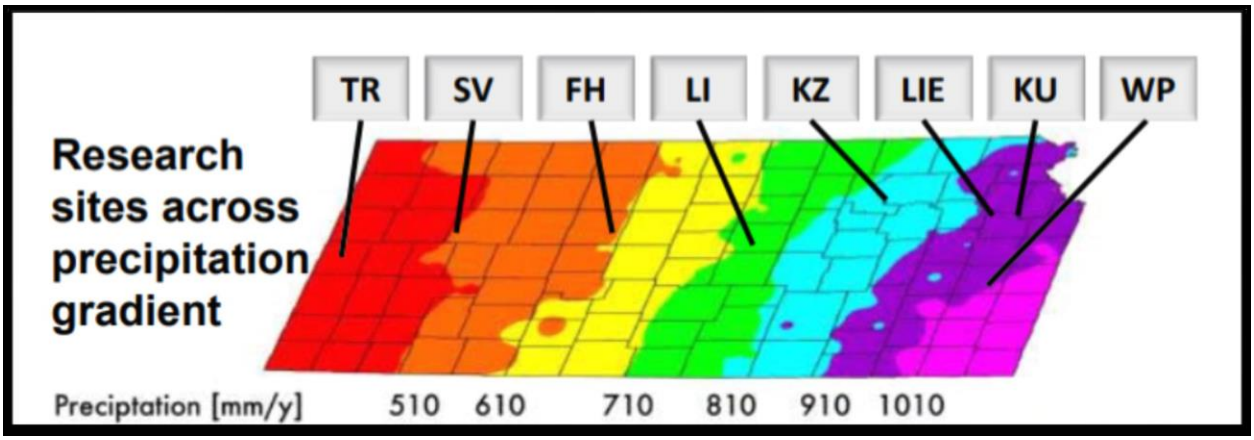

Figure 2

This map shows the precipitation gradient across Kansas with the driest parts on in the west and wettest in the east. Each site is within its own zone of average precipitation measured in mm per year. Sites are marked as Smoky Valley being SV, Fort Hays as FH, Konza Prairie as KZ, and KU Field Station as KU. Map provided by EPSCOR.

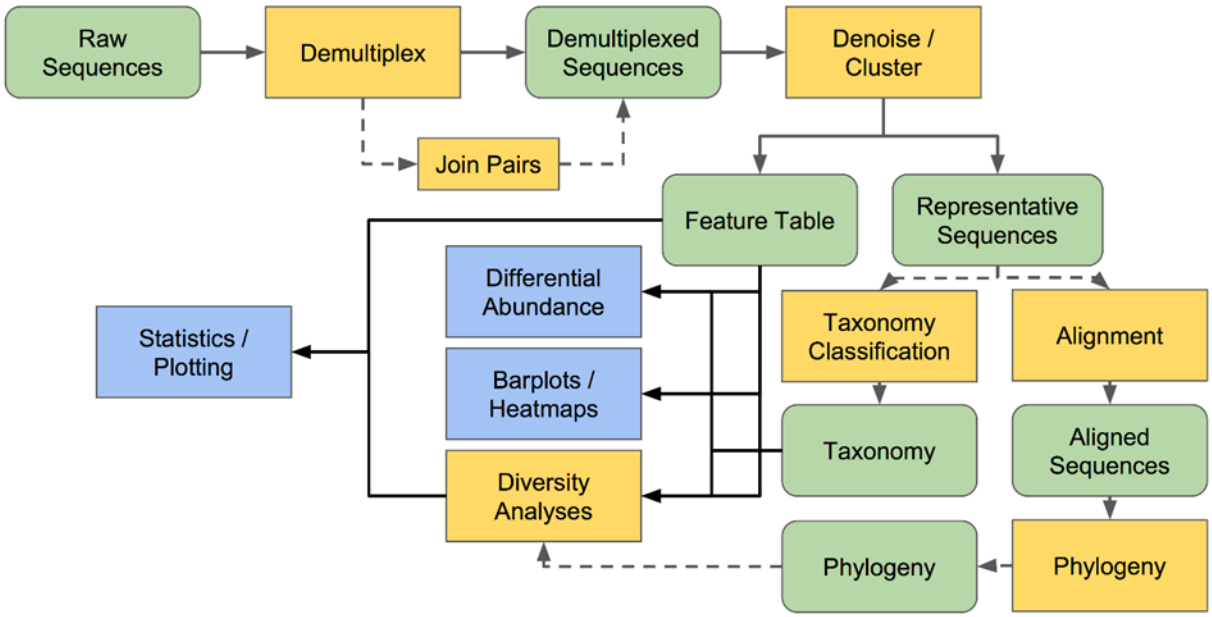

Figure 3

This flowchart provides a visual for the generalized overview of the processes performed during this study. Provided by the QIIME2 development team.

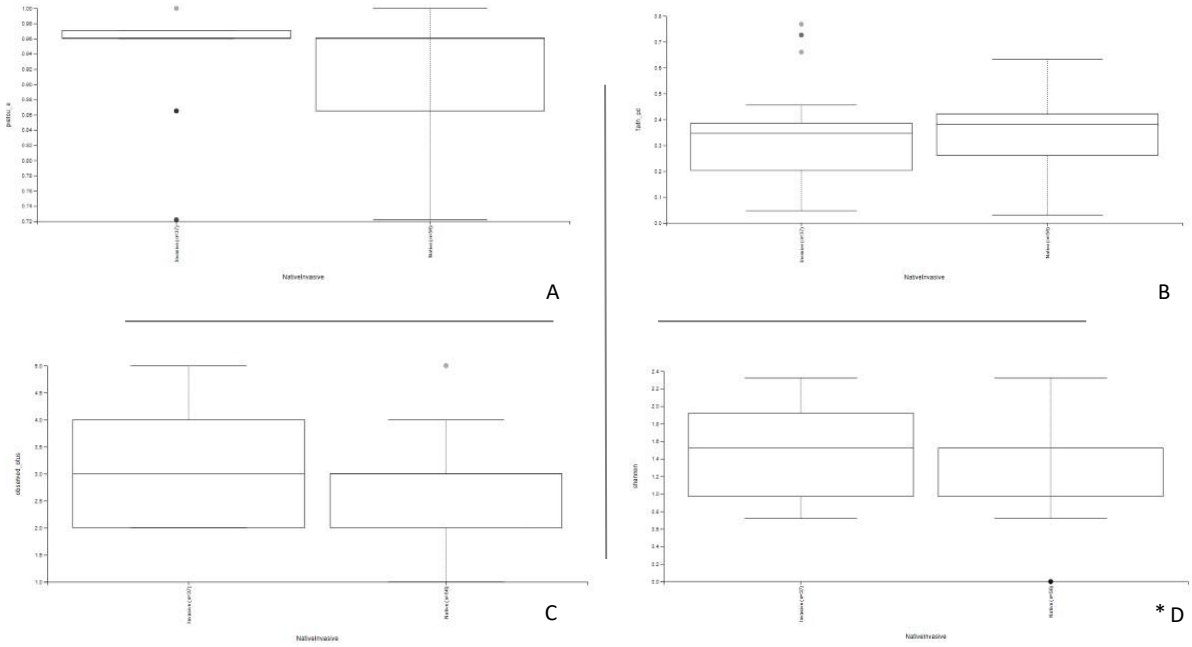

Figure 4: Box & whisker plots from each 16S alpha diversity metric testing nativity of soil & root microbiome of various native and Old-World Bluestem invaded grasslands across Kansas. (A) Evenness showing native samples having wider variation than invasive samples. (B) Faiths Phylogenetic Diversity showing native samples having a higher amount of diversity. (C) number of OTUs in each sample showing native samples with less variation than invasive samples. (D) Shannon's Index showing native samples having less variation in abundance and richness. (Groupwise * p-values < 0.05)

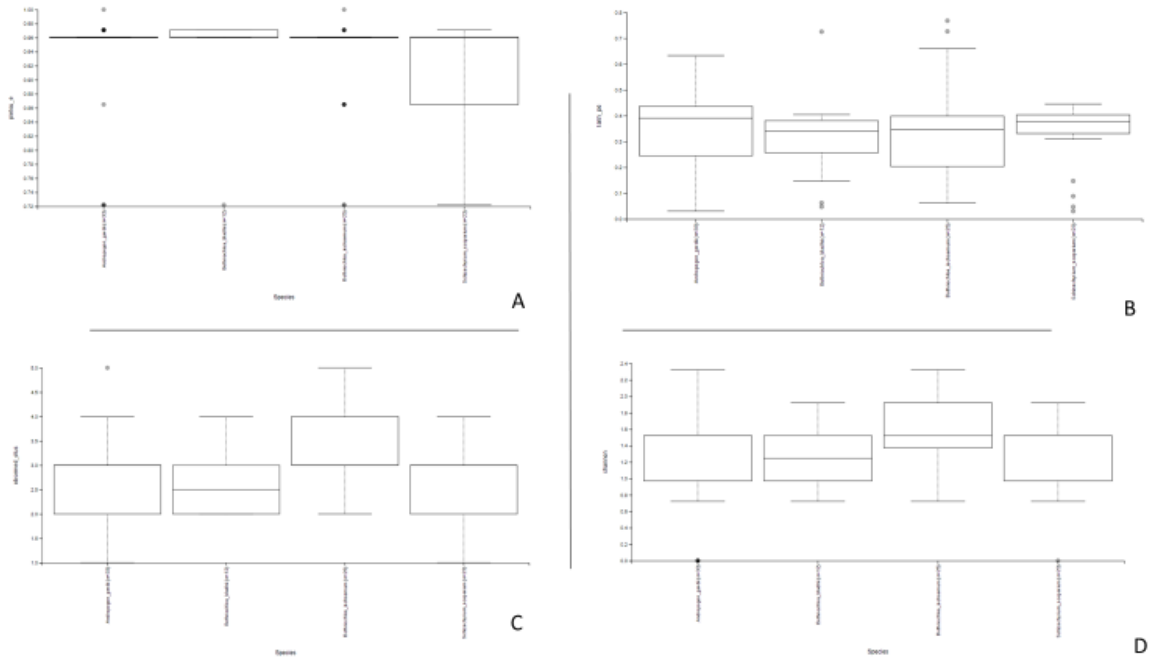

Figure 5: Box & whisker plots from each 16S alpha diversity metric testing species. (A) Evenness showing equivalent medians with only *S. scoparium* having any meaningful variation. (B) Faiths Phylogenetic Diversity showing similar medians and *S. scoparium* having the least variation. (C) Number of unique OTUs in each sample showing similar variation between species. (D) Shannon's Distance similar variation with varying medians between species. (Groupwise * p-values < 0.05)

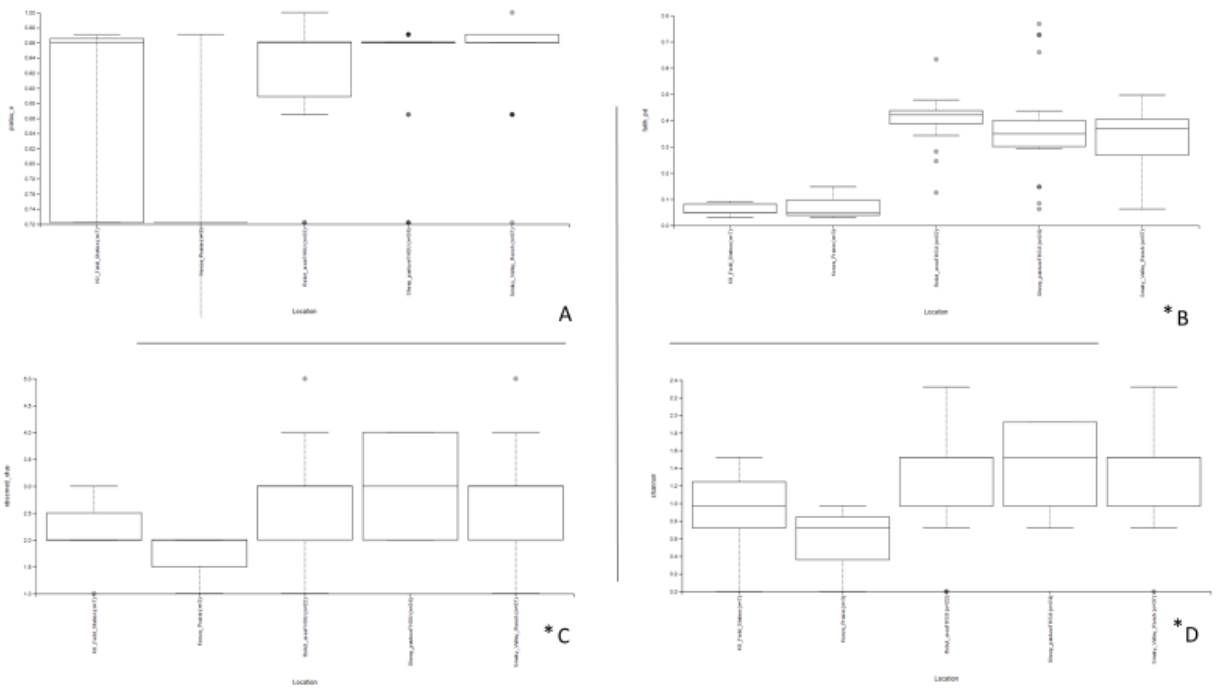

Figure 6: Box & whisker plots from each 16S alpha diversity metric testing location. (A) Evenness showing similar medians except for Konza Prairie. Variation among locations is highly variable. (B) Faiths Phylogenetic Diversity showing similar medians between eastern and western locations. Variation is narrow and within similar range based on eastern or western location (C) Number of unique OTUs in each sample showing similar variation between species. (D) Shannon's Distance similar variation, the Sheep Pasture being an exception, and varying medians between species. (Groupwise * p-values < 0.05)

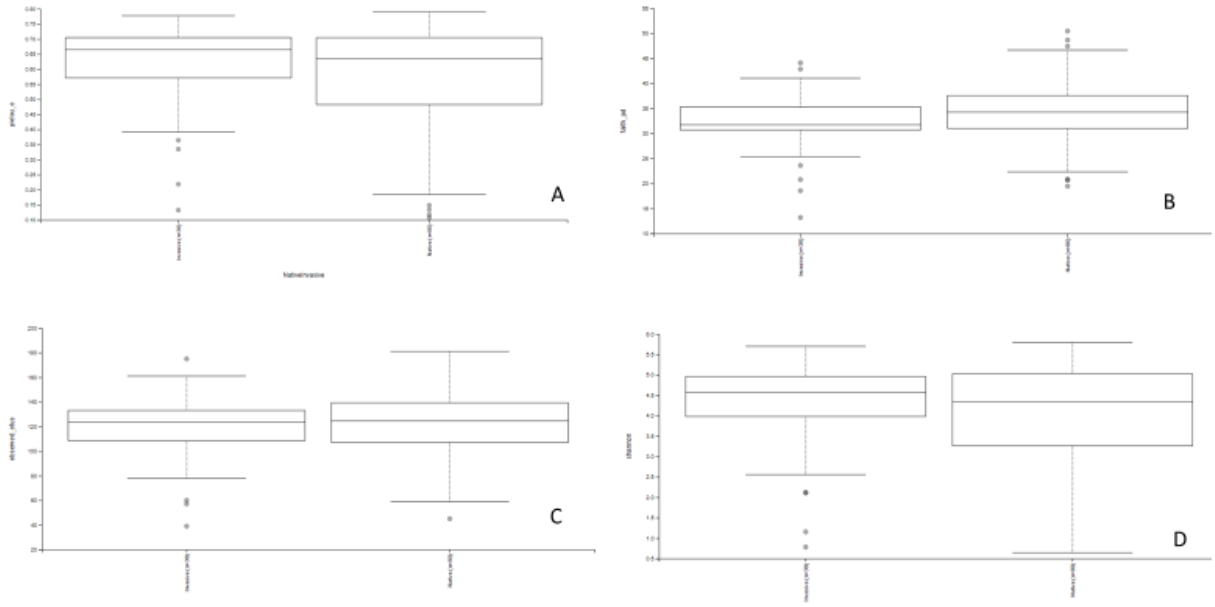

Figure 7: Box & whisker plots from each ITS alpha diversity metric testing nativity. (A) Evenness showing similar medians with less variation in the invasive samples. (B) Faiths Phylogenetic Diversity showing a lower median and less variation in the invasive samples. (C) Number of unique OTUs in each sample showing similar medians with less variation in the invasive samples (D) Shannon's Distance with invasive having a higher median and lower variation. (Groupwise * p-values < 0.05)

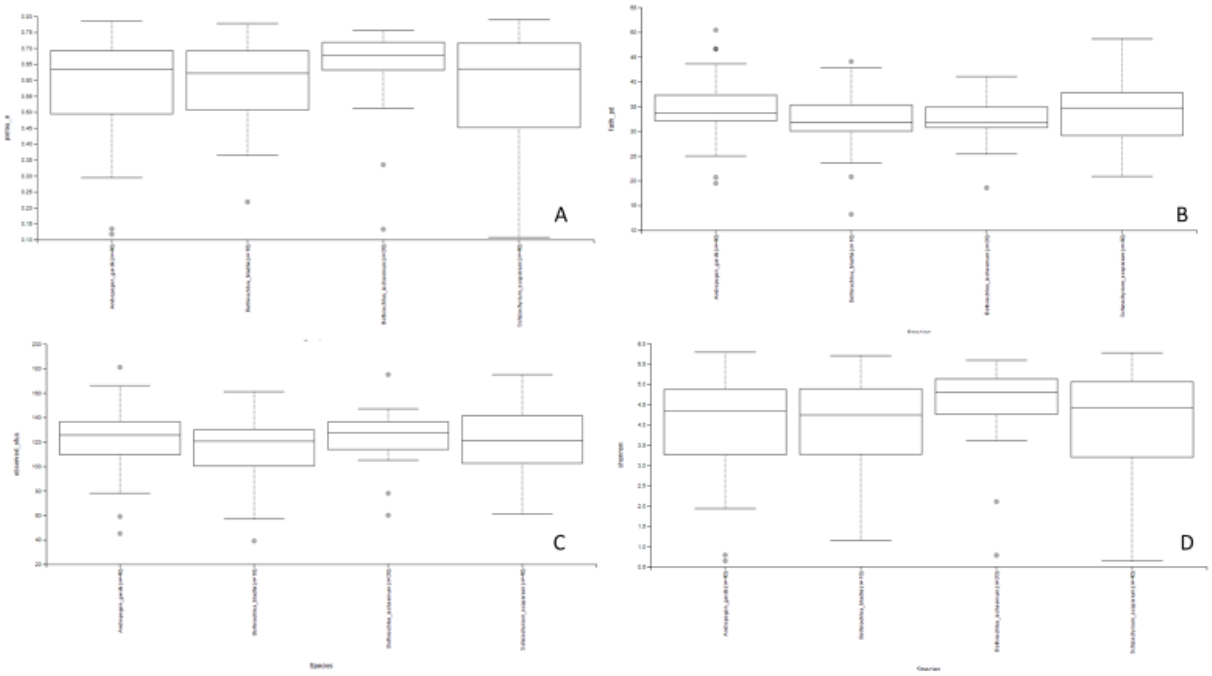

Figure 8: Box & whisker plots from each ITS alpha diversity metric testing species. (A) Evenness showing similar medians and variation with *B. ischaemum* being an exception having less. (B) Faiths Phylogenetic Diversity each species having a different median with *A. gerdii* and *B. bladhii* having similar variation. (C) Number of unique OTUs in each sample showing medians in a narrow range with less variation in the *B. ischaemum* samples and more variation in the *S. scoparium* samples. (D) Shannon's Distance shows *B. ischaemum* with less variation and a higher median (Groupwise * p-values < 0.05)

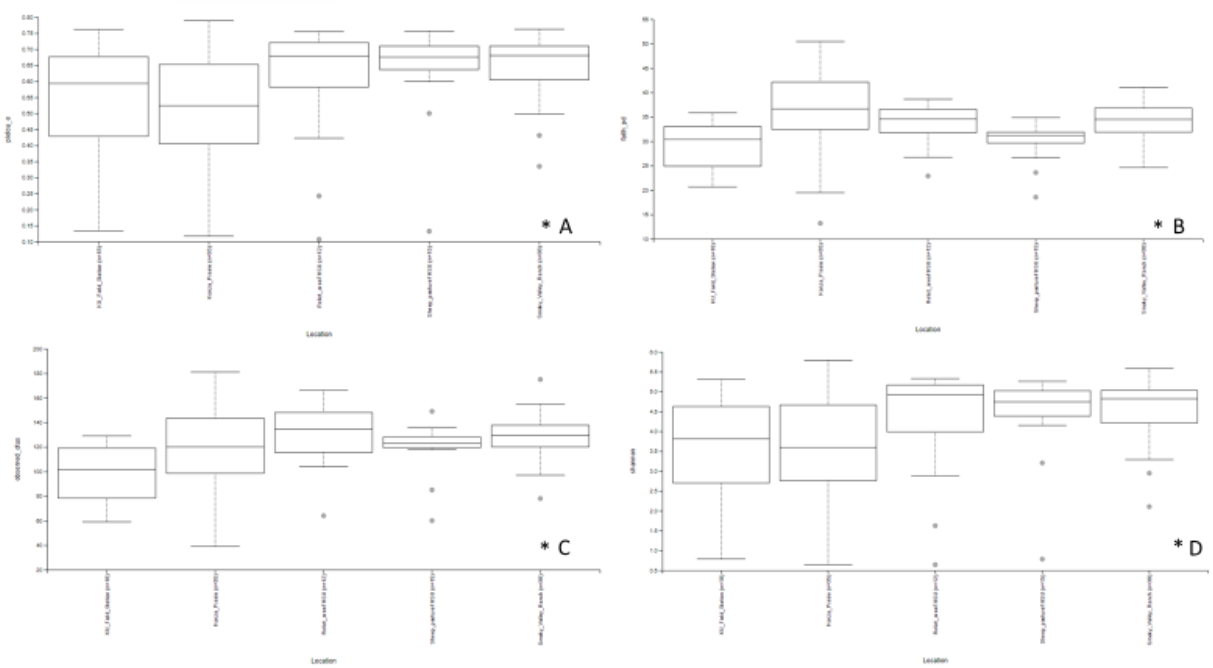

Figure 9: Box & whisker plots from each ITS alpha diversity metric testing location. (A) Evenness showing a clear divide between the eastern locations and western location with west having higher medians and lower variation. (B) Faiths Phylogenetic Diversity does not have a consistent pattern between east and west. Konza Prairie has the highest median and highest variation with KU having the lowest median and the Sheep Pasture having the lowest variation. (C) Number of unique OTUs in each sample showing no pattern. KU has the lowest median, the Relict Area has the highest, while the Sheep Pasture has the lowest variation. (D) Shannon's Distance shows the previous east versus west pattern with KU and Konza having wider variation and lower medians with the western sites being the reverse. (Groupwise * p-values < 0.05)

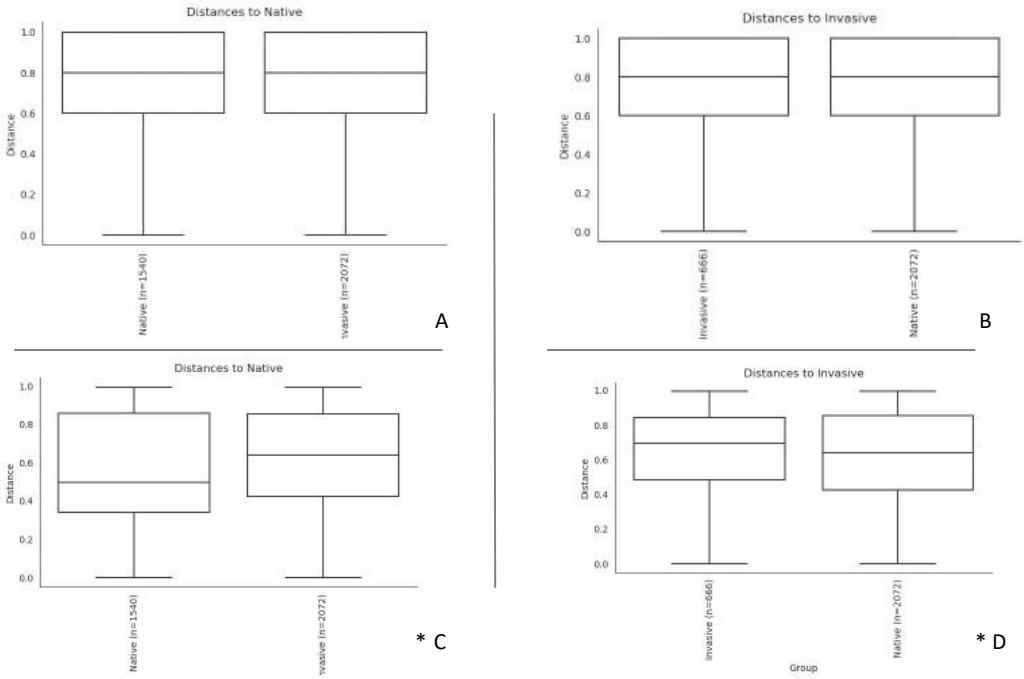

Figure 10: Box & whisker plots from the 16S PERMANOVA analysis. (A) is Bray-Curtis distance to invasive and shows less dissimilarity in the invasive samples. (B) is Bray-Curtis distance to native, it contrasts figure A and shows no dissimilarity between native and invasive samples beyond a higher spread of outliers in the native samples. (C) shows Unweighted UniFrac distance to invasive native samples having a higher median and narrower variance indicating the invasive samples have less diversity. (D) is Unweighted UniFrac distance to native and shows no difference between native and invasive samples. (Groupwise * p-values < 0.05)

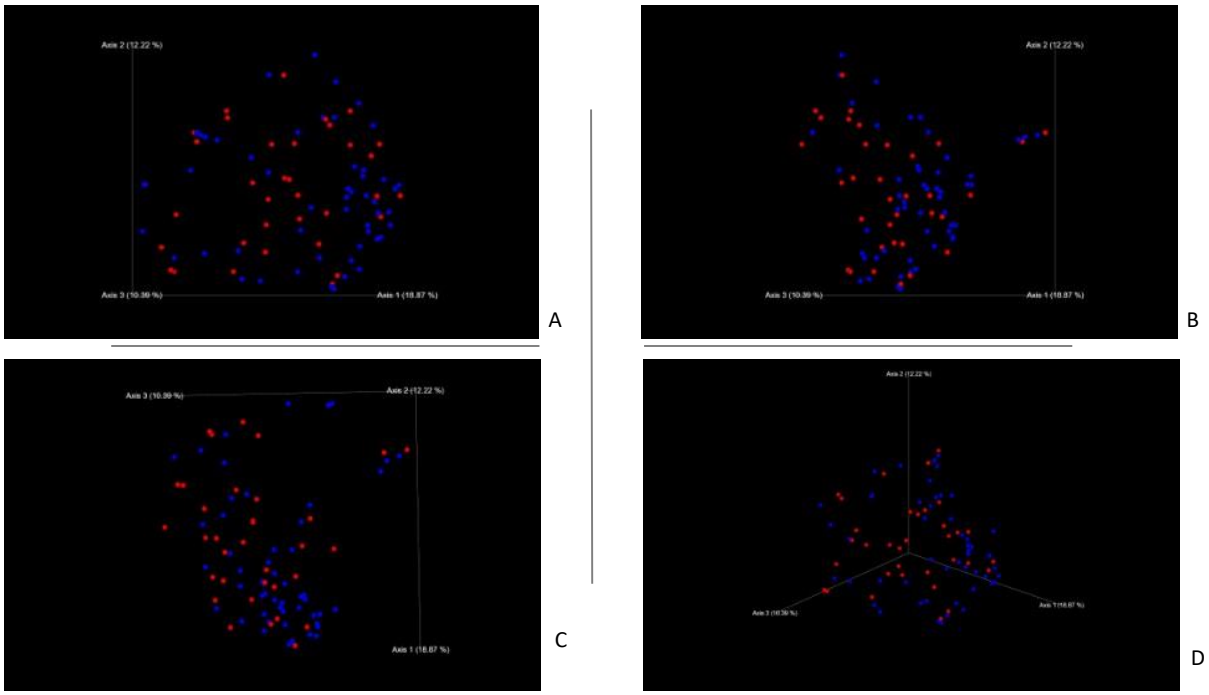

Figure 11: 16S Bray-Curtis Emperor Plots Showing Nativity (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. There are no groupings based on nativity.

Blue: Native

Red: Invasive

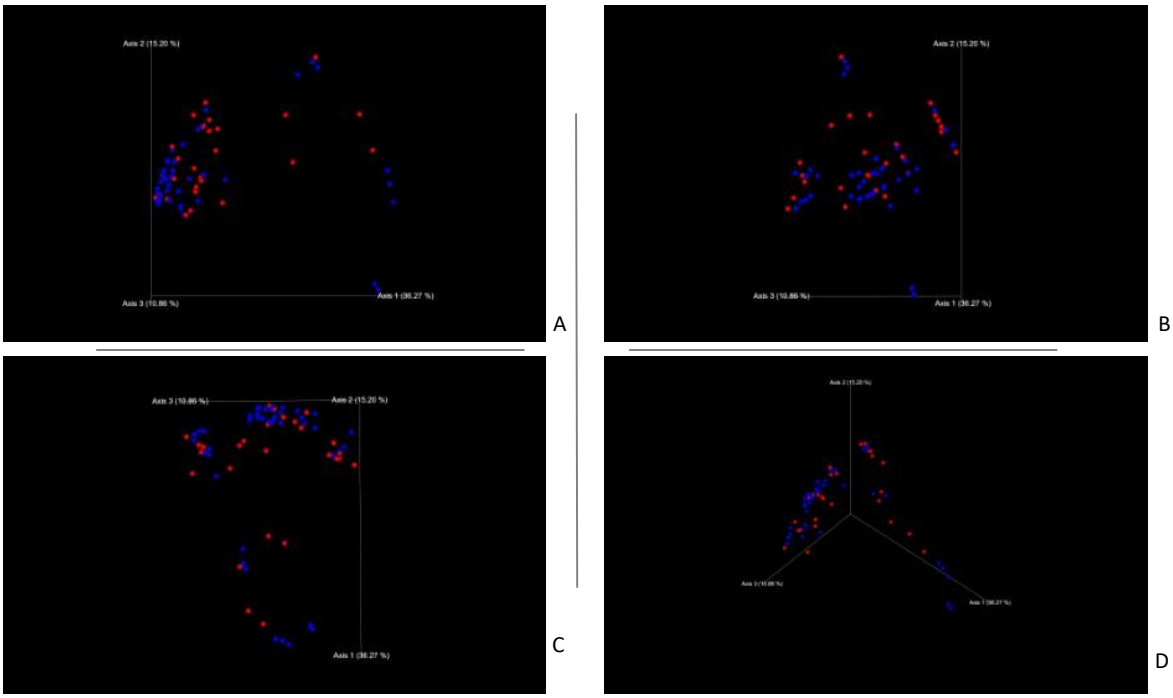

Figure 12: 16S Unweighted UniFrac Emperor Plots Showing Nativity (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. We see a single grouping of native species along axis 3.

Blue: Native

Red: Invasive

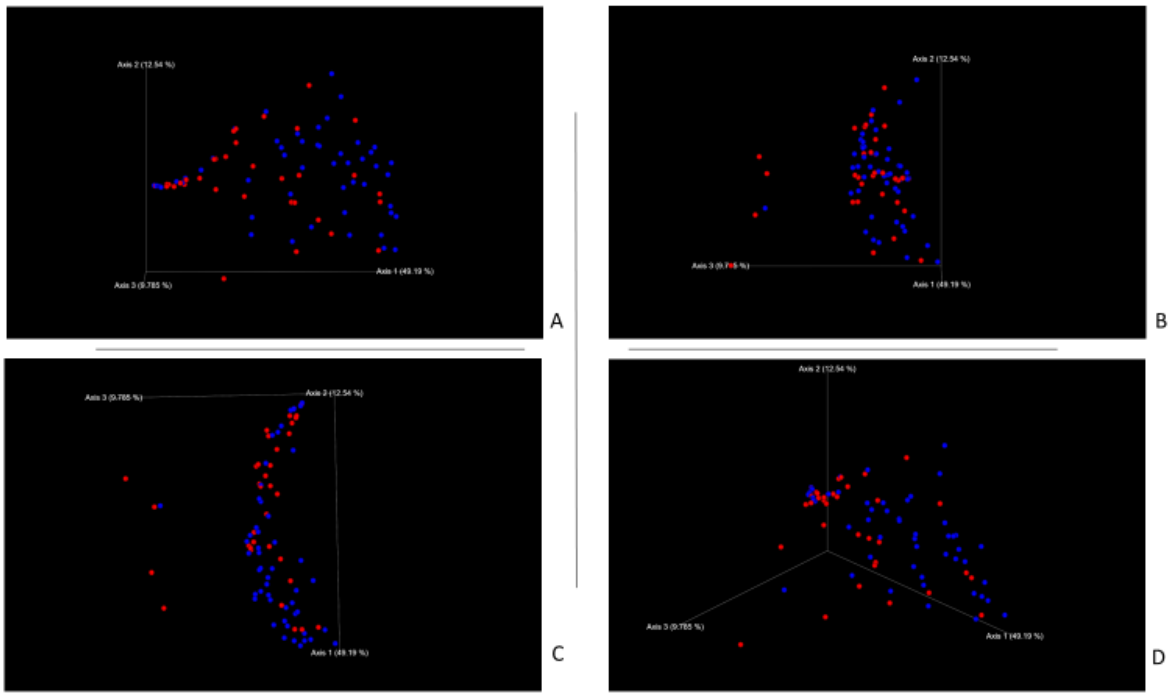

Figure 13: 16S Weighted UniFrac Emperor Plots Showing Nativity. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. For nativity we see no patterns in these plots. In figure 13c we see a very prominent horseshoe effect indicating the secondary gradient is not well represented.

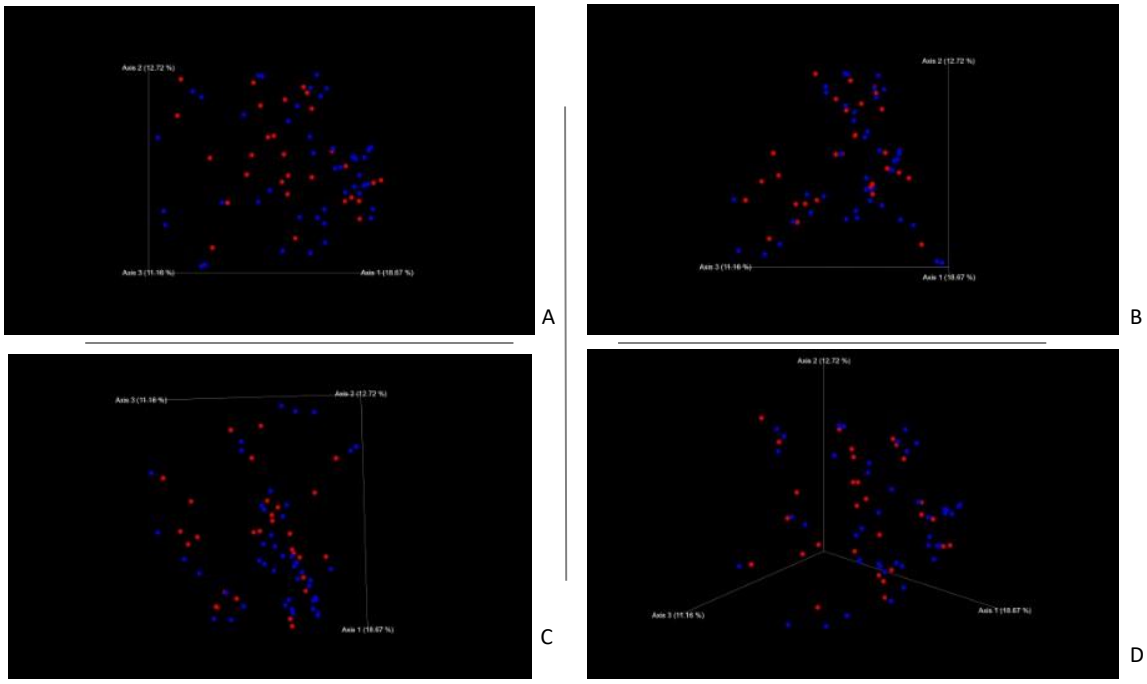

Figure 14: 16S Jaccard Distance Emperor Plots Showing Nativity. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. For nativity we see no patterns formed.

Blue: Native

Red: Invasive

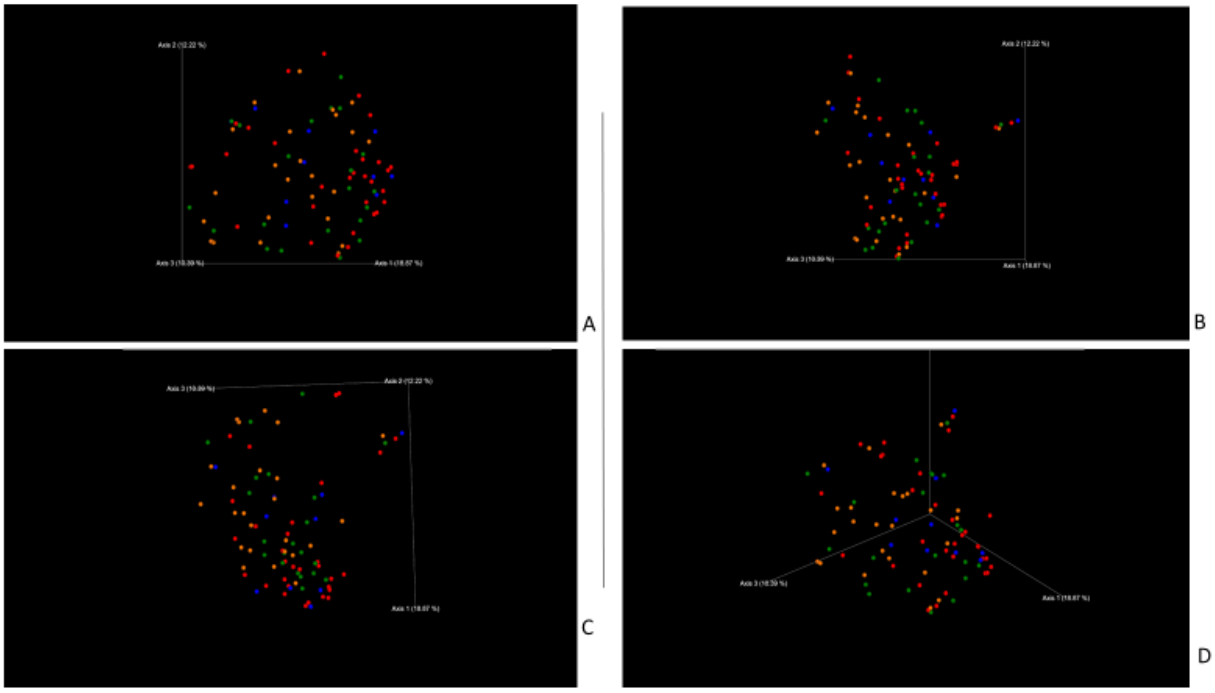

Figure 15: 16S Bray-Curtis Emperor Plots Showing Species (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. There is no grouping in the plots.

Red: *Andropogon gerdii*

Blue: *Bothriochloa bladhii*

Orange: *Bothriochloa ischaemum*

Green: *Schizachyrium scoparium*

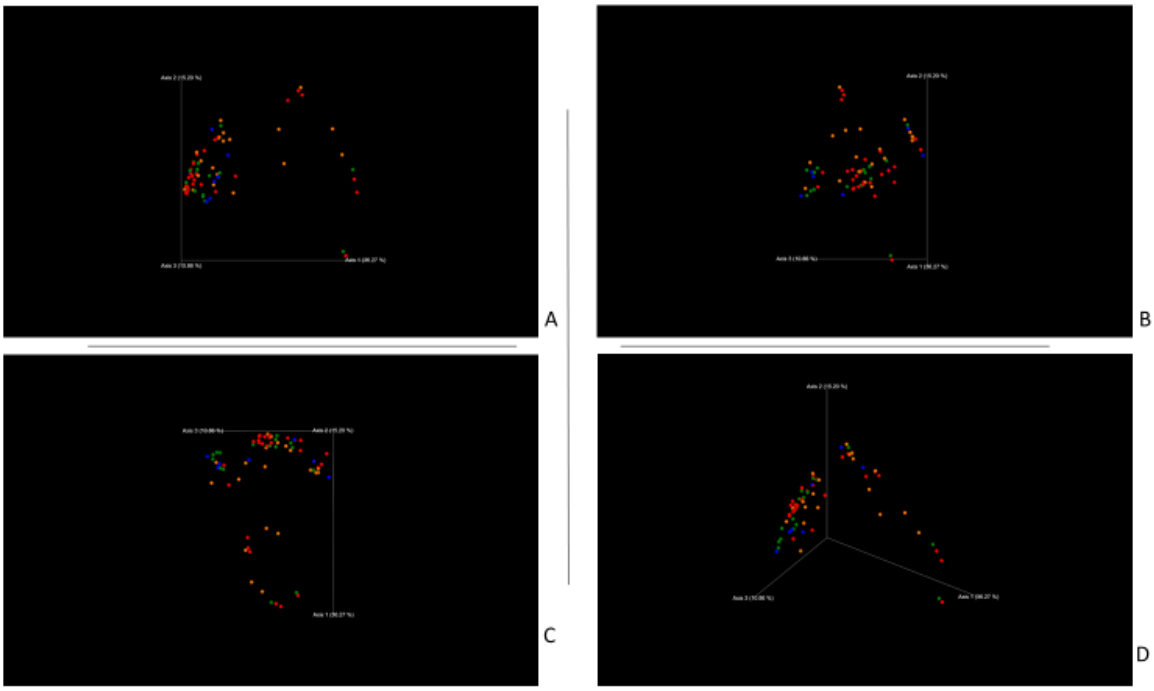

Figure 16: 16S Unweighted UniFrac Emperor Plots Showing Species (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. We see a small grouping of *A. gerdii* within the native grouping that follows axis 3.

Red: *Andropogon gerdii*

Blue: *Bothrochloa bladhii*

Orange: *Bothriochloa ischaemum*

Green: *Schizachyrium scoparium*

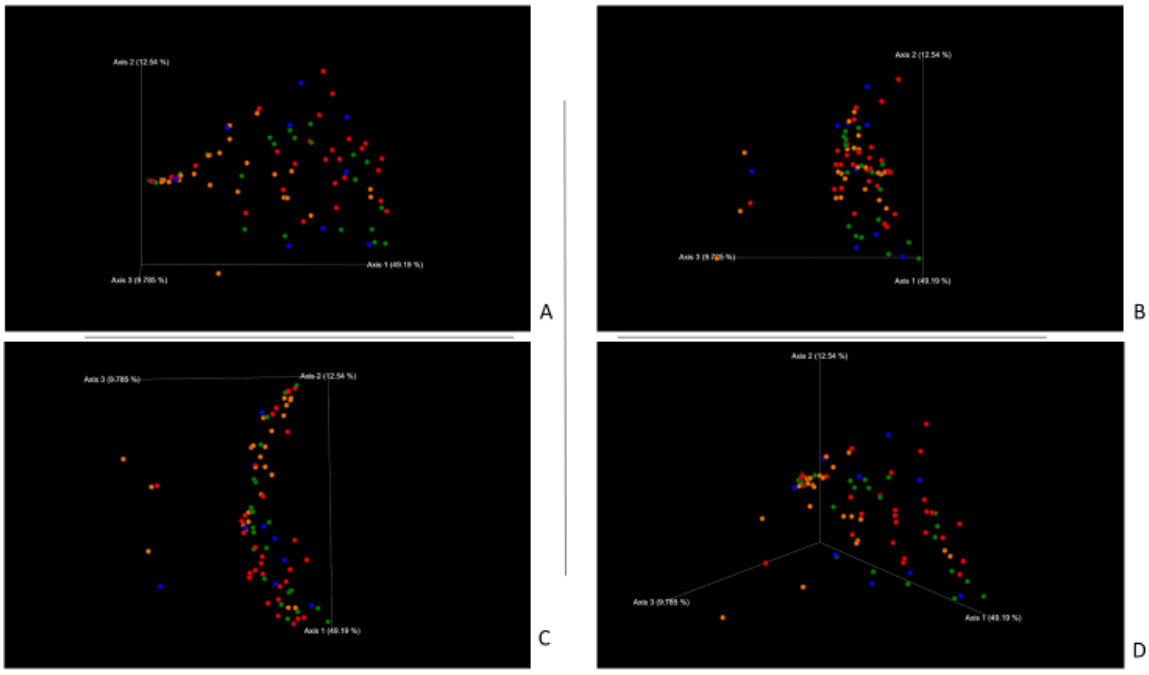

Figure 17: 16S Weighted UniFrac Emperor Plots Showing Species. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. For species we see no patterns in these plots. In figure 15c we see a very prominent horseshoe effect indicating the secondary gradient is not well represented.

Red: *Andropogon gerdii*

Blue: *Bothrochloa bladhii*

Orange: *Bothriochloa ischaemum*

Green: *Schizachyrium scoparium*

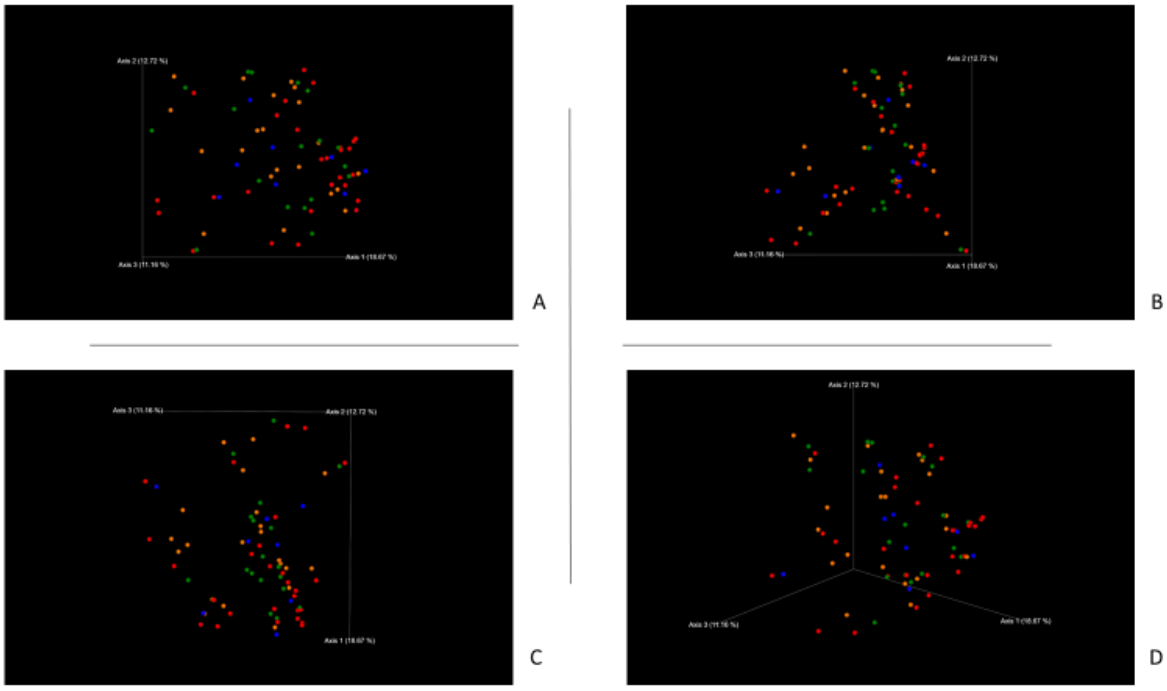

Figure 18: 16S Jaccard Distance Emperor Plots Showing Species. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. For species we see no patterns formed.

Red: *Andropogon gerdii*

Blue: *Bothrochloa bladhii*

Orange: *Bothriochloa ischaemum*

Green: *Schizachyrium scoparium*

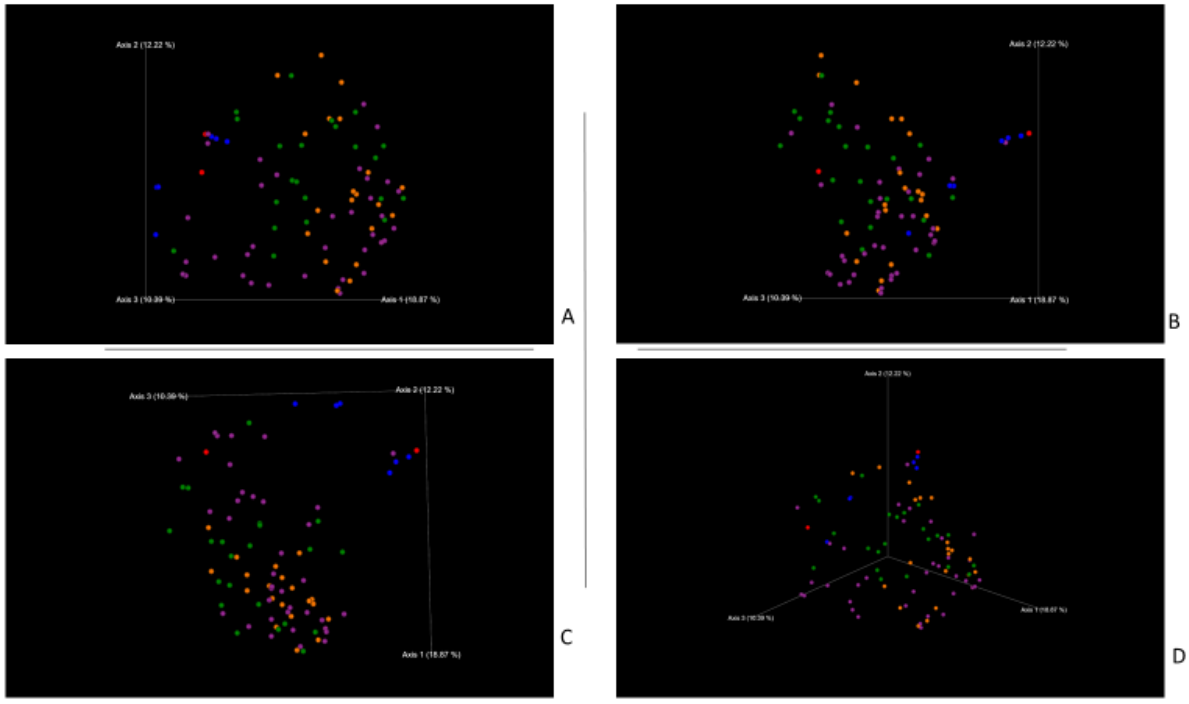

Figure 19: 16S Bray-Curtis Emperor Plots Showing Location (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. The only group in these plots is a pocket of samples from KU Field Station.

Red: Konza Prairie

Blue: KU Field Station

Orange: FHSU Relict Area

Green: FHSU Sheep Pasture

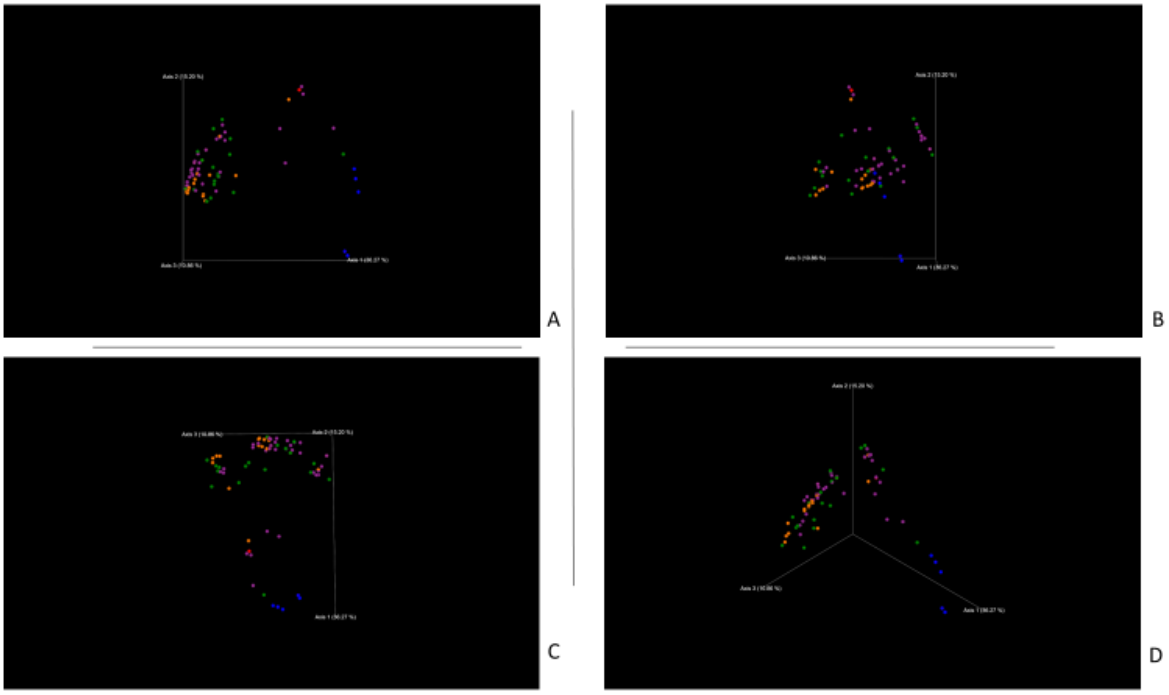

Figure 20: 16S Unweighted UniFrac Emperor Plots Showing Location. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. We see KU Field Station occupying its own area, the rest of the locations are mixed throughout.

Red: Konza Prairie

Blue: KU Field Station

Orange: FHSU Relict Area

Green: FHSU Sheep Pasture

Purple: Smoky Valley Ranch

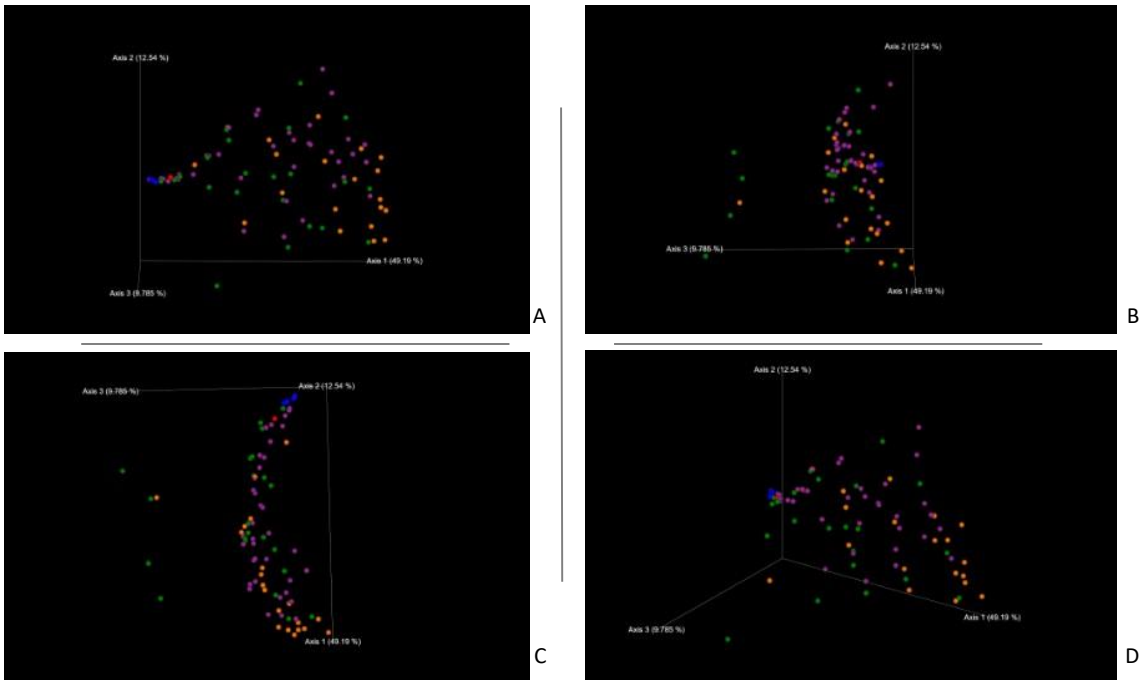

Figure 21: 16S Weighted UniFrac Emperor Plots Showing Location. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. For location we see no patterns in these plots. In figure 16c we see a very prominent horseshoe effect indicating the secondary gradient is not well represented.

Red: Konza Prairie

Blue: KU Field Station

Orange: FHSU Relict Area

Green: FHSU Sheep Pasture

Purple: Smoky Valley Ranch

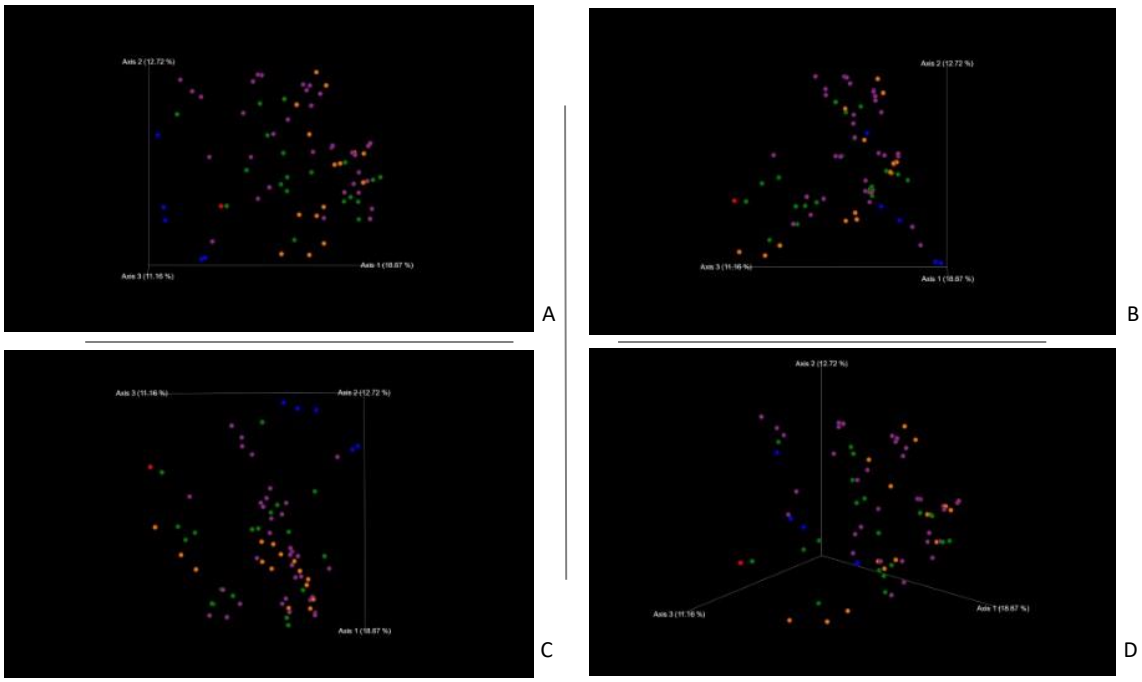

Figure 22: 16S Jaccard Distance Emperor Plots Showing Location. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. For location we see no patterns formed.

Red: Konza Prairie

Blue: KU Field Station

Orange: FHSU Relict Area

Green: FHSU Sheep Pasture

Purple: Smoky Valley Ranch

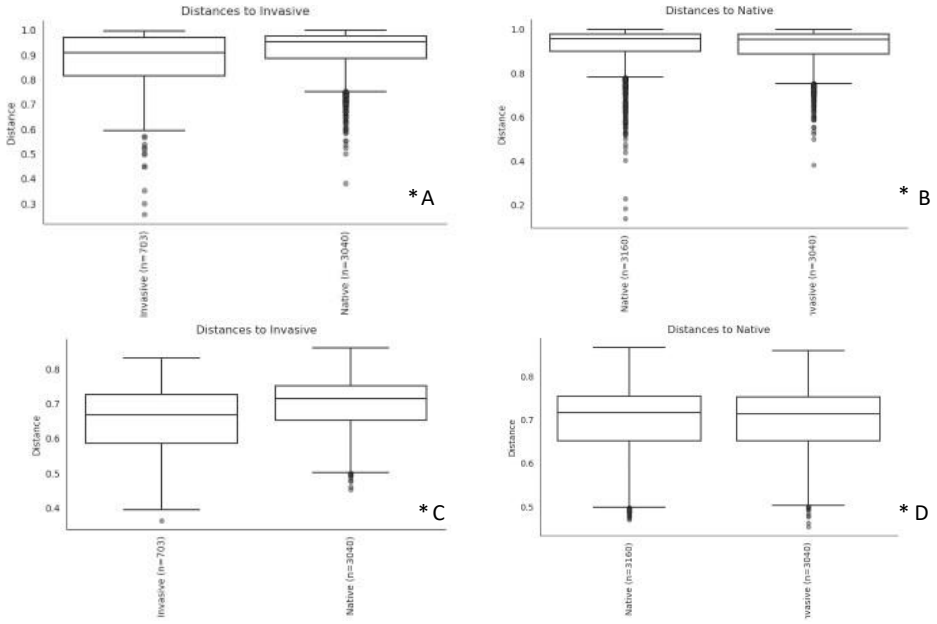

Figure 23: Box & whisker plots from the ITS PERMANOVA analysis. (A) the Bray-Curtis distance to invasive plot shows less dissimilarity in the invasive samples. (B) the Bray-Curtis distance to native plot shows no difference in contract to figure 13a beyond a higher spread of outliers in the native samples. (C) Unweighted UniFrac distance to invasive shows native samples having a higher median and narrower variance. (D) shows no difference beyond the spread of outliers. (* p-values < 0.05)

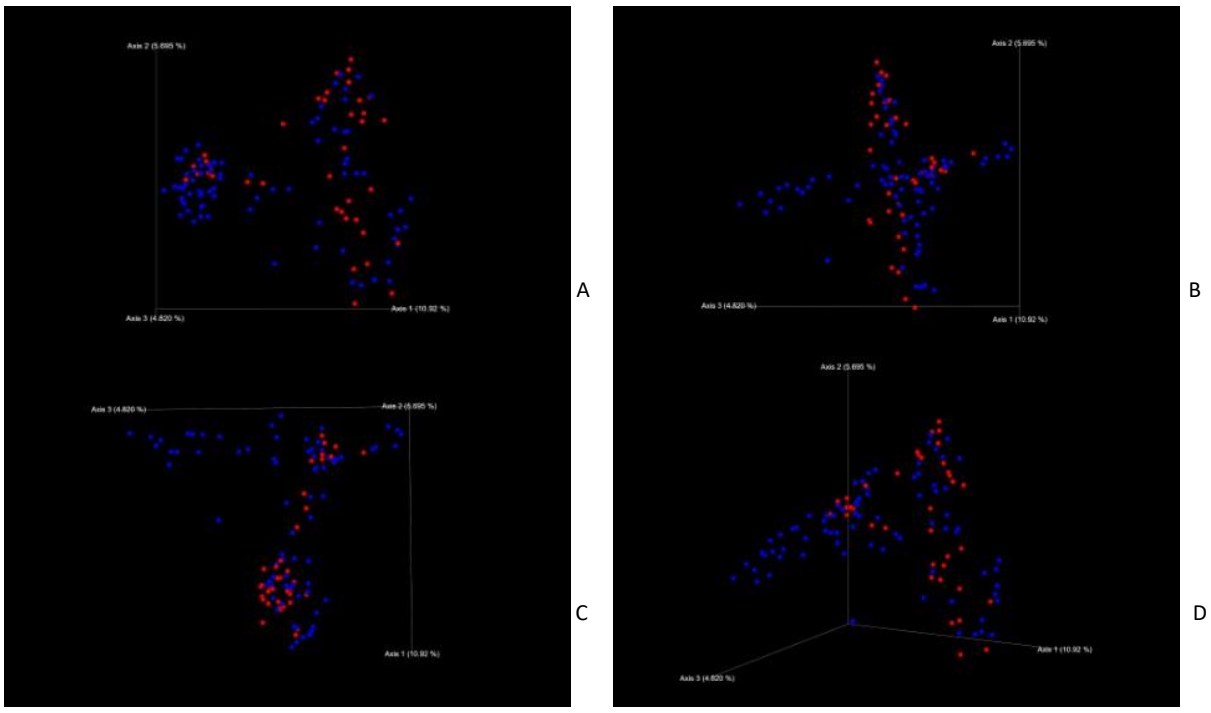

Figure 24: ITS Bray-Curtis Dissimilarity Emperor Plots Showing Nativity. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. For location we see no patterns formed. We see two distinct structures in these plots visible in figures B, C, and D. There is no pattern when visualizing nativity.

Blue: Native

Red: Invasive

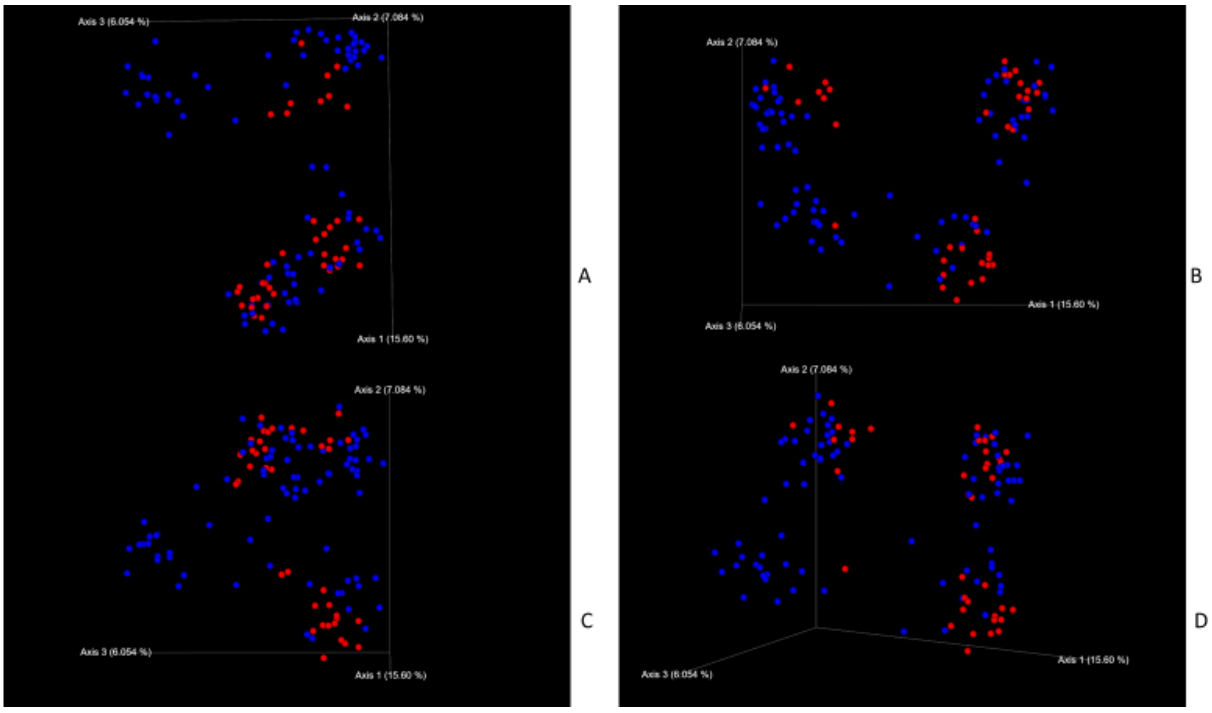

Figure 25: ITS Unweighted UniFrac Emperor Plots Showing Nativity. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. Here we see four distinct clusters of mixed nativity. In figure A we see some invasive samples grouping in the top left cluster in figure A. In figure D there is some sub-grouping visible separating native from invasive in the bottom right group. The rest are mixed.

Blue: Native

Red: Invasive

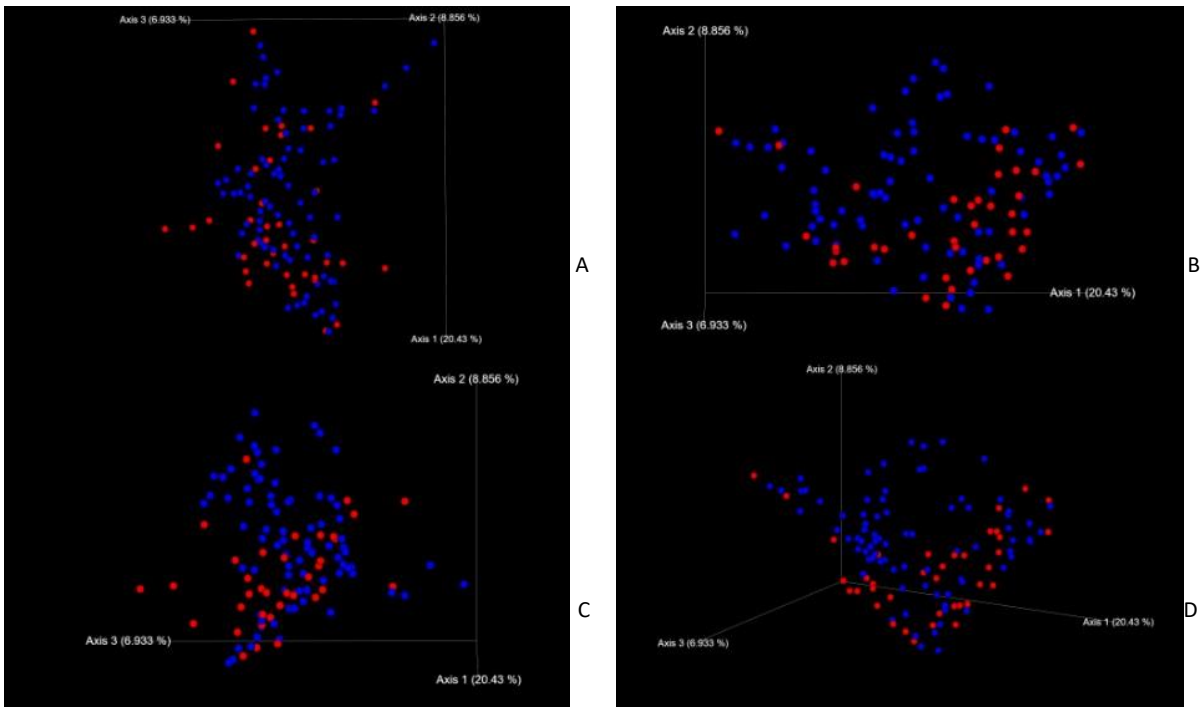

Figure 26: ITS Weighted UniFrac Emperor Plots Showing Nativity. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. Looking at nativity we see no patterns or groupings of points. In figure D we see a potential horseshoe, however if present it is very weak.

Blue: Native

Red: Invasive

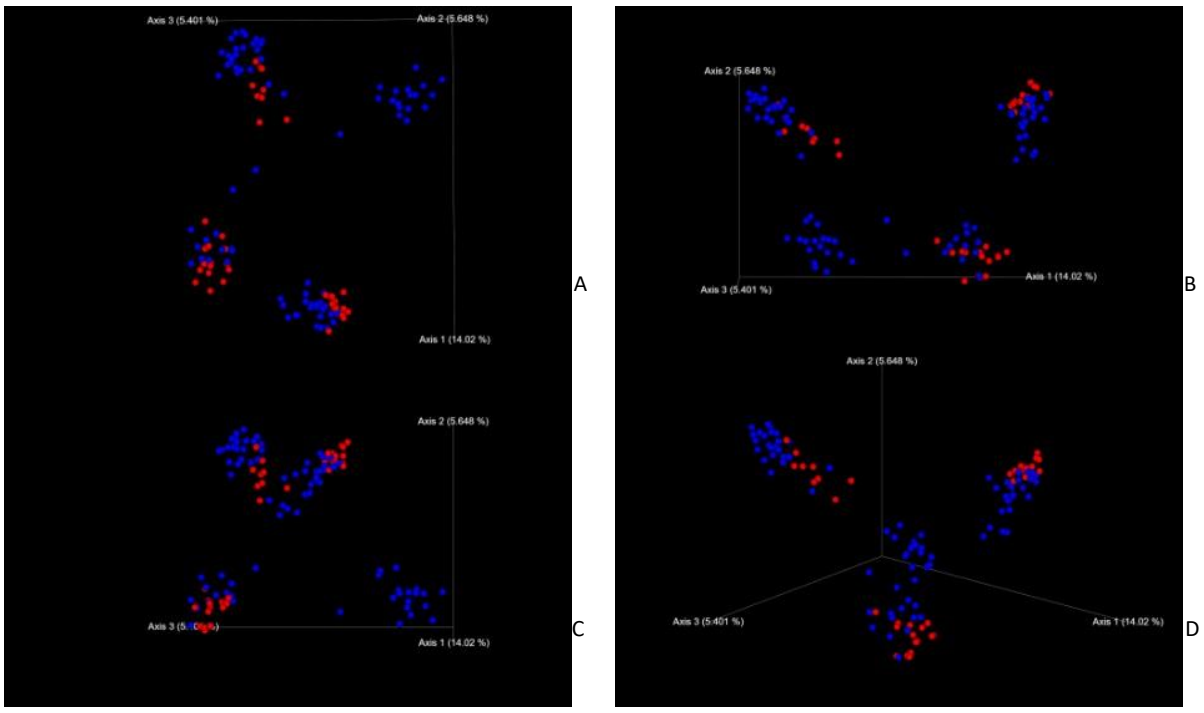

Figure 27: ITS Jaccard Distance Emperor Plots Showing Nativity. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. Here we see 4 distinct groups with subgrouping based on nativity. We see in figure A three of the groups having invasive groupings with the last lacking any. Figure C further shows these invasive subgroups within the larger groupings.

Blue: Native

Red: Invasive

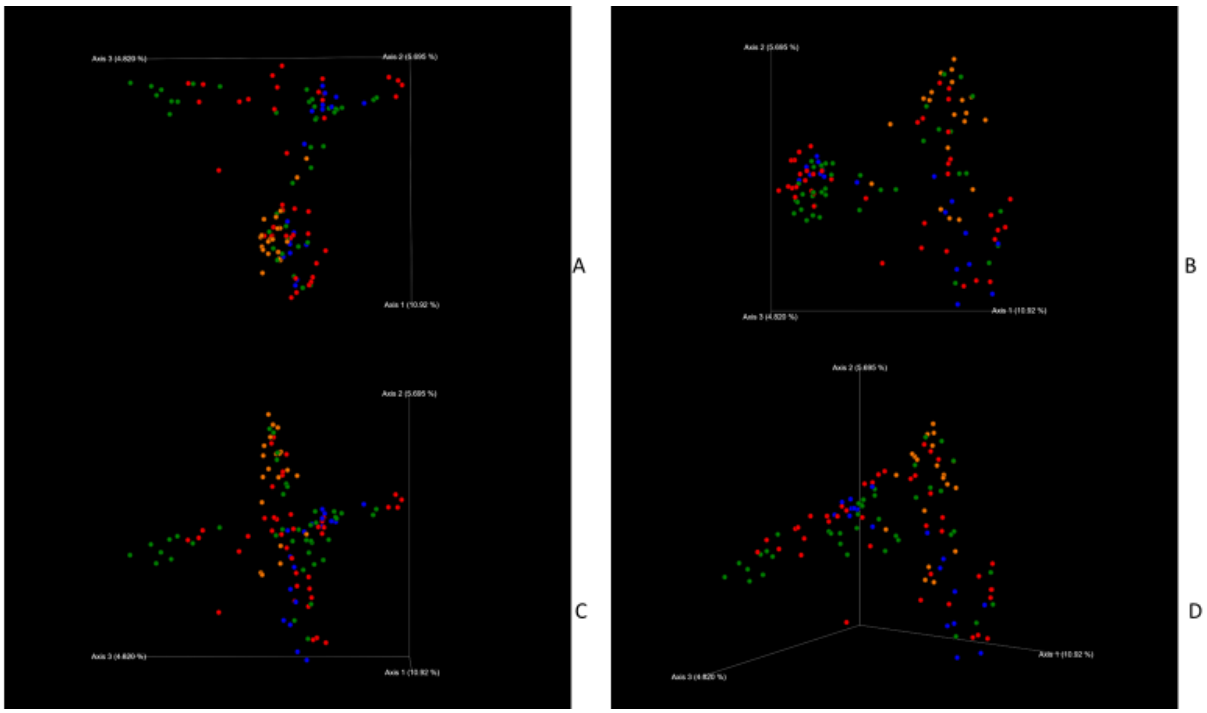

Figure 28: ITS Bray-Curtis Dissimilarity Emperor Plots Showing Species. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. For location we see no patterns formed. We see two distinct structures in these plots visible in figures E, F, and G. Looking at species we see a grouping of *B. ischaemum* and two groups of *B. bladhii*. Other species have some weak single clusters forming.

Red: *Andropogon gerdii*

Blue: *Bothrochloa bladhii*

Orange: *Bothriochloa ischaemum*

Green: *Schizachyrium scoparium*

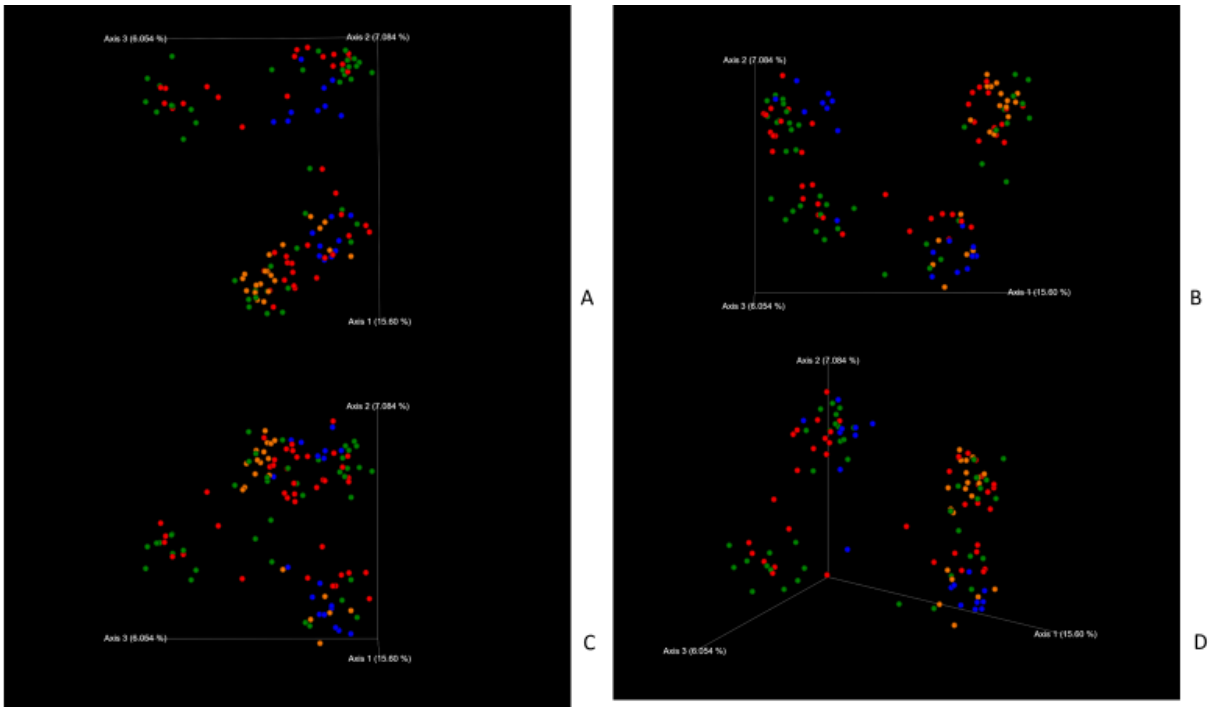

Figure 29: ITS Unweighted UniFrac Emperor Plots Showing Species. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. Here we see four distinct clusters of species forming weak clusters.

Red: *Andropogon gerdii*

Blue: *Bothrochloa bladhii*

Orange: *Bothriochloa ischaemum*

Green: *Schizachyrium scoparium*

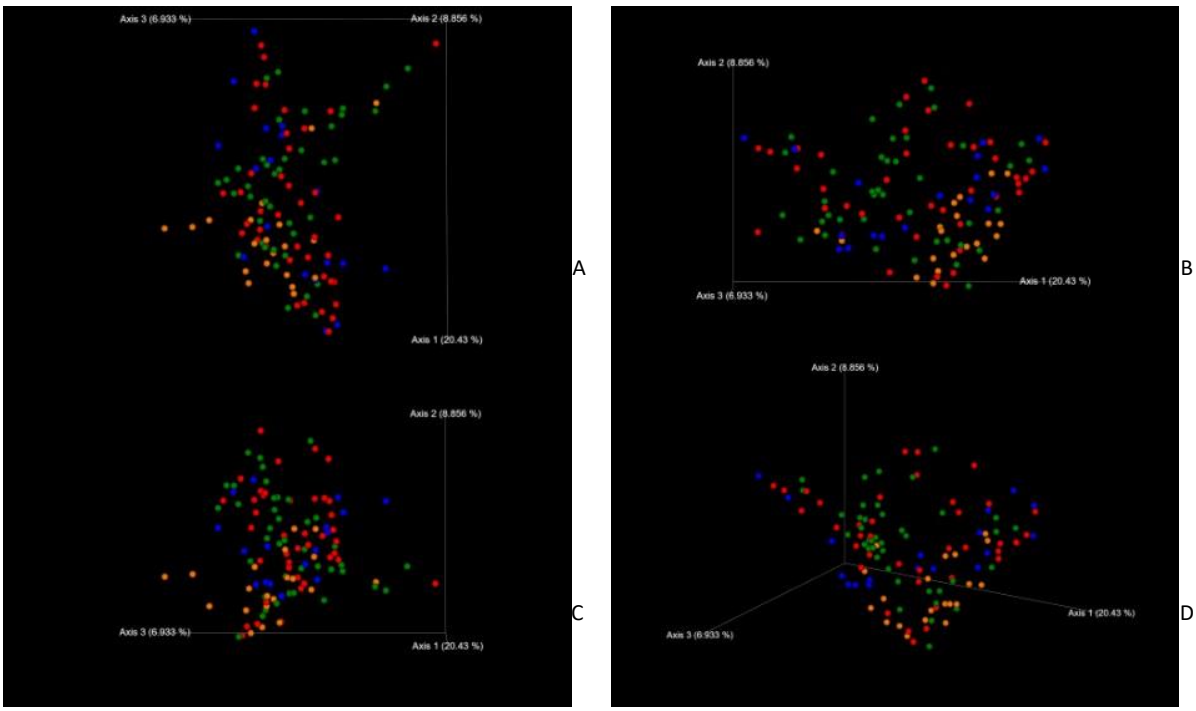

Figure 30: ITS Weighted UniFrac Emperor Plots Showing Species. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. Looking at species we see no distinct clustering. In figure D we see a potential horseshoe, however if present it is very weak.

Red: *Andropogon gerdii*

Blue: *Bothrochloa bladhii*

Orange: *Bothriochloa ischaemum*

Green: *Schizachyrium scoparium*

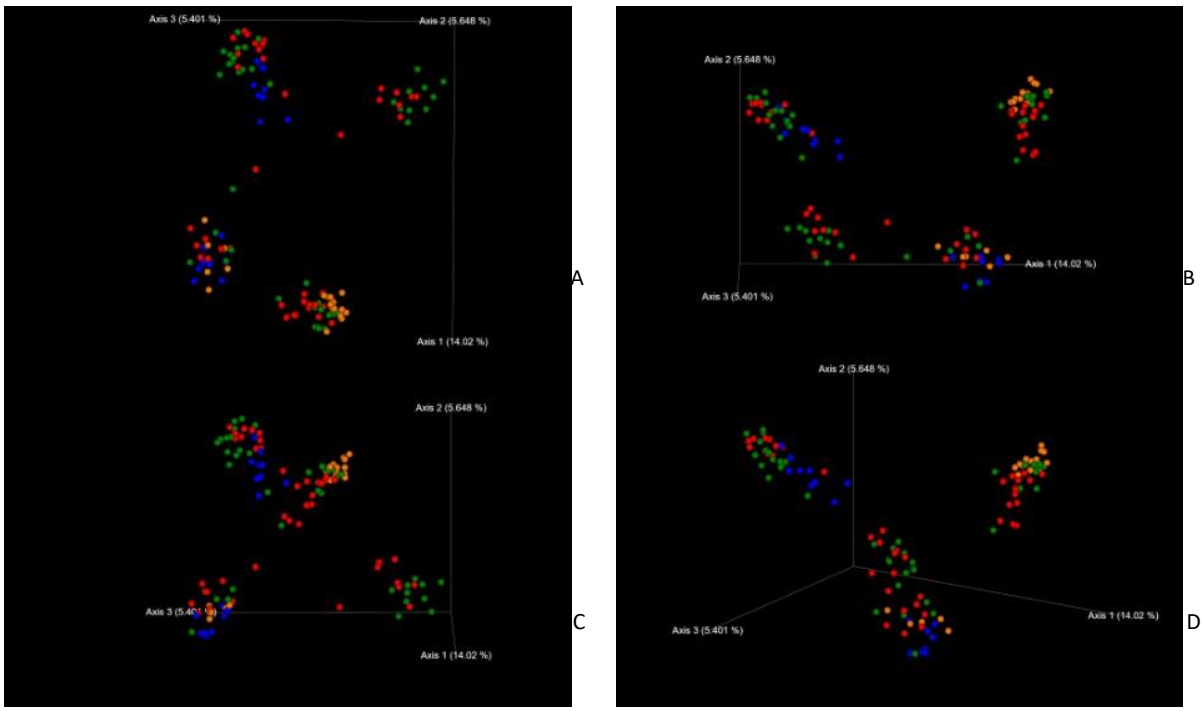

Figure 33: ITS Jaccard Distance Emperor Plots Showing Species. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. Looking at species we see a grouping of various species within the larger groups. Each species has formed its own subgroup within the four larger clusters.

Red: *Andropogon gerdii*

Blue: *Bothrochloa bladhii*

Orange: *Bothriochloa ischaemum*

Green: *Schizachyrium scoparium*

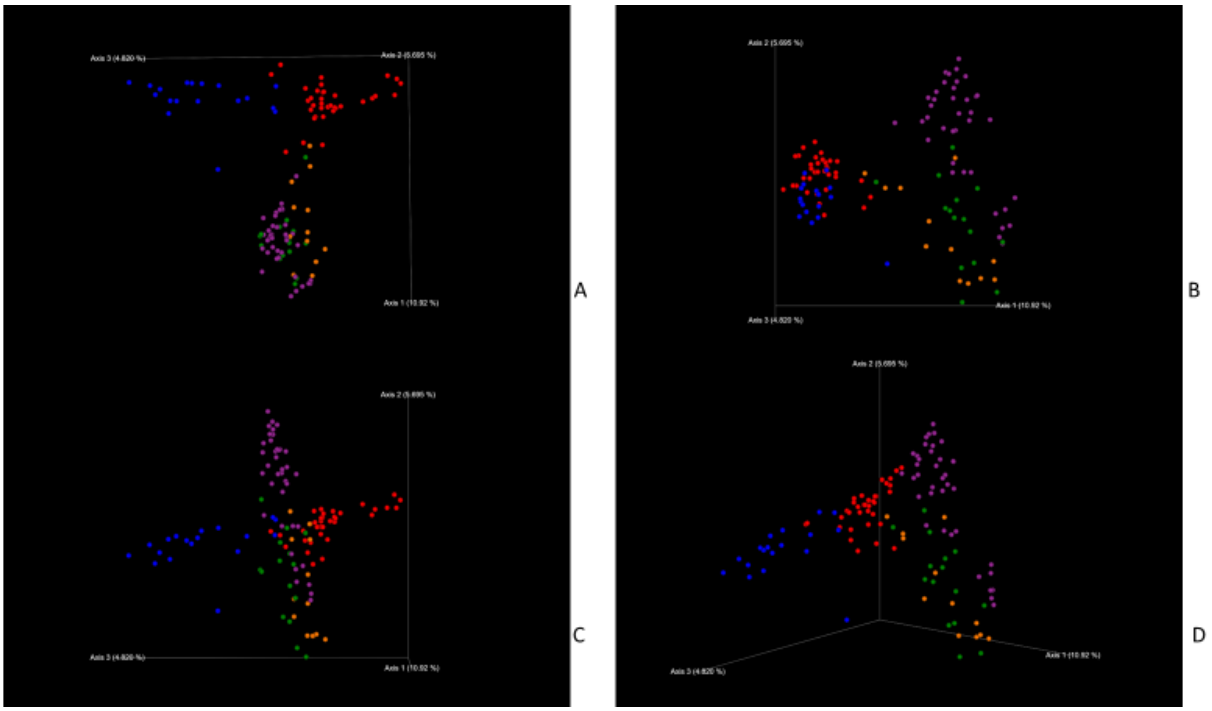

Figure 32: ITS Bray-Curtis Dissimilarity Emperor Plots Showing Location. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. For location we see no patterns formed. We see two distinct structures in these plots visible in figures J, K, and L. There is no pattern when visualizing nativity. For location, we see very clear grouping with the FHSU sites mixing. There is some mild bleed over between sites but eastern sites don't appear in western clusters and vice versa.

Red: Konza Prairie

Blue: KU Field Station

Orange: FHSU Relict Area

Green: FHSU Sheep Pasture

Purple: Smoky Valley Ranch

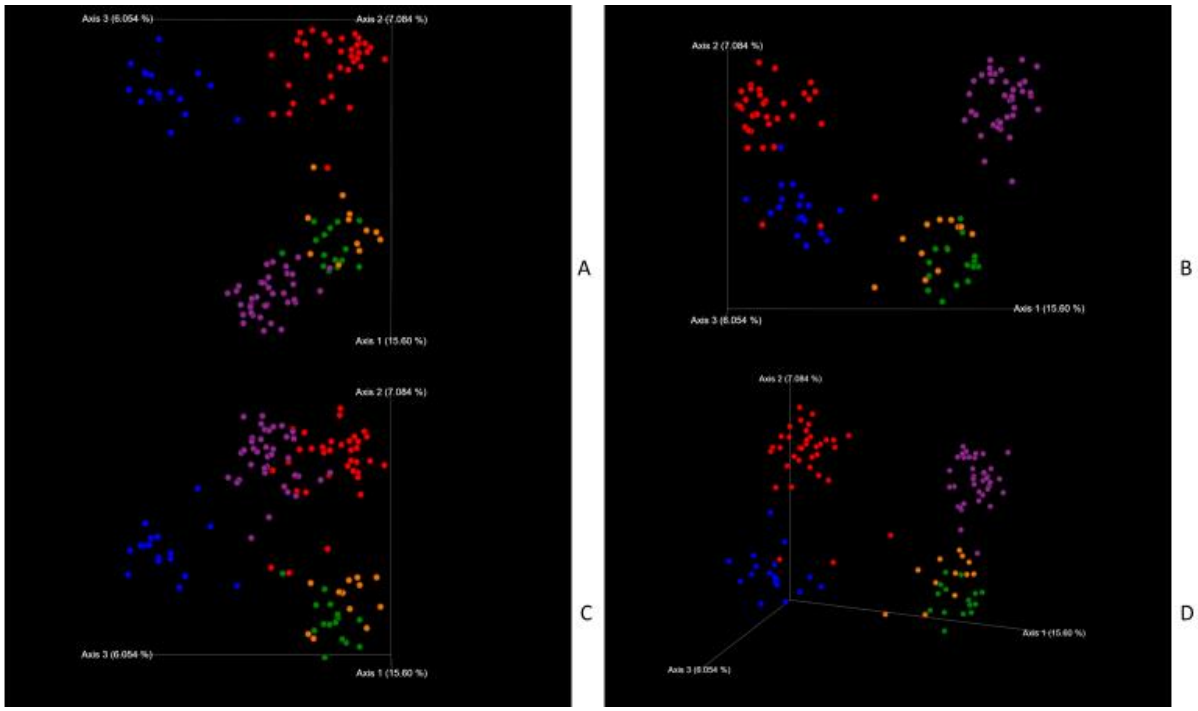

Figure 33: ITS Unweighted UniFrac Emperor Plots Showing Location. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. Here we see each of the four groups being related to specific locations with the FHSU sites mixing. There is no bleed over between sites that are in different counties.

Red: Konza Prairie

Blue: KU Field Station

Orange: FHSU Relict Area

Green: FHSU Sheep Pasture

Purple: Smoky Valley Ranch

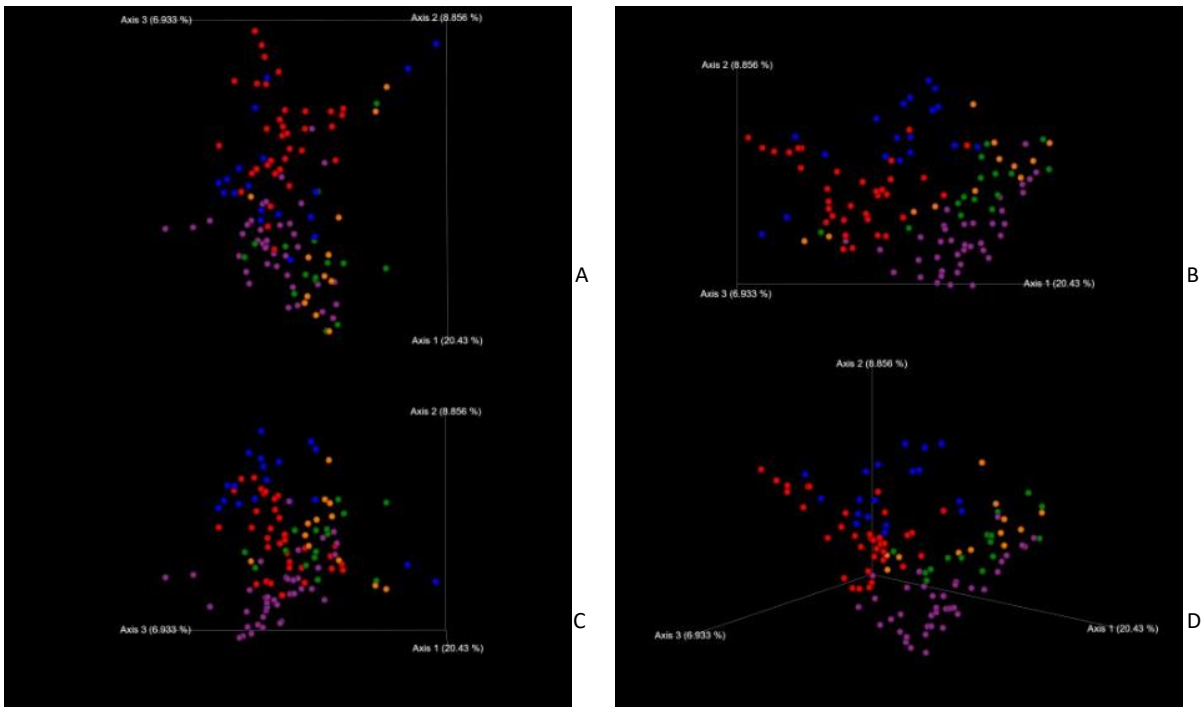

Figure 31: ITS Weighted UniFrac Emperor Plots Showing Location. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. For location, we see distinct clustering with the FHSU sites mixing. The separation of clustering is weaker here than in the other diversity metrics with some bleed through, but the groups are still distinct. In figure C we see a potential horseshoe, however if present it is very weak.

Red: Konza Prairie

Blue: KU Field Station

Orange: FHSU Relict Area

Green: FHSU Sheep Pasture

Purple: Smoky Valley Ranch

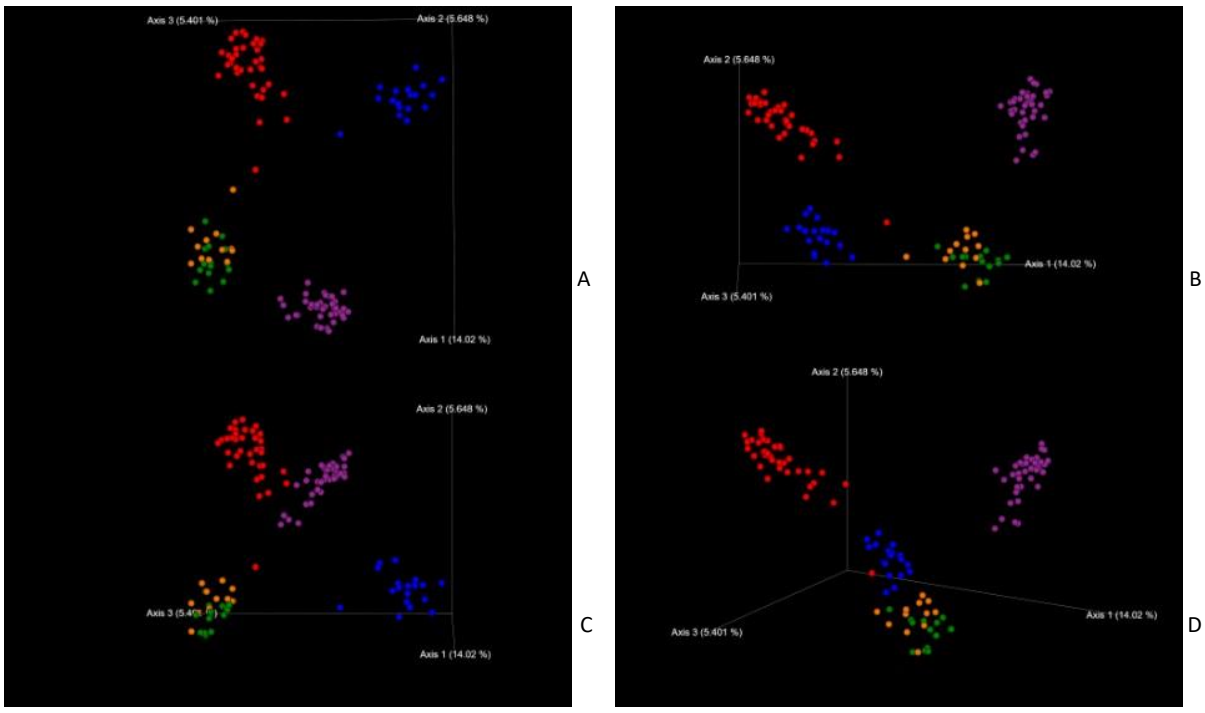

Figure 34: ITS Jaccard Distance Emperor Plots Showing Location. (A) emperor plot viewing axis 1 & 2. (B) emperor plot viewing axis 2 & 3. (C) emperor plot viewing axis 1 & 3. (D) emperor plot with an overview of all axis. Visualizing location we see those four groups are made up entirely of specific location except for the FHSU sites being mixed. Very clear separation is seen with no bleed over.

Red: Konza Prairie

Blue: KU Field Station

Orange: FHSU Relict Area

Green: FHSU Sheep Pasture

Purple: Smoky Valley Ranch

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