ANALYSES AND COMPARISONS OF BACTERIAL COMMUNITIES WITH DIFFERENT LAND MANAGEMENTS

by

RIMA ANN UPCHURCH

(Under the Direction of William B. Whitman)

ABSTRACT

16S rRNA gene libraries were constructed to evaluate the effects of natural and human disturbances on soil communities. The first study compared bacterial communities from agricultural and forest soils in the Southeast United States. Agricultural soils included conventional till or no-till regimes. Results showed that tillage regimes affect community structure, with conventional till populations being a subset of no-till. Forest soils from abandoned pasture communities were similar to nearby old-growth forest. Both forest communities differed from those of the agricultural plots. Bacterial communities from geographically distant forest soils at a second site resembled old-growth forest, but not the successional forest. Therefore, the impact of agriculture may be long lasting on bacterial populations.

A second study compared the bacterial communities of soils associated with a rice-fallow rotation system. Community comparisons significantly differed across all treatments, showing both the flooded rice and pasture to be a subset of the never-tilled control soil.

KEY WORDS: 16S rRNA, LIBSHUFF, Agriculture, Rice paddy, Bacterial diversity

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RIMA ANN UPCHURCH

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ANALYSIS AND COMPARISONS OF BACTERIAL COMMUNITIES WITHIN SOILS UNDER DIFFERING LAND MANAGEMENT PRACTICES USING 16S rRNA GENE LIBRARIES

by

RIMA ANN UPCHURCH

Major Professor:	William B. Whitman
Committee:	Mary Ann Moran Joy Peterson

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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CHAPTER

I.INTRODUCTION AND LITERATURE REVIEW

1. General introduction

Prokaryotes have had billions of years to adapt and evolve to almost every habitat on the planet. Analysis of prokaryotic diversity has traditionally consisted of cultivating microorganisms from the environment, but this technique is limited because of the inability of a wide range of prokaryotes to grow in the laboratory (Torsvik and Ovreas, 2002; Torsvik et al., 2002). Worldwide bacterial species estimates far exceed that of any other kingdom, with as many as half a million species found within 30 grams of soil using reassociation kinetics at less than 70% DNA reassociation to define a species (Dykhuizen, 1998). Estimates of worldwide numbers have shown that as many as 4-6 X 10^{30} prokaryotes inhabit the earth with the majority of these within the soil (Whitman et al., 1998). Considering these numbers, soil prokaryotes may represent a substantial fraction of the diversity on the planet (Magurran, 2005; Hughes et al., 2001; Gotelli and Colwell, 2001; Hill et al., 2003).

Soil microbial communities, with their vast amount of metabolic and genetic diversity, are often difficult to characterize (Ovreas and Torsvik, 1998). Even within a soil, there are variations in communities through depth (Ovreas and Torsvik, 1998). Microbial communities significantly impact the soil because they perform essential processes, such as nutrient cycling, soil formation and decomposition (Torsvik and Ovreas, 2002; Whitman et al., 1998; Borneman et al., 1997; Balota et al., 2003). Therefore, understanding prokaryotic communities and diversity is central to discerning how ecological processes function in the soil and what mechanisms exist to control this diversity (Torsvik et al., 2002; Hughes et al., 2001).

1.1 16S rRNA gene libraries

The now widespread use of molecular techniques to study environmental samples (marine, freshwater, soil, etc.) has led to new understanding about prokaryotic diversity (Singleton et al., 2001; von Wintzingerode et al., 1997). Using only plate culturing methods, less than 1% of the total bacterial population has been cultured (Ovreas and Torsvik, 1998). There have been improvements in culturing techniques since the realization that many of the abundant soil bacteria have few or no characterized isolates. Specifically, use of minimal growth substrates and longer incubation times greatly affects the amount of bacteria that can be isolated from soil (Sait et al., 2002). However, molecular techniques, especially 16S rRNA gene libraries, allow microbiologists to study microbial communities without culturing biases and to gain valuable information about the compositional and functional diversity within an environment (McCaig et al., 1999).

Although clone libraries allow researchers to analyze communities without culturing, a number of factors influence the results of these studies. The first step in making clone libraries involves extracting DNA from the environment. A major concern is whether or not lysis is complete for all organisms (More et al., 1994). Many techniques, both chemical and physical, have been developed for extraction (freeze-thaw, bead-beating, lysozyme treatment), with comparisons revealing definite effects on results depending on extraction methods (Martin-laurent et al., 2001). Often a combination of physical and chemical methods is used to minimize this problem, especially bead-beating with sodium dodecyl sulfate (More et al., 1994; Furlong et al., 2002). The initial sample size of soil used for extraction, however, does not seem to have an effect on the community analysis (Ranjard et al., 2003).

Once extracted, the polymerase chain reaction (PCR) is used to amplify the 16S rRNA gene. This is a highly conserved gene which all living organisms must have to survive. There are many problems associated with using PCR in environmental samples (see von Wintzingerode et al. 1997 for a review). PCR may be inhibited by substances found in soil that were not removed during the extraction process (e.g. humic acids) (Wilson, 1997). Preferential annealing between primers also presents a concern for PCR studies.

Universal primers, such as 27F and 1392/1492R, are chosen to try and amplify all bacterial taxa (Hongoh et al., 2003). Recently their efficiency has been examined. Marchesi et al. (1998) saw a lower efficiency of these primers as compared to 63F-1387R. Hongoh et al. (2003), however, found that 63F introduced serious bias from primer mismatch. Primer mismatch may be suppressed by using lower annealing temperatures during PCR (Hongoh et al., 2003; Suzuki and Giovannoni, 1996; von Wintzingerode et al., 1997). Since these methods are used to estimate diversity in environments, the creation of PCR artifacts and chimeras remains important. PCR artifacts from *Taq* polymerase errors and chimeras increase the number of "rare" sequences found within libraries (Acinas et al., 2005; von Wintzingerode et al., 1997). The occurrence of these artificially rare sequences cannot be fully eliminated, but lowering the number of cycles during PCR significantly reduces this problem (Acinas et al., 2005). Programs have been developed to detect chimeras, such as CHIMERA_CHECK and Bellerophon (Maidak et al., 2001; Huber et al., 2004). Even so, these problems cannot be totally eliminated, so each sequence should be examined carefully before further analysis such as phylogenetic determinations, diversity measurements and library comparisons.

1.2 Abundant phyla within soil

Libraries constructed from 16S rRNA genes do not contain information about the function of different species within an environment, especially when there is not a close relative in cultivation (Janssen, 2006; Dojka et al., 2000; Rappe and Giovannoni , 2003) The phylogenetic composition of these libraries, however, provides a useful comparison tool (Janssen, 2006; Hughes et al., 2001; Rappe and Giovannoni , 2003). The taxonomy of 16S rRNA gene libraries is determined by comparing sequence data to nucleotide databases, such as GenBank or the Ribosomal Database Project (RDP), or by making phylogenetic trees with reference sequences. There is no standard for classifying bacteria into higher taxa, but frequently sequences with at least 75-80 % identity are assigned to the same phylum (Schloss and Handelsman, 2005; Borneman et al., 1997; Hugenholtz et al., 1998). As more bacteria are cultured from the environment, the 16S rRNA gene data begins to represent real organisms and therefore becomes more informative about how communities function and what role diversity plays in that function (Hughes et al., 2001).

At this time, however, some of the most abundant phyla lack more than a few cultured representatives. One study found that the dominant genera of soil bacteria in the American Type Culture Collection (ATCC) make up only 2.7-3.7 % of soil bacteria (Floyd et al., 2005; Janssen, 2006). A recent review by Janssen (2006) identified the dominant bacterial taxa from soil libraries. As indicated in Figure 1.1, nine phyla dominated the soil libraries. Overall the Proteobacteria make up an average of 39 % of soil library communities, about twice as many as any other phylum (Janssen, 2006). A large number of cultured representatives exist for this phylum. The large diversity in physiology of this group, however, means generalizations about their function are not possible (Janssen, 2006).

The Acidobacteria average about 20 % of soil libraries. Only three bacteria have been isolated for this phylum, so their function in the soil has not been determined (Janssen, 2006; Garrity et al., 2004). This phylum is widespread in terrestrial environments, and possibly contains the most genetically and metabolically diverse lineage of the domain Bacteria (Barns et al., 1999). The Actinobacteria make up an average of 13 % of the soil libraries. This group has many isolates in the databases with a large phenotypic diversity, many of which are known for their antibiotic production and role in litter decomposition (Basilio, 2003; Janssen, 2006; Dojka et al., 2000). Gremion et al. (2003) found that this phylum not only constituted an abundant part of the rDNA gene libraries, but it was also the major metabolically active phylum as seen though rRNA studies. The Verrucomicrobia comprise an average of 7 % of soil communities. This phylum is particularly abundant in soils and has few characterized isolates, several of which live with eukaryotes (Janssen, 2006; Wagner and Horn, 2006). The Bacteroidetes make up an average of 5 % of soil libraries. This group has many cultured bacteria from a range of habitats and displays a large phenotypic diversity (Gupta, 2004).

The remaining four phyla each make up 2-3 % of the soil communities. The Chloroflexi and Gemmatimonadetes lack many characterized isolates (Janssen, 2006; Zhang et al., 2003; Rappe and Giovannoni, 2003). The majority of Planctomycetes isolates are from aquatic sources, so these isolates may not represent the physiology of soil bacteria correctly (Janssen, 2006; Liesack and Stackebrandt, 1992). The Firmicutes contain both the genera Bacillus and Clostridia, generally considered soil bacteria, which encompass a variety of phenotypes (Janssen, 2006; Garrity et al., 2004). Both of these genera are spore-formers, which might be difficult to lyse and could affect how well-characterized they are in PCR-based studies (Janssen, 2006).

The number of bacterial phyla within all environments has greatly increased since 16S rRNA gene libraries have become a common practice (Hugenholtz et al., 1998). Candidate divisions based on clonal sequences have become a common occurrence with many sequences from soil libraries being unrelated to other phyla (Hugenholtz et al., 2003). While it is possible that some of these may be PCR artifacts or chimeras, more and more of these groups, once found, are seen in other libraries. This result suggests that they represent novel organisms.

2. Library analyses and comparisons

Once 16S rRNA gene libraries have been constructed, understanding the results may be difficult. A variety of techniques and tools have been developed to determine how bacterial communities are different and where those differences lie. Studying the taxa composition and diversity, measured using estimators and indices, are the two most common practices. In recent years computer programs have been developed that compare libraries, as well.

2.1 Defining a species, richness and evenness

Studies that use 16S rDNA broaden our view of biological diversity and the importance of microorganisms in the environment (Magurran, 2005; Hughes et al., 2001; Gotelli and Colwell, 2001; Hill et al., 2003; Curtis et al., 2002, Martin, 2002, Rodriguez-Valera, 2002). Diversity includes two elements: richness and evenness (Magurran, 2005; Hill et al., 2003; Hughes et al., 2001; Martin, 2002). In general terms, richness equals the number of species while evenness denotes the abundance of individual species within a community. Any operational taxonomic unit, or OTU, can be applied to any level of taxonomy, but commonly refers to species (Schloss and Handelsman, 2005; Hughes et al., 2001; Martin, 2002). In terms of 16S rRNA gene analyses, a unique sequence represents a species within the environment (Schloss and Handeslman, 2005; Hughes et al., 2001). The definition of uniqueness, however, varies

(Schloss and Handelsman, 2005; Hughes et al., 2001, Martin, 2002; Rodriguez-Valera, 2002). Electrophoresis and sequence similarity of 16S rRNA genes are the most commonly used techniques for determining OTU's. Similarity provides more precision than electrophoresis because sequences may share the same electrophoretic pattern but have more than 3 % difference or have a 3 % difference and not share the same pattern (Schloss and Handelsman, 2005; Dunbar et al., 1999; Felske et al., 1997). Frequently sequences with at least 97 % identity constitute an OTU (Schloss and Handelsman, 2005; Hughes et al., 2001; Borneman et al., 1997; Hugenholtz et al., 1998). This is not a strict rule, but rather a convention (Schloss and Handelsman, 2005). Manually assigning OTU's involves examining a distance matrix, which is time-consuming and can be confusing when complex interactions exist (Schloss and Handeslman, 2005). In recent years computer programs have arisen to quickly and reproducibly assign OTU's, such as FastGroup, EstimateS and DOTUR (Schloss and Handelsman, 2005). These programs also calculate various measures of richness and evenness based on how they determine OTU's.

2.2 Diversity calculations

Soil ecologists use many different calculations and indices to determine the values of both richness and evenness (see Hill 2003 for a review). These differ in the weight given to observable species or OTU's (*S*), number sampled (*N*) and unique/rare sequences (singletons). If coverage is low, S does not account for the total number of species within an environment (total richness). This is often the case with soil libraries with most studies showing incomplete coverage from sampling (Hughes et al., 2001; Schloss and Handelsman, 2005). This can be visualized by comparing rarefaction curves (Figure 1.2). Rarefaction curves contain information about the success of the sampling by comparing randomized accumulation curves (Hughes et al., 2001). As communities are fully sampled and OTU's become less rare, curves approach an

asymptote. Linear rarefaction curves, which soil studies frequently show at distances of 1-3 %, indicate that a site has been insufficiently sampled (Hughes et al., 2001; Schloss and Handelman, 2005). Richness estimators, such as Chao1, define a lower limit for the expected number OTU's in a fully sampled community (Hill et al., 2003, Magurran, 2005; Hughes et al., 2001). On the other hand, general richness indices, such as Margalef (D_{mg}) compare the *S* and *N* and may be useful for comparing relative diversity (Hughes et al., 2001; Hill et al., 2003). Evenness measurements are just as important as richness, but are often overlooked or not as well understood. For example, if two sites have the same *N* and *S* but one OTU represents an overwhelming majority in one community, then the other community would be considered more diverse (Magurran, 2005). Interestingly, the capacity for soils to recover from disturbance seems to be linked to its evenness (Magurran, 2005). Evenness indices give weight to either the rare or dominant OTU's. Two of the most commonly used calculations, the Shannon evenness index (*E*) and Simpson's index (*D*), represent each of these categories (Hill et al., 2003). The value of *E* depends on the value of the Shannon diversity and is discussed below. By summing the square of the proportion of OTU's, *D* yields the probability of two sequences being from the same species (Hill et al., 2003, Magurran, 2005). The advantage of utilizing *D* may depend on the importance or relevance of dominant species (Hill et al., 2003).

Probably the most widely used diversity measurement is the Shannon index, or *H'* (Hill et al., 2003; Magurran, 2005; Hughes et al., 2001). The prevalence of *H'* in community studies results from its combining both richness and evenness in its calculation (Hill et al., 2003, Magurran, 2005). Rare sequences, then, influence this measurement because with more OTU's the sum grows. The significance of *H'*, however, may be unclear because it does not represent a probability or number of OTU's. Furthermore, it is difficult to compare *H'* between sites or

libraries with differing values of *N* (Hill et al., 2003). The Shannon evenness index, defined as the ratio of *H'* to H_{max}, simplifies this problem. As *H'* approaches its maximum, the ratio grows closer to 1, indicating a large diversity. Therefore, even when *N* differs between libraries, the value of *E* still holds relevance for comparison between libraries.

2.3 Computer programs to compare libraries

One issue with 16S rRNA gene libraries is how to determine if there is a significant difference in their composition (Singleton et al., 2001). Contrasting the taxa present in libraries is commonly used. This can be done manually or using programs such as UniFrac or the RDP's Library Compare. Unfortunately, as mentioned above, classification into phylogenetic groups is not consistent between researchers, and the abundance of groups may be affected by PCR conditions. LIBSHUFF, a computer program, was the first to compare coverages between libraries independent of the phyla. The statistical method used by LIBSHUFF to analyze diversity in clone libraries starts by calculating the homologous and heterologous coverages, defining coverage as by Good, for increments of evolutionary distances (*D*) (Good, 1953, Singleton et al., 2001). Figure 1.3 shows these coverages, as determined by LIBSHUFF. The difference between these curves is calculated at each point using the Cramer-von Mises test (Good, 1953; Singleton et al.; Pettitt, 1982). LIBSHUFF then randomly assigns sequences to "new" libraries and redoes this comparison. The original and randomized values are then ranked, largest to smallest to give a p-value. The forward (X to Y) and reverse (Y to X) comparisons are not equivalent (Singleton et al., 2001; Furlong et al., 2002). One "run" of LIBSHUFF, then, is really two comparisons. Therefore, the experiment-wise error must be corrected for using another calculation. In Figure 1.3, the X to Y comparison shows similar libraries, while the reverse does not. This indicates that the X library is a subset of the Y library.

Schloss et al. (2004) found an error in LIBSHUFF that has since been corrected. This group also wrote a computer program, called ∫-LIBSHUFF, that computes the integral form of the Cramer-von Mises test statistic. Furthermore, it can do multiple comparisons at smaller increments of *D* than LIBSHUFF.

3. Molecular studies of bacterial communities associated with agriculture

Though recent molecular studies have shown the staggering diversity of soil bacteria, very little is understood about how communities respond to disturbances within their environment (Whitman et al., 1998; Buckley and Schmidt, 2003). Both natural and human disturbances on soil cause changes in microbial communities and can be long lasting (Degrood et al., 2005; Buckley and Schmidt, 2003; Mummey et al., 2002). These disturbances impact the community through physical and chemical changes in the soil (Wright and Coleman, 2002; Sohlenius, 1982; Greenberg and McNab, 1998; Ulanova, 2000). Specifically the mineralization of nutrients, lower water holding capacity, and aggregate destabilization affect the microbial biomass in soils (Bossio and Scow, 1995; Balota et al., 2003; Wienhold et al., 2004; Bossio et al., 2005; Haynes, 1999). Reversing these changes can be difficult, with lasting consequences such as erosion and lower biodiveristy (Bossio and Scow, 1995; Sanchez et al., 2003).

Natural disturbances include wind/storm damage and landslides, which affect the plant flora and topsoil (Degrood et al., 2005; Wright and Coleman, 2002). Human activities, such as timber harvesting, mining, and agriculture, influence the soil communities, as well (Kennedy, 1999; Tilman et al., 2001; Matson et al., 1997; Dunbar et al., 2002). Of all of these disturbances, agriculture exerts global influences on the soil and the bacteria that reside there (Ovreas and Torsvik, 1998).

3.1 Effects of tillage

Approximately 1/3 of the world's land area is used for agriculture (Tilman et al., 2001). Previous culture-independent studies comparing agricultural and natural soils showed differences between the structure of the communities (Borneman et al., 1997; Buckley and Schmidt, 2003; Nusslein and Tiedje, 1999). Agricultural management practices, such as tillage or crop rotations, affect the soil differently, as well. Tillage regimes vary in the impact on both physical and chemical properties in soil (Haynes, 1999; Balota et al., 2003). Conventional tillage (CT) uses plowing and disking for cultivation, while no-tillage (NT) uses direct seed drilling to minimize soil disturbance (Dominy and Haynes, 2002; Balota et al., 2003; Haynes, 1999). NT lessens the loss of soil organic matter (SOM), an important soil quality indicator and sustainability factor, associated with cultivating land (Dominy and Haynes, 2002; Balota et al., 2003; Haynes, 1999). Furthermore, microbial biomass and respiration was higher in NT (Balota et al., 2003; Dominy and Haynes, 2002). Few studies, however, have been done to analyze the effects of agriculture on the diversity of bacterial communities (Kennedy, 1999). Ovreas and Torsvik (1998) found that frequently tilled land exhibited a lower diversity in Biolog phenotypes and gradient gel banding patterns than untilled land. Crop-fallow rotations have been shown to lessen these effects on diversity and soil properties (Bossio et al., 2005). Specifically, continuously farmed land showed a lower SOM and microbial biomass than improved wooded land (Bossio et al., 2005). Crop-fallow systems, however, might not be as effective in offsetting damages from tillage as continuous farming with less rigorous cultivation methods (Wienhold and Halvorson, 1998; Wienhold et al., 2004).

The effects of agriculture on the soil are also long-lasting (Buckley and Schmidt, 2003). Buckley and Schmidt (2003) examined the microbial communities between conventionally tilled

(CT), successional for >9 and >45 years (HCS and LS), and never cultivated fields (NCS). The composition of the soil community in the NCS field differed from both the CT and HCS fields, while the communities between the CT and HCS remained similar. The LS site, however, resembled the NCS field but differed from both the CT and HCS fields. These results indicated that the effects of agriculture are long lasting, but these soils can be improved.

3.2 Flooded rice paddies

One especially harsh agricultural practice is flooded rice farming. Continuous rice farming, along with the general problems associated with agriculture, leads to soil compaction, lower levels of nitrogen, and an accumulation of SOM (Folgarait et al., 2003; Lal, 2000). In addition to these factors, the flooding itself influences the bacterial communities. The saturated soil causes an oxygen gradient to form with three sections: surface soil, bulk soil, and rhizosphere (Liesack et al., 2000). This gradient forms within a few hours of flooding, creating a shift in dominant members as the oxygen is consumed until it stabilizes about a month later (Liesack et al., 2000, Noll et al., 2005). Figure 1.4 shows a schematic of a cross-section through rice paddy soil. Oxygen enters the system through the surface/water interface, which is depleted quickly with microscale amounts of oxygen in the rhizoplane (Liesack et al., 2000). The bulk soil is anoxic with bacteria using alternate electron acceptors as dictated by redox potential (Liesack et al., 2000; Ponnamperuma, 1972). As seen in other anoxic environments, methane is the final product (Liesack et al., 2000; Chin et al., 1999).

References

Acinas, S.G., R. Sarma-Rupavtarm, V. Klepac-Ceraj and M.F. Polz. 2005. PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. Appl Environ Microbiol 71:8966-9.

Balota, E.L., A. Colozzi-Filho, D.S. Andrade and R.P. Dick. 2003. Microbial biomass in soils under different tillage and crop rotation systems. Biology and Fertility of Soils 38:15.

Barns, S.M., S.L. Takala and C.R. Kuske. 1999. Wide distribution and diversity of members of the bacterial kingdom Acidobacterium in the environment. Appl Environ Microbiol 65:1731-7.

Basilio, A. I. Gonzalez, M.F. Vicente, J. Gorrochategui, A. Cabello, A. Gonzalez and O. Genilloud. 2003. Patterns of antimicrobial activities from soil actinomycetes isolated under different conditions of pH and salinity. J Appl Microbiol 95: 814-823.

Borneman, J., and E.W. Triplett. 1997. Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl Environ Microbiol 63:2647-53.

Bossio, D.A., and K.M. Scow. 1995. Impact of Carbon and Flooding on the Metabolic Diversity of Microbial Communities in Soils. Appl Environ Microbiol 61:4043-4050.

Bossio, D.A., M.S. Girvan, L. Verchot, J. Bullimore, T. Borelli, A. Albrecht, K.M. Scow, A.S. Ball, J.N. Pretty and A.M. Osborn. 2005. Soil microbial community response to land use change in an agricultural landscape of western Kenya. Microb Ecol 49:50-62.

Buckley, D.H., and T.M. Schmidt. 2003. Diversity and dynamics of microbial communities in soils from agro-ecosystems. Environ Microbiol 5:441-52.

Chin, K.J., D. Hahn, U. Hengstmann, W. Liesack and P.H. Janssen. 1999. Characterization and identification of numerically abundant culturable bacteria from the anoxic bulk soil of rice paddy microcosms. Appl Environ Microbiol 65:5042-9.

Curtis, T.P., W.T. Sloan and J.W. Scannell. 2002. Estimating prokaryotic diversity and its limits. Proc Natl Acad Sci U S A 99:10494-9.

DeGrood, S.H., V.P. Claassen and K.M. Scow. 2005. Microbial community composition on native and drastically disturbed serpentine soils. Soil Biology and Biochemistry 37:1427.

Dojka, M.A., J.K. Harris and N.R. Pace. 2000. Expanding the known diveristy and environmental distribution of an uncultured phylogenetic division of bacteria. Appl Environ Microbiol 66:1617-1621.

Dominy, C.S., and R.J. Haynes. 2002. Influence of agricultural land management on organic matter content, microbial activity and aggregate stability in the profiles of two Oxisols. Biology and Fertility of Soils 36:298.

Dunbar, J., S.M. Barns, L.O. Ticknor and C.R. Kuske. 2002. Empirical and theoretical bacterial diversity in four Arizona soils. Appl Environ Microbiol 68:3035-45.

Dunbar, J., S. Takala, S.M. Barns, J.A. Davis and C.R. Kuske. 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Appl Environ Microbiol 65:1662-9.

Dykhuizen, D. E., 1998. Santa Rosalia revisited:Why are there so many species of bacteria?. Antonie van Leeuwenhoek. **73:** 25–33.

Felske, A., H. Rheims, A. Wolterink, E. Stackebrandt and A. Akkermans. 1997. Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils. Microbiology 143:2983-2989.

Floyd, M.M., J. Tang, M. Kane and D. Emerson. 2005. Captured diversity in a culture collection: case study of the geographic and habitat distributions of environmental isolates held at the american type culture collection. Appl Environ Microbiol 71:2813-23.

Folgarait, P., F. Thomas, T. Desjardins, M. Grimaldi, I. Tayasu, P. Curmi and P. Lavelle. 2003. Soil properties and the macrofauna community in abandoned irrigated rice fields of northeastern Argentina. Biology and Fertility of Soils 38:349.

Furlong, M.A., D.R. Singleton, D.C. Coleman and W.B. Whitman. 2002. Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and the burrows and casts of the earthworm Lumbricus rubellus. Appl Environ Microbiol 68:1265-79.

Garrity, G. M., J.A. Bell and T. G. Lilburn. 2004. Taxonomic outline of the prokaryotes, release 5.0. Springer-Verlag, New York, N.Y.

Good, I.J. 1953. The population frequencies of species and the estimation of population parameters. Biometrika 40:237-264.

Gotelli, N.J., and R.K. Colwell. 2001. Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. Ecol Lett 4:379-391.

Greenberg, C.H., and W.H. McNab. 1998. Forest disturbance in hurricane-related downbursts in the Appalachian mountains of North Carolina. For. Ecol. Manag. 104, 179-191.

Gremion, F., A. Chatzinotas and H. Harms. 2003. Comparative 16S rDNA and 16S rRNA sequence analysis indicates that Actinobacteria might be a dominant part of the metabolically active bacteria in heavy metal-contaminated bulk and rhizosphere soil. Environ Microbiol 5:896-907.

Gupta, R.S. 2004. The phylogeny and signature sequences characteristics of Fibrobacteres, Chlorobi and Bacteroidetes. Crit Rev Microbiol 30:123-43.

Haynes, R.J. 1999. Size and activity of the soil microbial biomass under grass and arable management. Biology and Fertility of Soils 30:210.

Hill, T.C.J., K.A. Walsh, J.A. Harris and B.F. Moffett. 2003. Using ecological diversity measures with bacterial communities. FEMS Microbiology Ecology 43:1.

Hongoh, Y., H. Yuzawa, M. Ohkuma and T. Kudo. 2003. Evaluation of primers and PCR conditions for the analysis of 16S rRNA genes from a natural environment. FEMS Microbiol Lett 221:299-304.

Huber, T., G. Faulkner and P. Hugenholtz. 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics 20:2317-9.

Hugenholtz, P., B.M. Goebel and N.R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J Bacteriol 180:4765-74.

Hugenholtzt, P., and T. Huber. 2003. Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. Int J Syst Evol Microbiol 53:289-93.

Hughes, J.B., J.J. Hellmann, T.H. Ricketts and B.J. Bohannan. 2001. Counting the uncountable: statistical approaches to estimating microbial diversity. Appl Environ Microbiol 67:4399-406.

Janssen, P.H. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl Environ Microbiol 72:1719-28.

Kennedy, A.C. 1999. Bacterial diversity in agroecosystems. Agriculture, Ecosystems & Environment 74:65.

Lal, R. 2000. Physical management of soils of the tropics: priorities for the 21st century. Soil Science 165:191-207.

Liesack, W., and E. Stackebrandt. 1992. Occurrence of novel groups of the domain Bacteria as revealed by analysis of genetic material isolated from an Australian terrestrial environment. J Bacteriol 174:5072-5078.

Liesack, W., S. Schnell and N.P. Revsbech. 2000. Microbiology of flooded rice paddies. FEMS Microbiol Rev 24:625-45.

McCaig, A.E., L.A. Glover and J.I. Prosser. 1999. Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. Appl Environ Microbiol 65:1721-30.

Magurran, A.E. 2005. Biological diversity. Curr Biol 15:R116-8.

Maidak, B.L., J.R. Cole, T.G. Lilburn, C.T. Parker, Jr., P.R. Saxman, R.J. Farris, G.M. Garrity, G.J. Olsen, T.M. Schmidt and J.M. Tiedje. 2001. The RDP-II (Ribosomal Database Project). Nucleic Acids Res 29:173-4.

Marchesi, J.R., T. Sato, A.J. Weightman, T.A. Martin, J.C. Fry, S.J. Hiom, D. Dymock and W.G. Wade. 1998. Design and Evaluation of Useful Bacterium-Specific PCR Primers That Amplify Genes Coding for Bacterial 16S rRNA. Appl Environ Microbiol 64:2333.

Martin, A.P. 2002. Phylogenetic approaches for describing and comparing the diversity of microbial communities. Appl Environ Microbiol 68:3673-82.

Martin-Laurent, F., L. Philippot, S. Hallet, R. Chaussod, J.C. Germon, G. Soulas and G. Catroux. 2001. DNA extraction from soils: old bias for new microbial diversity analysis methods. Appl Environ Microbiol 67:2354-9.

Matson, P.A., W.J. Parton, A.G. Power and M.J. Swift. 1997. Agricultural intensification and ecosystem properties. Science 277, 504-508.

More, M., J. Herrick, M. Silva, W. Ghiorse and E. Madsen. 1994. Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. Appl Environ Microbiol 60:1572-1580.

Mummey, D.L., P. Stahl and J. Buyer. 2002. Soil microbiological properties 20 years after surface mine reclamation: spatial analysis of reclaimed and undisturbed sites. Soil Biology and Biochemistry. 34:1717-1725.

Noll, M., D. Matthies, P. Frenzel, M. Derakshani and W. Liesack. 2005. Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. Environ Microbiol 7:382-95.

Nusslein, K., and J.M. Tiedje. 1999. Soil bacterial community shift correlated with change from forest to pasture vegetation in a tropical soil. Appl Environ Microbiol 65:3622-6.

Ovreas, L., and V. Torsvik. 1998. Microbial Diversity and Community Structure in Two Different Agricultural Soil Communities. Microb Ecol 36:303-315.

Pettitt, A.N. 1982. Cramer von mise test statistic Wiley-Interscience, New York, N.Y.

Ponnamperuma, F.N. 1972. The chemistry of submerged soils. Adv Agron 24:29-96.

Ranjard, L., D.P. Lejon, C. Mougel, L. Schehrer, D. Merdinoglu and R. Chaussod. 2003. Sampling strategy in molecular microbial ecology: influence of soil sample size on DNA fingerprinting analysis of fungal and bacterial communities. Environ Microbiol 5:1111-20.

Rappe, M., and S. Giovannoni. 2003. The uncultured microbial majority. Annu Rev Microbiol 57:369-394.

Rodriguez-Valera, F. 2002. Approaches to prokaryotic biodiversity: a population genetics perspective. Environ Microbiol 4:628-33.

Sait, M., P. Hugenholtz and P.H. Janssen. 2002. Cultivation of globally distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. Environ Microbiol 4:654-66.

Sanchez, P., C. Palm and S. Buol. Fertility capability soil classification: a tool to help assess soil quality in the tropics. Geoderma. 114:157-185.

Schloss, P.D., and J. Handelsman. 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl Environ Microbiol 71:1501-6.

Schloss, P.D., B.R. Larget and J. Handelsman. 2004. Integration of microbial ecology and statistics: a test to compare gene libraries. Appl Environ Microbiol 70:5485-92.

Singleton, D.R., M.A. Furlong, S.L. Rathbun and W.B. Whitman. 2001. Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. Appl Environ Microbiol 67:4374-6.

Sohlenius, B., 1982. Short-term influence of clear-cutting on abundance of soil-microfauna (Nematoda: Rotatoria and Tardigrada) in a Swedish pine forest soil. J. Appl. Ecol. 19, 349-359.

Suzuki, M.T., and S.J. Giovannoni. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl Environ Microbiol 62:625-30.

Tilman, D., J. Fargione, B. Wolff, C. D'Antonio, A. Dobson, R. Howarth, D. Schindler, W.H. Schlesinger, D. Simberloff and D. Swackhamer. 2001. Forecasting agriculturally driven global environmental change. Science 292:281-4.

Torsvik, V., and L. Ovreas. 2002. Microbial diversity and function in soil: from genes to ecosystems. Curr Opin Microbiol 5:240-5.

Torsvik, V., L. Ovreas and T.F. Thingstad. 2002. Prokaryotic diversity--magnitude, dynamics, and controlling factors. Science 296:1064-6.

Ulanova, N.G., 2000. The effects of windthrow of forest at different spatial scales: a review. For. Ecol. Manag. 135, 155-167.

von Wintzingerode, F., U.B. Gobel and E. Stackebrandt. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev 21:213-29.

Wagner, M., and M. Horn. 2006. The Planctomycetes, Verrucomicrobia, Chlamydiae and sister phyla comprise a superphylum with biotechnological and medical relevance. Curr Opin Biotechnol 17:241-9.

Whitman, W.B., D.C. Coleman and W.J. Wiebe. 1998. Prokaryotes: the unseen majority. Proc Natl Acad Sci U S A 95:6578-83.

Wienhold, B.J., and A.D. Halvorson. 1998. Cropping system influences on several soil quality attributes in the northern Great Plains. J Soil Water Conserv 53:254-258.

Wienhold, B.J., S.S. Andrews and D.L. Karlen. 2004. Soil quality: a review of the science and experiences in the USA. Environ Geochem Health 26:89-95.

Wilson, I.G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol 63:3741-51.

Zhang, H., Y. Sekiguchi, S. Hanada, P. Hugenholtz, H. Kim, Y. Kamagata and K. Nakamura. 2003. Gemmatimonas aurantiaca gen. nov., sp. nov., a gram-negative, aerobic, polyphosphate-accumulating micro-organism, the first cultured representative of the new bacterial phylum Gemmatimonadetes phyl. nov. Int J Syst Evol Microbiol 53:1155-63.

Figure 1.1
Contributions of 16S rRNA and 16S rRNA genes from members of different phyla in libraries prepared from soil bacterial communities (2,920 clones in 21 libraries). The horizontal line in the middle of each block indicates the mean, the block represents 1 standard deviation on either side of the mean, and the vertical lines extending above and below each block indicate the minimum and maximum contributions of each phylum (Reproduced from Janssen, 2006).

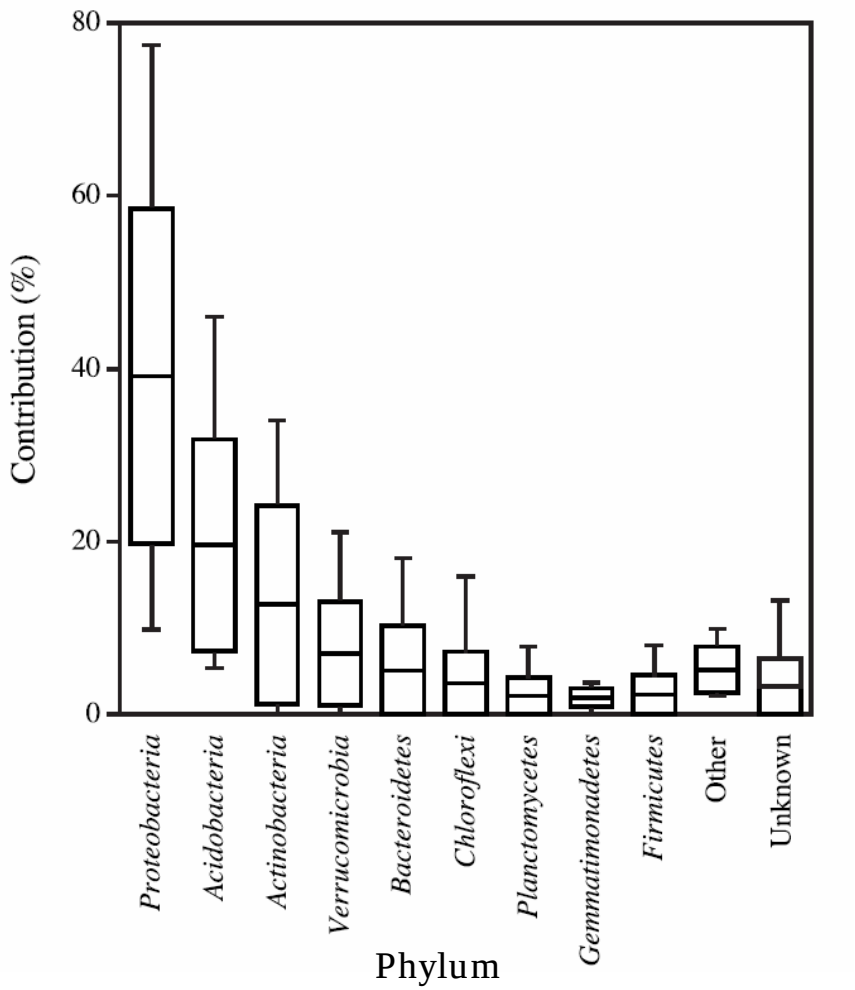

80
60
40
20
0
Contribution (%)
Proteobacteria
Acidobacteria
Actinobacteria
Verrucomicrobia
Bacteroidetes
Chloroflexi
Planctomycetes
Gemmatimonadetes
Firmicutes
Other
Unknown
Phylum

Figure 1.2
Rarefaction curves of observed OTU richness in human mouth (○) and gut (●) bacterial samples. The error bars are 95% confidence intervals (CI's) and were calculated from the variance of the number of OTU's drawn in 100 randomizations at each sample site (Reproduced from Hughes et al., 2001).

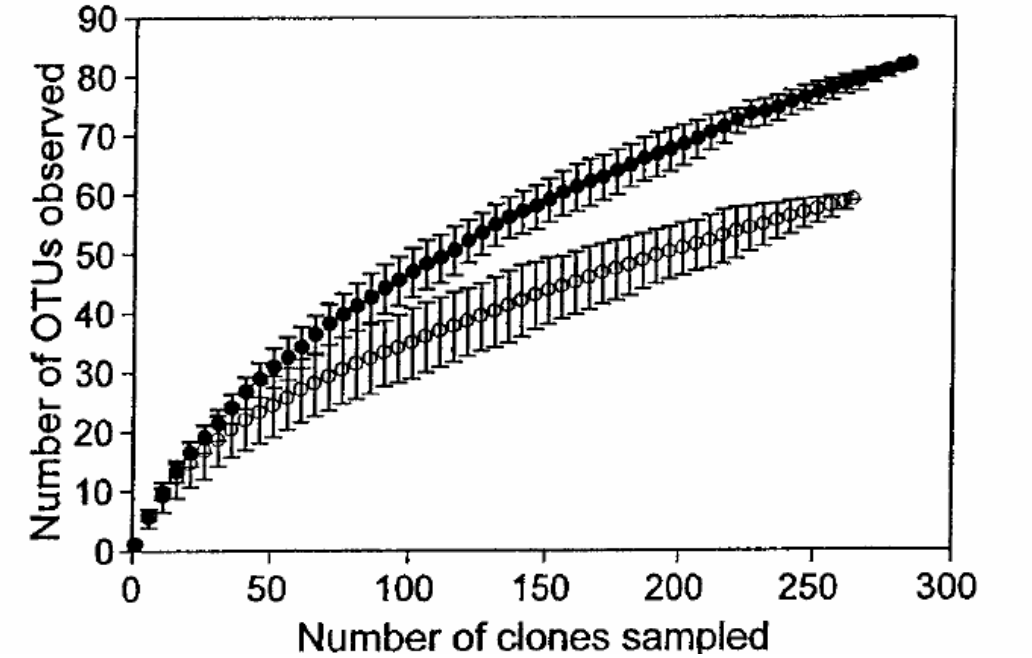

Number of OTUs observed
0
10
20
30
40
50
60
70
80
90
0
50
100
150
200
250
300
Number of clones sampled

Figure 1.3
LIBSHUFF comparison of soil and cast clones. Homologous coverage curves are shown with open circles (○) and heterologous coverage curves with closed circles (●). Solid lines indicate the difference between the homologous and heterologous coverage curves at each value of *D* as determined by the Cramér-von Mises test statistic, and broken lines denote the 95% value of the random shuffles. (A) Comparison of the cast clones (X) to the soil clones (Y); (B) comparison of the soil clones (X) to the cast clones (Y) (Reproduced from Furlong et al., 2002).

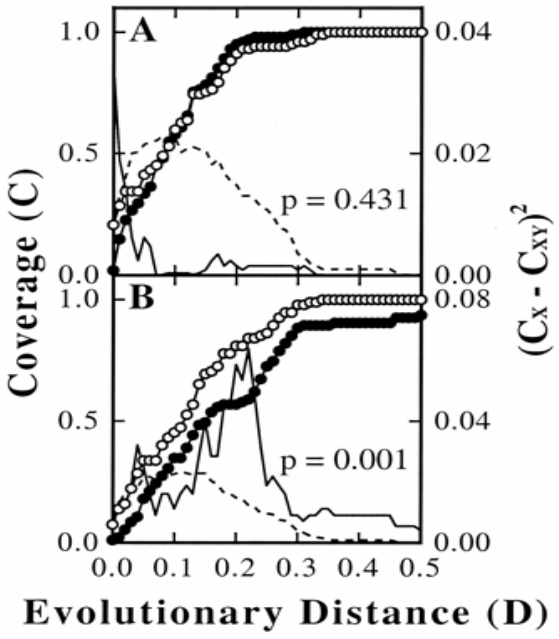
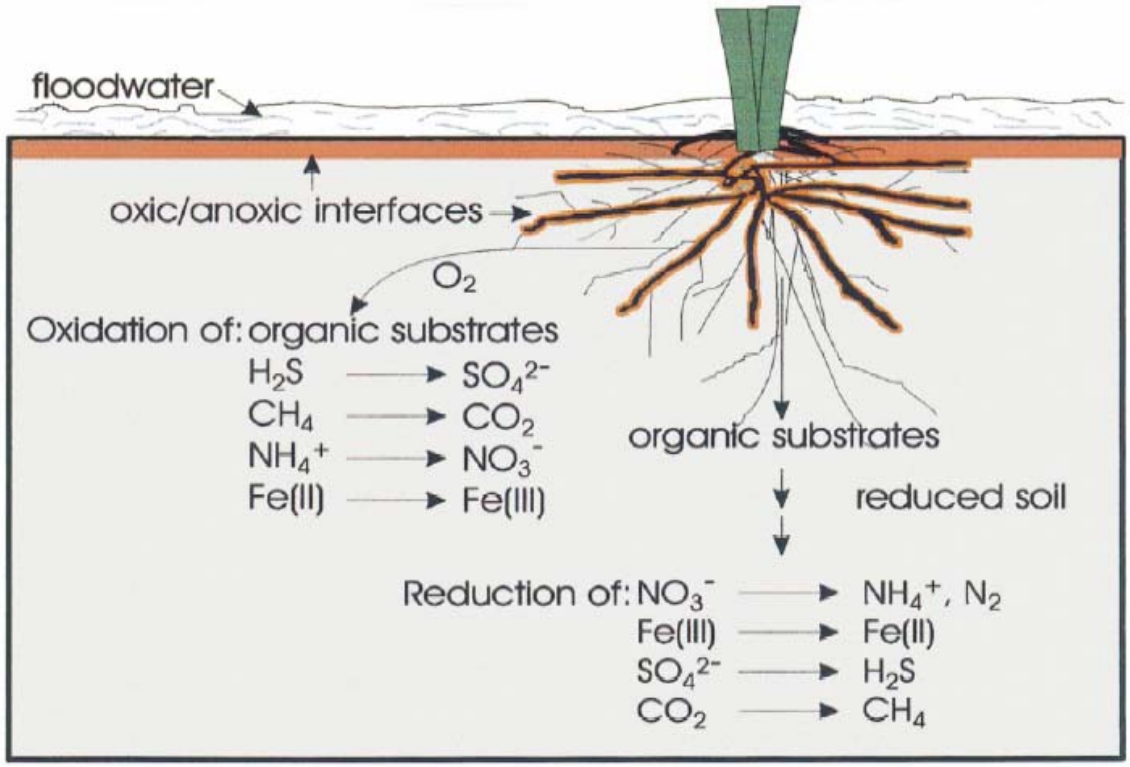

A
B
p = 0.431
p = 0.001
Coverage (C)
$(C_X - C_{XY})^2$
Evolutionary Distance (D)
1.0
0.5
0.0
0.04
0.02
0.00
0.08
0.04
0.00
0.0 0.1 0.2 0.3 0.4 0.5

Figure 1.4
Schematic cross-section through the compartmentalized rice paddy soil. Distinct compartments: oxic/anoxic interfaces (uppermost mm of the surface soil and the narrow regions around the root system) versus anoxic bulk soil (reduced soil). Redox reactions characteristic of oxic and anoxic zones, respectively, are shown. (Reproduced from Liesack et al., 2000)


floodwater
oxic/anoxic interfaces
O_2
Oxidation of: organic substrates
$H_2S \rightarrow SO_4^{2-}$
$CH_4 \rightarrow CO_2$
$NH_4^+ \rightarrow NO_3^-$
Fe(II) → Fe(III)
organic substrates
reduced soil
Reduction of: $NO_3^- \rightarrow NH_4^+, N_2$
Fe(III) → Fe(II)
$SO_4^{2-} \rightarrow H_2S$
$CO_2 \rightarrow CH_4$

II. MOLECULAR ANALYSES OF PROKARYOTIC COMMUNITIES FROM AGRICULTURAL AND FOREST SOILS[1]

[1]Upchurch, R., Chih-Yu Chiu, Karin Everett, Glen Dyszynski, David C Coleman, William B Whitman. *To be submitted* Soil Biology and Biochemistry.

Abstract:

The effects of disturbance and land management on the bacterial communities in soil were studied using 16S rRNA gene libraries from conventional till (CT) and no-till (NT) land, successional (new) forest (NF), and old forest (OF) at the Horseshoe Bend (HSB) research site in Athens, Georgia. The NF and OF were compared to the communities of forested lands in western North Carolina at the Coweeta (CWT) Long Term Ecological Research Site. The Proteobacteria and Actinobacteria were the most abundant groups within in the total clone data. LIBSHUFF analysis revealed that the CT and NT communities significantly differed from each other as well as from both the forest communities at HSB, while the NF and OF communities were similar to one another. The agricultural soil communities, however, were subsets of the NF community but not the OF community. When compared to the CWT communities 160 km away, the OF community appeared similar but the NF did not. The NF plot represented a transitional phase in the soil regimes and properties, with the effects of agriculture on the NF bacterial community still discernible after 30 years.

Key Words: Community structure, Phylogenetic, 16S rRNA, LIBSHUFF

1. Introduction

The effects and longevity of disturbances on soil prokaryotic diversity have not been well documented (Kennedy, 1999; McCaig et al., 2001). Increases in agriculturally utilized land, up to 1/3 of the total land area on earth, have caused increases in erosion, losses of soil organic matter, and changes within prokaryotic communities in the soil (Tilman et al., 2001; Matson et al., 1997). In forest soils, timber harvesting and damage caused by wind are another set of common disturbances (Wright and Coleman, 2002).

Soil disturbances caused by natural or human activities have direct impacts on ecosystem properties and function, such as nutrient cycling and physical and chemical complexity (Wright and Coleman, 2002; Sohlenius, 1982; Greenberg and McNab, 1998; Ulanova, 2000). Soil, one of the largest reservoirs for prokaryotes, and its processes are greatly influenced by bacterial community structure, activity, and stability (McCaig et al., 2001; Whitman et al.., 1998; Dunbar et al., 2002).

Analysis of prokaryotic communities and diversity in soil has traditionally consisted of cultivating microorganisms from the environment. This technique is limited because of the inability of a wide range of prokaryotes to be efficiently cultured in the laboratory (Torsvik and Ovreas, 2002; Torsvik et al., 2002). The use of molecular, culture-independent based techniques has led to a new understanding of prokaryotic diversity (for a review see von Wintzingerode, 1997). A commonly used molecular method to study communities utilizes 16S rRNA gene clone libraries constructed from DNA extracted from whole soil communities (Dunbar et al., 2002). Libraries have the potential to determine the composition and diversity within soil microbial environments, which is essential to understanding the role of these communities and their effects on ecosystem processes (Dunbar et al., 2002; McCaig et al., 2001). In this study, the effects of

various human and natural disturbances was studied on agricultural and forest soil from two research sites, Horseshoe Bend in northern Georgia and Coweeta Hydrologic Laboratory in western North Carolina. The effects of different tillage regimes, namely conventional deep moldboard plowing and no-tillage agriculture were compared to the effects of disturbance by previous agricultural practices, hurricane wind damage, and canopy damage from cutting on forest soils.

2. Materials and Methods

2.1 *Sample site description*

2.1.1 *Horseshoe Bend Site*

The Horseshoe Bend (HSB) research site, located in Athens, Georgia on the floodplain of the Oconee River, consisted of a sandy loam (typic Kanhapludult) soil (Furlong et al., 2002). Winter cover crops consisted of rye (*Secale cereale* L.), and crimson clover (*Trifolium pratense* L.), followed by summer crops of maize (*Zea mays* L.) and since 1998, cotton (*Gossypium hirsutum* L.) (Hendrix et al., 2001). Annual precipitation was 1010 mm. (30 y average). Soil organic matter ranged from 0.7-1.0 % in CT and 1.7-2.1 % in NT. The pH in the upper horizons averaged 5.7.

HSB samples were taken from four locations. Two of them were within eight 0.1 ha. field plots that have been agriculturally managed using deep moldboard plowing followed by disking or conventional tillage (CT) and plots managed by direct seed drilling or no-till agriculture (NT). A third location, called new forest or NF, denoted a transect 10 meters from the edge of the CT and NT plots in an area of successional forest. This forest developed from pasture that was abandoned in 1973 (Odum et al., 1973). Old forest, or OF, refered to a strip of land adjacent to the river and within 100 m of the NF that was forested since before 1938. Both new and old

forests are dominated by water oak (*Quercus nigra*) and sweetgum (*Liquidambar styraciflua* L.). Three cores at each HSB location were collected on March 28, 2002. Libraries were constructed using the 5-10 cm layer of each soil core. These libraries were labeled NT5, CT5, NF5 and OF5 depending on the location. At the locations NT and OF, libraries were also constructed from the 2-5 cm layer and labeled NT2 and OF2, respectively.

2.1.2 *Coweeta LTER Site*

The Coweeta Hydrologic Laboratory (CWT) is a Long Term Ecological Research site (LTER) located in Otto, North Carolina in the Southern Appalachians. It consisted of Ultisol soils at lower elevations (760 m) with silty loam texture, Typic Hapuldult (near transects) and Inceptisols, Typic Haplumbrept at higher elevations (Maxwell and Coleman, 1995). The pH in the upper horizons averaged 5.7. Samples were taken from three plots. Cut (CU) and Storm (ST) were in the chestnut oak (*Quercus prinus* L.) and tulip poplar (*Liriodendron tulipifera* L.) dominated riparian forest with a rosebay rhododendron (*Rhododendron maximum* L.) understory that comprised Watershed 55. In the CU plot, stems were cut in 1995 (Maxwell and Coleman, 1995; Wright and Coleman, 2002). The ST plot was damaged by Hurricane Opal in 1995, which knocked down most of the overstory (Wright and Coleman, 2002). Summit (SM) was at 1340 m elevation and dominated by northern hardwoods (*Betula alleghaniensis* and *Quercus rubra*) (Knoepp et al., 2000). Three CWT samples were collected at each location by coring on April 20, 2002. For these samples soils from the 0-10 cm depths were utilized.

2.2 *DNA extraction and preparation for 16S rRNA gene libraries*

Soils from sections of each core were mixed, and large roots, rocks, and animals were removed. Total environmental DNA was extracted from 1 g of soil using an UltraClean Soil DNA Extraction kit (MoBio Industries). 16S rRNA genes were amplified using low-cycle PCR.

Each reaction consisted of 1 Ready-To-Go bead (Amersham Biosciences), 1 µl of DNA (about 10-100 ng), 2 µl of 27F primer (10µmol) [5'-AGA GTT TGA TCM TGG CTC AG-3'], 2 µl of 1492R primer (10µmol) [5'-ACG GYT ACC TTG TTA CGA CTT-3'], and enough dH_2O to bring the total volume to 25 µl. Each PCR included 1 cycle of 95 ^oC for 5 min followed by 15 cycles of 95 ^oC for 1 min, 62 ^oC for 1 min, and 72 ^oC for 2 min with a final cycle of 72 ^oC for 4 min on a Mastercycler Gradient (Eppendorf).

PCR products were immediately cloned using a TOPO TA cloning kit (Invitrogen) using the pCR2.1 vector. Plates were incubated overnight at 37 ^oC and then 24 hours at 4 ^oC. Well-isolated white colonies were picked into 96-well blocks with Luria-Bertani broth and kanomycin and grown overnight. Sterile glycerol was added to a final concentration of 10 %, and an aliquot of this was transferred to a 96-well sequencing block. Both the sequencing and the original culture blocks with LB broth and glycerol were stored at –80 ^oC.

2.3 *Sequence and alignment viewing and editing*

Sequencing plates were sent to Iowa State Plant Sciences sequencing facility or Seqwright, Inc., in Houston, Texas. The 16S rRNA genes were partially sequenced using the primer 27F. The full 16S rRNA gene for selected clones was determined by further sequencing using 533F [5'-GTG CCA GCM GCC GCG GTA A-3'] and 1492R at the University of Michigan's sequencing facility. Chromatographs of sequences were viewed and manually edited for quality using Sequencer version 2.0 (Gene Codes Corporation) and ChromasPro (Technelysium Pty Ltd). Sequences that were less than 410 base pairs were resequenced or not included in further analyses.

Sequence alignments were constructed using PILEUP in the GCG Wisconsin package or Clustal W. Alignments were viewed and manually edited using version 2.6.02 of the program GeneDoc using the guidelines of Furlong et al. (2002).

2.4 *Library compairsons*

Edited alignments were used to calculate distance matrices using the Jukes-Cantor algorithm in the program DNADIST from the Phylip package (Felsenstein, 2004). These matrices were then used as the input file for the program PRELIBSHUFF. This practical extraction and report language (PERL) program formats a distance matrix into a sample file for use by the program LIBSHUFF version 1.2 (Singleton et al., 2001). The Bonferroni correction was used to correct for experiment-wise error when doing multiple LIBSHUFF comparisons. Two diversity measurements were used to compare clone libraries independent of their phylogenetic composition. The Shannon diversity index was used as a measure of general diversity, including richness and evenness (Shannon and Weaver, 1967). The Chao1 estimator was calculated as an alternative to Shannon diversity (Chao, 1984). Both of these calculations were determined at sequence similarity values of 97 % and 99 %. To analyze the distribution of abundant taxa within libraries, groups were constructed using DOTUR at a distance of less than or equal to 0.03 (Schloss et al., 2005). These groups were then analyzed using the binomial test (Siegel, 1956).

2.5 *Taxonomic assessment*

Taxonomic identifications were made using the program RDPquery written by Wade Sheldon and Glen Dyszynski at the University of Georgia (http://simo.marsci.uga.edu/public_db/rdp_query. htm). This program compared clone sequences to type species in the RDP, calculated the percent similarity between individual sequences and the closest-related type species, and

used these similarities to make taxonomic assessments (Lasher et al., submitted). The taxonomic ranks were assigned using similarity values for species, genus, family, order, class and phylum as 100 %, 95 %, 92 %, 91 %, 85 % and 80 %, respectively.

2.6 *Tree construction and analysis*

Phylogenetic trees were constructed from the edited alignments of the soil libraries. Distance matrices were calculated using DNADIST (Felsenstein, 2004). These matrices were used to construct neighbor-joining trees or Fitch-Margoliash with bootstrap values in the Phylip package (Felsenstein, 2004). Trees shown were constructed using a representative clone sequence for groups with multiple sequences.

3. Results

3.1 *Construction of bacterial libraries*

Between 87-114 clones were sequenced from the 16S rRNA gene libraries prepared from HSB and CWT cores (Table 2.1). Each location was represented by two to three replicate cores that were each prepared independently. Comparisons from these cores were not significantly different from one another, as determined by LIBSHUFF analysis, which indicated that the methods for construction were valid and reproducible and that the soil microbiota is fairly homogenious (data not shown). Replica cores were then grouped together according to location for further analyses. All libraries contained sequences from bacterial groups that are difficult to lyse (e.g., Actinobacteria), so cell lysis during the extraction was considered complete (More et al., 1994). Although few duplicate sequences were found, there were many closely related sequences within each library.

3.2 *Phylogeny of clone libraries*

Overall the phyla present in the libraries were in both the HSB and CWT clones (Table 2.1). This was true even when comparing cropland (CT and NT) and forest (NF, OF, CU, SM, and ST) soils. The Acidobacteria were not found in the libraries which was unusual because this group is commonly found as a large percentage of clones in other 16S rDNA library studies (Dunbar et al., 1999; Dunbar et al., 2002; Furlong et al., 2002). This group was recently named, however, and with only a few cultured representatives the cut-off values for analysis may have been too stringent.

The most abundant phylum in all the libraries was the Proteobacteria, which consisted of 32 % of the total number of clones. The distribution of Proteobacteria within the HSB and CWT samples was similar (removing NT2 and OF2). The largest group of Proteobacteria was the Alpha-proteobacteria. This was the most abundant phylogenetic group after the unclassified bacteria. This group of bacteria included many common soil bacteria, such as nitrifying bacteria and Rhizobiaceae. In order to get a better understanding of clone relationhips, a phylogenetic tree was constructed from the 16S rRNA gene sequences of the Alpha-proteobacteria, as shown in Figure 2.1. This tree is constructed with representative clones, as explained by the figure legend. Overall, the clones were not closely related to reference sequences.

The second most abundant phylogenetic group was the Actinobacteria with about 7 % of the clones. The group of bacteria has been recognized to be important in decomposition of plant matter and humus formation and therefore is one of the most abundant phyla found within soil. A phylogenetic tree was constructed the same as the Alpha-Proteobacteria (Figure 2.2). As before the clones did not group with reference sequences.

The third largest phylum, about 6 % of clones, came from another well known soil group, Verrucomicrobia. This group of bacteria was also recently named and appears to be ubiquitous in soil even though the exact character of this group has not been elucidated.

The remaining phyla present within the libraries each consisted of less than 3 % of the clones. Three of these less abundant phyla include the Planctomycetes, commonly found in aquatic systems and in soil, the Bacteroidetes, commonly found in soil, and the Gemmatimonadetes, another recently named phylum for which a cultured isolate was obtained in 2003.

Close to one-half of the sequences within each library fell into the "unclassified" group, indicating that they possessed less than 80 % sequence similarity to a type stain in the RDP database. This number dramatically dropped when non-type strains and clone sequences from other studies in the database were included in this comparison. In Figure 2.3 the comparison of two cumulative curves indicated that a larger fraction of clones had a higher percent similarity to sequences within the database when clone sequences were included. Therefore, the unclassified group is not wholly unique but rather under-represented by cultured isolates. Furthermore, the unclassified group did not represent any single phylum, but rather many different taxa. In the unclassified bacteria tree (Figure 2.4), a slight majority of the clone sequences grouped near a reference sequence, but most of these references are for candidate phyla or from other clone library studies. This indicated that the unclassified bacteria are found within other soil libraries and are not from PCR artifacts.

Thirty sequences with low similarity to previously described soil clones were chosen for full length sequence analysis. A consensus sequence for each of these clones was obtained from 27F, 533F, and 1492R. These sequences were then used to construct a phylogenetic tree (Figure

2.5) with ~1000 base pair and the reference sequences from the unclassified bacteria tree. As in the unclassified tree, the majority of clones that grouped near a reference sequence were to candidate phyla or other clone sequences.

3.3 *Diversity indices*

The soil bacterial community appeared to be diverse. Evenness and Shannon indices were all close to their maximum values (Table 2.2). Although not as high, the richness index was 0.49-0.77 of its maximum values. All these indices were high, with values from the agricultural plots and NF exceeding the OF and CWT libraries. The Chao1 estimator followed a similar pattern (Table 2.2). Analyses were performed with an operational taxonomic unit (OTU) of greater than or equal to 97 % sequence similarity as assigned by DOTUR. Both NT2 and NT5 plots had a large Chao1 estimator, especially NT5, compared to the other libraries. Table 2.3 shows how rare ribotypes dominated this environment. This affected the calculations of the diversity index and species estimator. The CT Chao1 value is rather low considering it has a Shannon index approaching maximum. At an OTU defined as greater than or equal to 99 % sequence similarity, however, the Chao1 for CT increased to 676.9. This indicated that there was a large number of closely related organisms present within the libraries because with a small change in the distance used to define an OTU there was a large change in the estimated Chao1 richness.

Rarefaction curves, which compared the observed richness of unequally sampled sites, remained linear at the 0.03 distance level for all libraries (data not shown). Furthermore, combining similar libraries, e.g. all the Coweeta sequences, did not affect this observation (data not shown). These results indicate that the libraries have not been sampled fully at this time. Under-sampled sites may have inaccurate richness estimations. The Chao1 estimator, however, is

often more sensitive than rarefaction curves, and so can still be considered valid (Kemp and Aller, 2004).

3.5 *Library similarity measurements*

There were multiple hypotheses being tested for this study. First, the hypothesis that soils from conventional tillage and no tillage regimes were not different was tested using LIBSHUFF (Singleton, 2001). The heterologous coverage of the CT by the NT library was not significantly different ($p=0.643$) from the homologous coverage of the CT library by itself. This implies that the sequences within CT were well represented by the NT library (Table 2.4). However, the reciprocal comparison was significantly different ($p=0.013$). Thus the NT library contained sequences that were absent from CT, and CT appeared to be a subset of NT.

The second comparison tested the hypothesis that agricultural and forest soil communities from HSB were different (Table 2.4). Comparisons of agricultural to NF communities showed that they were not significantly different (p-value >0.159) while the reciprocal comparisons were different (p-value <0.012). Therefore both CT and NT communities appeared to be subsets of NF. In contrast the OF community was significantly different to those of CT and NT in both directions which suggested that there were portions of the bacterial community in both the agricultural and OF plots that were absent from the other libraries. The Bonferroni correction for comparison of all the HSB libraries was 0.012, which indicated that the communities overall were significantly different from one another. The results of the LIBSHUFF can be viewed in light of the effect of multiple rare ribotypes with low abundance within the soil (Table 2.3). LIBSHUFF comparisons were affected by more distantly related organisms; the greater the phylogenetic difference then the fewer the number of sequences within the library needed to cause the libraries to be significantly different from one another.

The next hypothesis was that the various forest soil plots with different above-ground variation, CU, ST, and SM, were not significantly different from one another (Table 2.5). Individual LIBSHUFF comparisons were not significantly different and the experiment-wise p-value of 0.34 confirmed these results. The last hypothesis to be tested involved the comparison of the forest soils from the two different research sites (Table 2.6). When each Coweeta plot was compared to the OF plot, none of the LIBSHUFF p-values were significant, with an overall p-value of 0.23.

The NF plot, which stopped being used for agriculture in 1938, appeared to have an intermediate community between those of the OF and Coweeta. In contrast to the OF results, the Coweeta and NF communities were significantly different from one another, with an experiment-wise p-value of 0.006. The only community with which NF shared any similarity was that of CU (p-value = 0.074). Therefore, NF was a subset of CU, while CU was significantly different from NF. These measurements were further supported by the LIBSHUFF results from the comparison of the unclassified bacteria and the HSB Proteobacteria. The unclassified bacteria results mimicked the overall results, while the Proteobacteria showed an interesting result. Specifically, the NF was similar to the agricultural plots while differing from the OF plot. This contradicts the results from the overall LIBSHUFF comparison of the NF to OF, yet supports those of the NF to Coweeta comparisons. The Proteobacteria may be affected by agricultural practices, which have lasting effects as seen in the clone libraries.

3.6 *Abundant groups and binomial testing*

Phylogenetic groups, as determined by taxonomy, were analyzed to determine whether distribution of clones within these groups were specific for agriculture or forest clones (Table 2.7). The phylogenetic groups are labeled with the phylum name. The rest of clones were placed

into a fifth group labeled “other”. Significance was determined using the binomial test. There were no groups in the Actinobacteria that were significantly different for agriculture or forest. There were groups in the Alpha-proteobacteria and unclassified bacteria that were significantly different. The one group in the Other category that was significant consisted of Beta-proteobacteria. This supported earlier LIBSHUFF analyses which determined there were differences within the libraries.

4. Conclusions

This study had similar results compared to previous studies in soil where phylogenetic groups were examined (Diallo et al., 2004; McCaig et al., 1999; Lipson and Schmidt, 2004; Borneman et al., 1996; Smit et al., 2001). Actinobacteria and proteobacteria were the most abundant groups within the clones. The large number of alpha-proteobacteria is similar to other observations in the literature (McCaig et al., 1999; Smit et al., 2001). Gamma-proteobacteria, however, have also been found to comprise a large percentage of soil clones, even up to twice as much as the alpha-proteobacteria in some soils (Zhou et al., 2004; Diallo et al., 2004; Furlong et al., 2002). It is also unusual that so few Acidobacteria were found because they are often a large percentage of clones in other 16S rDNA library studies (Dunbar et al., 1999; Dunbar et al., 20002; Furlong et al., 2002). Thus the low percentage of Acidobacteria was unexpected. Previous studies from this same sample site have shown this group to comprise a large portion of the total sequences, as well as many other studies since the discovery of this bacterial group (Dunbar et al., 1999; Furlong et al., 2002; Dunbar et al., 2002). Furthermore, the bacterial communities obtained from different depths at sites with similar land management (forest) have been shown to contain different percentages of various phylogenetic groups, namely Acidobacteria, Alpha-proteobacteria, Planctomyces, Bacteroides, and high G+C Gram-positive

bacteria (Hackl et al., 2004). Of course, many of these groups are relatively new (Acidobacteria and Verrucomicrobia) and were not considered in the literature until recently.

The effect of agriculture on the bacterial communities in soil was two-fold. First was the analysis of how agriculture affects bacteria communities as seen through the LIBSHUFF comparisons of the agricultural plots, CT and NT, with the old and new forest plots. The new forest plot represents a transitional phase in the soil regimes and properties. Even after a substantial amount of time (>30 y), the effects of agriculture on bacterial communities are still discernable. The agriculture libraries were subsets of the NF library, but were very different from the OF library. Furthermore, the NF and OF libraries were similar to one another, but the Coweeta libraries, CU, ST, and SM were different from the NF. Figure 2.6 shows a schematic of how the HSB libraries are related to one another. The NF plot, then, still has changes in the soil bacterial community structure as a result of agriculture that are not present in the statistically similar non-farmed plots (OF, CU, ST, SM).

References

Borneman, J., P. Skroch, K.M. O'Sullivan, J. Palus, N. Rumjanek, J. Jansen, J. Nienhuis and E. Triplett. 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. 62, 1935-1943

Chao, A. 1984. Estimating the population size for capture-recapture data with unequal catchability. Biometrics 43, 783-791.

Diallo, M.D., M. Martens, N. Vloemans, S. Cousin, T. Vandekerckhove, M. Neyra, P. de Lajudie, A. Willems, M. Gillis, W. Vyverman and K. Van der Gucht. 2004. Phylogenetic analysis of partial bacterial 16S rDNA sequences of tropical grass pasture soil under *Acacia tortilis* subsp. *Raddiana* in Senegal. System. Appl. Microbiol. 27, 238-252.

Dunbar, J., S. Barns, L. Ticknor and C. Kuske. 2002. Empirical and theoretical bacterial diversity in four Arizona soils. Appl. Environ. Microbiol. 68, 3035-3045.

Dunbar, J., S. Takala, S. Barns, J. Davis, C. Kuske. 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Appl. Environ. Microbiol. 65, 1662-1669.

Felsenstein, J. 2004. Phylip (Phylogeny Inference Package) version 3.63. Distributed by author. Department of Genome Sciences, University of Washington, Seattle.

Furlong, M., D. Singleton, D.C. Coleman and W.B. Whitman. 2002. Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and the burrows and casts of the earthworm *Lumbricus rubellus*. Appl. Environl. Microbiol. 68, 1265-1279.

Greenberg, C.H., and W.H. McNab. 1998. Forest disturbance in hurricane-related downbursts in the Appalachian mountains of North Carolina. For. Ecol. Manag. 104, 179-191.

Hackl, E., S. Zechmeister-Boltenstern, L. Bodrossy and A. Sessitsch. 2004. Comparison of diversities and compositions of bacterial populations inhabiting natural forest soils. Appl. Environ. Microbiol. 70, 5057-5065.

Hugenholtz, P., B. Goebel and R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180, 4765-4774.

Kemp, P. F., and J.Y. Aller. 2004. Bacterial diversity in aquatic and other environments: what 16S rDNA libraries can tell us. FEMS Microbiol. Ecol. 47, 161-177.

Kennedy, A.C. 1999. Bacterial diversity in agroecosystems. Agric. Ecosyst. Environ. 74, 65-76.

Knoepp, J.D., D.C. Coleman, D.A. Crossley and J.S. Clark. 2000. Soil biological indicators: an ecosystem case study of their use. Forest Ecology and Management 138, 357-368.

Lipson, D.A., and S. Schmidt. 2004. Seasonal changes in an alpine soil bacterial community in the Colorado Rocky Mountains. Appl. Environ. Microbiol. 70, 2867-2879.

Matson, P.A., W.J. Parton, A.G. Power and M.J. Swift. 1997. Agricultural intensification and ecosystem properties. Science 277, 504-508.

Maxwell, R.A., and D.C. Coleman. 2002. Seasonal dynamics of nematode and microbial biomass in soils of riparian-zone forests of the Southern Appalachians. Soil Biol. Biochem. 1, 79-84.

McCaig, A., L.A. Glover and J. Prosser. 2001. Numerical analysis of grassland bacterial community structure under different land management regimens by using 16S ribosomal DNA sequence data and denaturing gradient gel electrophoresis banding patterns. Appl. Environ. Microbiol. 67, 4554-4559.

McCaig, A., A. Glover and J. Prosser. 1999. Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. Appl. Environ. Microbiol. 65, 1721-1730l.

More, M. I., J.B. Herrick, M.C. Silva, W.C. Ghiorse and E.L. Madsen. 1994. Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. Appl. Environ. Microbiol. 60, 1572-1580.

Odum, E.P., S.E. Pomeroy, J.C. Dickinson and K. Hutcheson. 1973. The effect of late winter litter burn on the composition , productivity and diversity of a 4-year old fallow-field in Georgia. Proc. Ann. Tall Timbers Fire Ecol.

Schloss, P. D., and J. Handelsman. 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl. Environ. Microbiol. 71, 1501-1506.

Shannon, C.E., and W. Weaver. 1963. The mathematical theory of communication. Univ. Illinois Press, Urbana.

Siegel, S., 1956. Nonparametric statistics for the behavioral sciences. McGraw-Hill, New York.

Singleton, D., M. Furlong, S. Rathburn and W.B. Whitman. 2001. Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. Appl. Environ. Microbiol. 67, 4374-4376.

Smit, E., P. Leeflang, S. Gommans, J. van den Broek, S. van Mil and K. Wernars. 2001. Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. Appl. Environ. Microbiol. 67, 2284-2291.

Sohlenius, B., 1982. Short-term influence of clear-cutting on abundance of soil-microfauna (Nematoda: Rotatoria and Tardigrada) in a Swedish pine forest soil. J. Appl. Ecol. 19, 349-359.

Tilman, D., J. Fargione, B. Wolff, C. D'Antonio, A. Dobson, R. Howarth, D. Schindler, W.H. Schlesinger, D. Simberloff and D. Swackhamer. 2001. Forecasting agriculturally driven global environmental change. Science. 292, 281-284.

Torsvik, V., and L. Ovreas. 2002. Microbial diversity and function in soil: from genes to ecosystems. Curr. Opin. Microbiol. 5, 240-245.

Torsvik, V., L. Ovreas and T. Thingstad. 2002. Prokaryotic diversity-magnitude, dynamics, and controlling factors. Science 296, 1064-1066.

Ulanova, N.G., 2000. The effects of windthrow of forest at different spatial scales: a review. For. Ecol. Manag. 135, 155-167.

von Wintzingerode, F., Gobel, U.B., Stackenbrandt, E., 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. 21, 213-229.

Whitman, W.B., D.C. Coleman and W.J. Wiebe. 1998. Prokaryotes: the unseen majority. Proc. Natl. Acad. Sci. U.S.A. 6578-6783.

Wright, C.J., and D.C. Coleman. 2002. Responses of soil microbial biomass, nematode trophic groups, N-mineralization, and litter decomposition to disturbance events in the Southern Appalachians. Soil Biol Biochem. 34, 13-25.

Zhou, J., B. Xia, H. Huang, A. Palumbo and J. Tiedje. 2004. Microbial diversity and heterogeneity in sandy subsurface soils. Appl. Environ. Microbiol. 70, 1723-1734.

Figure 2.1.

Neighbor joining phylogenetic tree of representative Alpha-proteobactiera clones from Horseshoe Bend and Coweeta soils and reference sequences with *Escherichia coli* as an outgoup. Clones are listed with the following guidelines: the first two letters indicate the library (CT, NT, NF, OF, CU, SM, or ST). The first number, 2 or 5, indicates depth, and the second number indicates the library replica. The last letter and number combination indicates the position in the sequencing block. Open circles (○) denote bootstrap values of ≥ 95 % and closed circles (●) denote bootstrap values of ≥ 50 % based upon 100 replicates. The scale bar represents the Jukes-Cantor evolutionary distance.

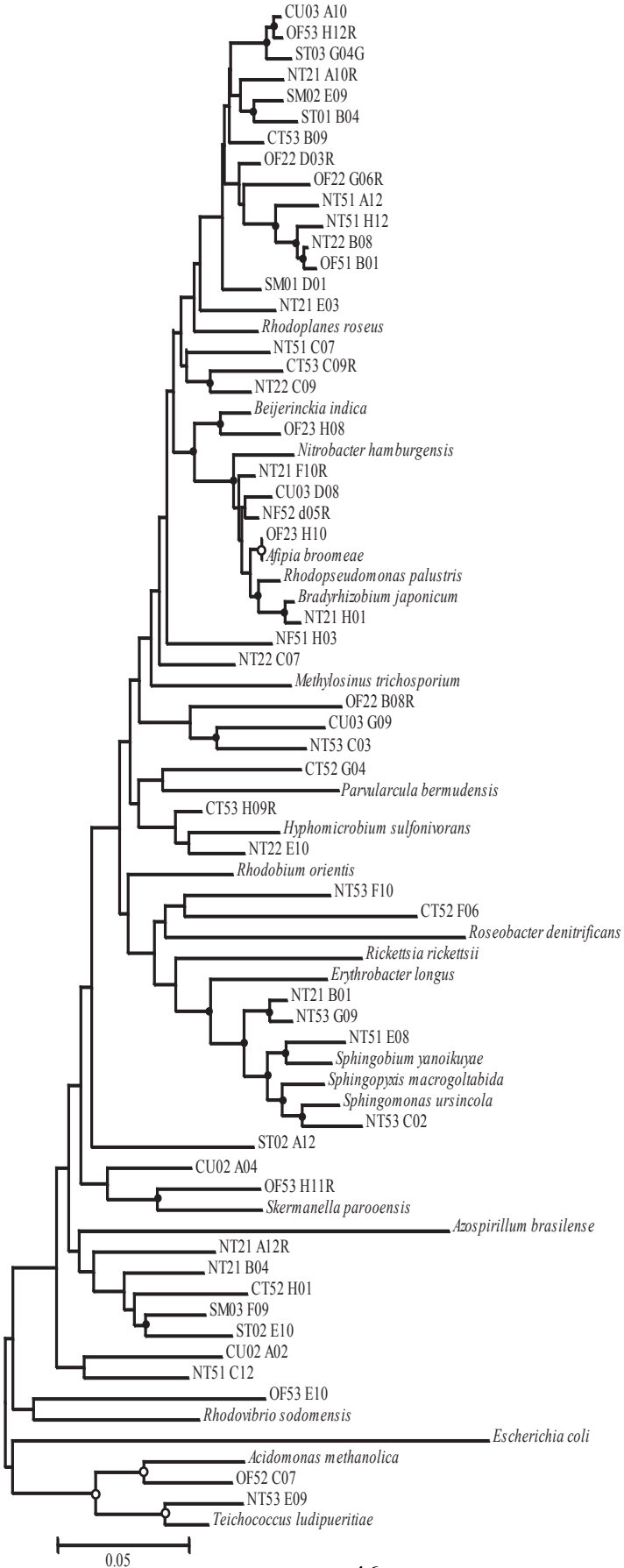

CU03 A10
OF53 H12R
ST03 G04G
NT21 A10R
SM02 E09
ST01 B04
CT53 B09
OF22 D03R
OF22 G06R
NT51 A12
NT51 H12
NT22 B08
OF51 B01
SM01 D01
NT21 E03
Rhodoplanes roseus
NT51 C07
CT53 C09R
NT22 C09
Beijerinckia indica
OF23 H08
Nitrobacter hamburgensis
NT21 F10R
CU03 D08
NF52 d05R
OF23 H10
Afipia broomeae
Rhodopseudomonas palustris
Bradyrhizobium japonicum
NT21 H01
NF51 H03
NT22 C07
Methylosinus trichosporium
OF22 B08R
CU03 G09
NT53 C03
CT52 G04
Parvularcula bermudensis
CT53 H09R
Hyphomicrobium sulfonivorans
NT22 E10
Rhodobium orientis
NT53 F10
CT52 F06
Roseobacter denitrificans
Rickettsia rickettsii
Erythrobacter longus
NT21 B01
NT53 G09
NT51 E08
Sphingobium yanoikuyae
Sphingopyxis macrogoltabida
Sphingomonas ursincola
NT53 C02
ST02 A12
CU02 A04
OF53 H11R
Skermanella parooensis
Azospirillum brasilense
NT21 A12R
NT21 B04
CT52 H01
SM03 F09
ST02 E10
CU02 A02
NT51 C12
OF53 E10
Rhodovibrio sodomensis
Escherichia coli
Acidomonas methanolica
OF52 C07
NT53 E09
Teichococcus ludipueritiae
0.05

Figure 2.2.

Neighbor-joining phylogenetic tree of representative Actinobacteria clones from Horseshoe Bend and Coweeta. Tree information is the same as described for Figure 2.1.

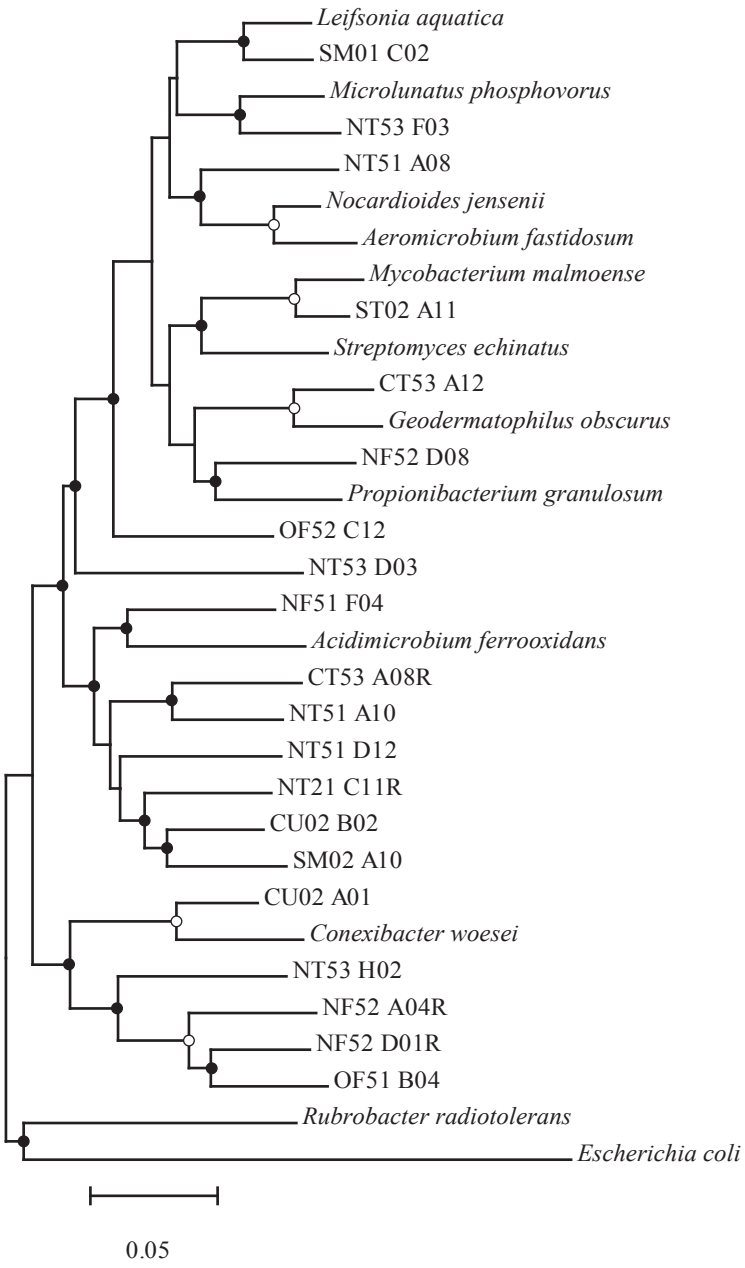

Leifsonia aquatica
SM01 C02
Microlunatus phosphovorus
NT53 F03
NT51 A08
Nocardioides jensenii
Aeromicrobium fastidosum
Mycobacterium malmoense
ST02 A11
Streptomyces echinatus
CT53 A12
Geodermatophilus obscurus
NF52 D08
Propionibacterium granulosum
OF52 C12
NT53 D03
NF51 F04
Acidimicrobium ferrooxidans
CT53 A08R
NT51 A10
NT51 D12
NT21 C11R
CU02 B02
SM02 A10
CU02 A01
Conexibacter woesei
NT53 H02
NF52 A04R
NF52 D01R
OF51 B04
Rubrobacter radiotolerans
Escherichia coli
0.05

Figure 2.3

Similarity of the soil clones to sequences in the Ribosomal Database Project (RDP). Cumulative curve of the fraction of clones versus percent similarity. Closed triangles (▲) represent the comparison of the clone sequences to the type sequences in the RDP with >1200 base pairs. Closed squares (■) represent the comparison of clone sequences to the entire RDP with lengths from <1200> base pair. The curve is cumulative for the fraction of clones with a minimum percent similarity.

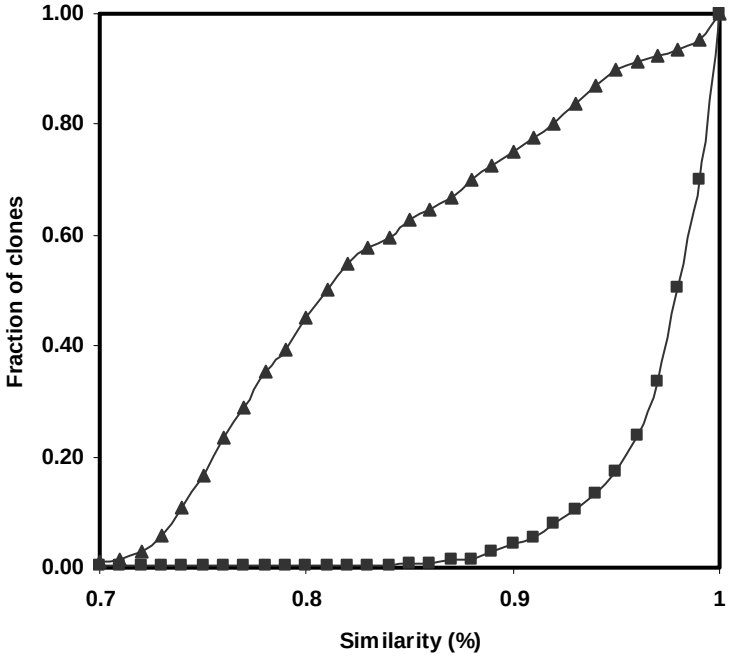

1.00
0.80
0.60
0.40
0.20
0.00
Fraction of clones
0.7
0.8
0.9
1
Similarity (%)

Figure 2.4.

Neighbor-joining phylogenetic tree of representative unclassified clones from Horseshoe Bend and Coweeta. Tree information is the same as described for Figure 2.

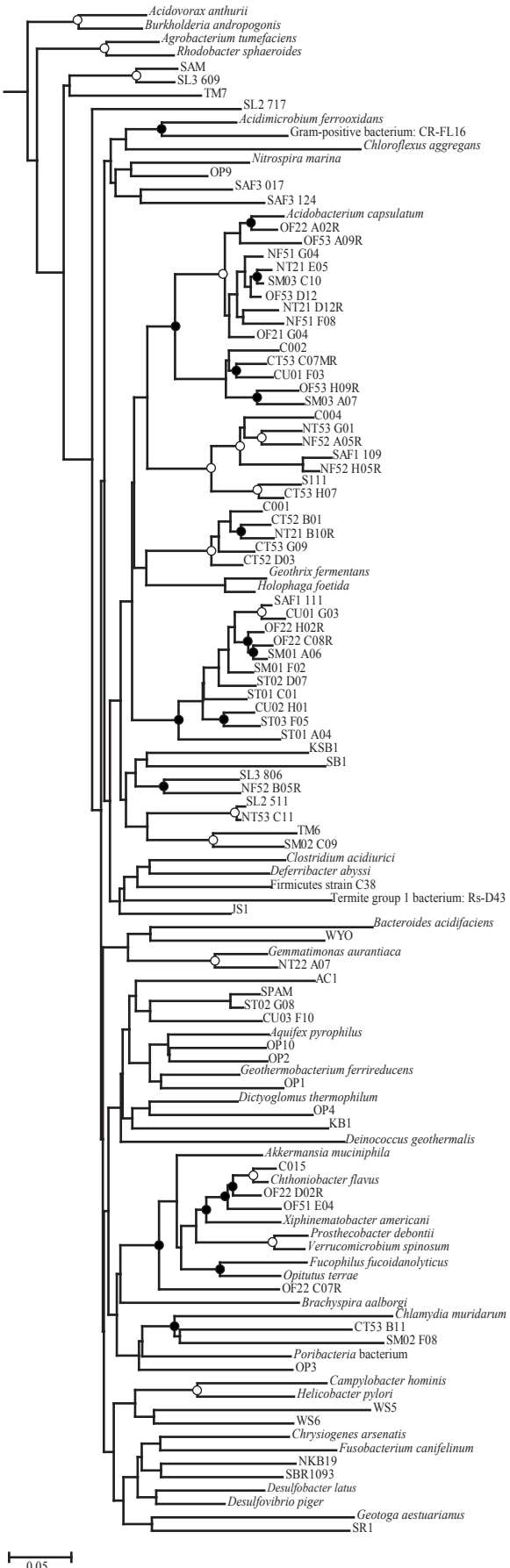

Acidovorax anthurii
Burkholderia andropogonis
Agrobacterium tumefaciens
Rhodobacter sphaeroides
SAM
SL3 609
TM7
SL2 717
Acidimicrobium ferrooxidans
Gram-positive bacterium: CR-FL16
Chloroflexus aggregans
Nitrospira marina
OP9
SAF3 017
SAF3 124
Acidobacterium capsulatum
OF22 A02R
OF53 A09R
NF51 G04
NT21 E05
SM03 C10
OF53 D12
NT21 D12R
NF51 F08
OF21 G04
C002
CT53 C07MR
CU01 F03
OF53 H09R
SM03 A07
C004
NT53 G01
NF52 A05R
SAF1 109
NF52 H05R
S111
CT53 H07
C001
CT52 B01
NT21 B10R
CT53 G09
CT52 D03
Geothrix fermentans
Holophaga foetida
SAF1 111
CU01 G03
OF22 H02R
OF22 C08R
SM01 A06
SM01 F02
ST02 D07
ST01 C01
CU02 H01
ST03 F05
ST01 A04
KSB1
SB1
SL3 806
NF52 B05R
SL2 511
NT53 C11
TM6
SM02 C09
Clostridium acidiurici
Deferribacter abyssi
Firmicutes strain C38
Termite group 1 bacterium: Rs-D43
JS1
Bacteroides acidifaciens
WYO
Gemmatimonas aurantiaca
NT22 A07
AC1
SPAM
ST02 G08
CU03 F10
Aquifex pyrophilus
OP10
OP2
Geothermobacterium ferrireducens
OP1
Dictyoglomus thermophilum
OP4
KB1
Deinococcus geothermalis
Akkermansia muciniphila
C015
Chthoniobacter flavus
OF22 D02R
OF51 E04
Xiphinematobacter americani
Prosthecobacter debontii
Verrucomicrobium spinosum
Fucophilus fucoidanolyticus
Opitutus terrae
OF22 C07R
Brachyspira aalborgi
Chlamydia muridarum
CT53 B11
SM02 F08
Poribacteria bacterium
OP3
Campylobacter hominis
Helicobacter pylori
WS5
WS6
Chrysiogenes arsenatis
Fusobacterium canifelinum
NKB19
SBR1093
Desulfobacter latus
Desulfovibrio piger
Geotoga aestuarianus
SR1
0.05

Figure 2.5.

Neighbor-joining phylogenetic tree of clones from full length sequence. Tree information is the same as described for Figure 2.

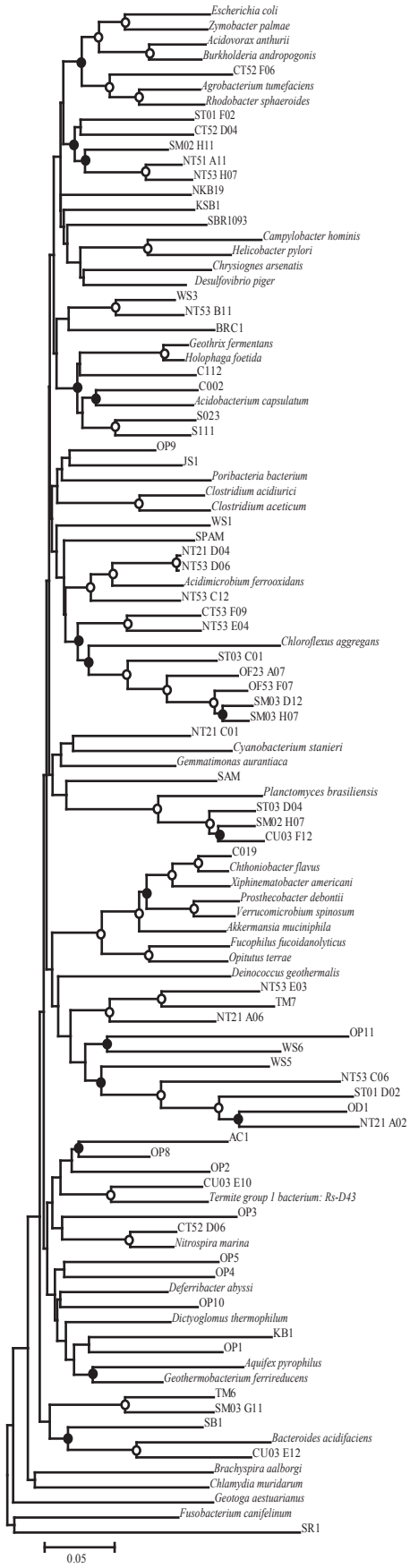

Escherichia coli
Zymobacter palmae
Acidovorax anthurii
Burkholderia andropogonis
CT52 F06
Agrobacterium tumefaciens
Rhodobacter sphaeroides
ST01 F02
CT52 D04
SM02 H11
NT51 A11
NT53 H07
NKB19
KSB1
SBR1093
Campylobacter hominis
Helicobacter pylori
Chrysiognes arsenatis
Desulfovibrio piger
WS3
NT53 B11
BRC1
Geothrix fermentans
Holophaga foetida
C112
C002
Acidobacterium capsulatum
S023
S111
OP9
JS1
Poribacteria bacterium
Clostridium acidiurici
Clostridium aceticum
WS1
SPAM
NT21 D04
NT53 D06
Acidimicrobium ferrooxidans
NT53 C12
CT53 F09
NT53 E04
Chloroflexus aggregans
ST03 C01
OF23 A07
OF53 F07
SM03 D12
SM03 H07
NT21 C01
Cyanobacterium stanieri
Gemmatimonas aurantiaca
SAM
Planctomyces brasiliensis
ST03 D04
SM02 H07
CU03 F12
C019
Chthoniobacter flavus
Xiphinematobacter americani
Prosthecobacter debontii
Verrucomicrobium spinosum
Akkermansia muciniphila
Fucophilus fucoidanolyticus
Opitutus terrae
Deinococcus geothermalis
NT53 E03
TM7
NT21 A06
OP11
WS6
WS5
NT53 C06
ST01 D02
OD1
NT21 A02
AC1
OP8
OP2
CU03 E10
Termite group 1 bacterium: Rs-D43
OP3
CT52 D06
Nitrospira marina
OP5
OP4
Deferribacter abyssi
OP10
Dictyoglomus thermophilum
KB1
OP1
Aquifex pyrophilus
Geothermobacterium ferrireducens
TM6
SM03 G11
SB1
Bacteroides acidifaciens
CU03 E12
Brachyspira aalborgi
Chlamydia muridarum
Geotoga aestuarianus
Fusobacterium canifelinum
SR1
0.05

Figure 2.6.

Schematic of the overlap suggested by LIBSHUFF analysis. CT is a subset of NT and NF. NT is a subset of NF. NF is a subset of OF, while CT and NT are different from OF.

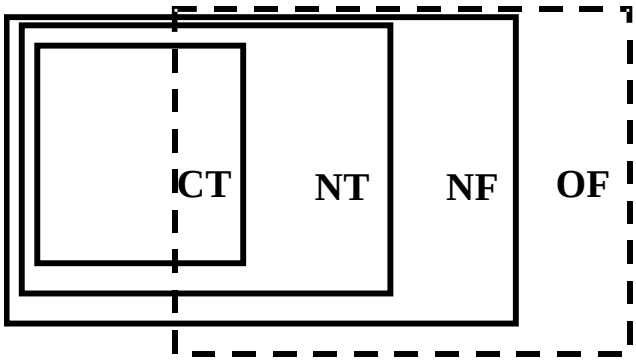

CT
NT
NF
OF

Table 2.1. Phylogenetic assignments of clones from replicate libraries grouped by plot.

	Number of clones in each group[a]								
Phylogenetic Group	Horseshoe Bend						Coweeta		
	CT5	NT2[b]	NT5	NF	OF2	OF5	CU	SM	ST
Actinobacteria	4	11	16	10	1	7	4	7	3
Bacteroidetes									
Flavobacteria	1								
Sphingobacteria	3	2	2	2	1			1	
Other				1	1		2		
Chloroflexi	1								
Firmicutes	1	2	1	4		1	2		1
Gemmatimonadetes	5	2	8	2	1	1	1		
Nitrospira	1			1		1		1	
Planctomycetes		8	1	2	1	3	3	7	3
Proteobacteria									
Alphaproteobacteria	20	20	28	17	16	12	25	21	37
Betaproteobacteria	8	8	5	4	2	3	4	1	
Deltaproteobacteria	1	1	1		1	1	1		
Gammaproteobacteria	2		3	3	3	1	2	1	1
Unclassified[d]		1	2	4	6	8	10	5	5
Verrucomicrobia	4	7	6	4	12	3	10	2	8
Unclassified bacteria[e]	42	47	43	45	46	46	48	54	48
Total (all taxa)	93	109	116	99	91	87	114	100	106

[a]Phylogenetic assignments were done using the Ribosomal Database Project (RDP) with sequence similarity cut-off values of 80 % for phylum and 85 % for class.
[b]NT2 and OF2 libraries were constructed from the upper 0-2 cm of the soil cores from the same plot as NT5 and OF5.
[c]Clones which did not share at least 85 % sequence similarity to a type species of Proteobacteria in the RDP.
[d]Clones which did not share at least 80 % sequence similarity to a type species in the RDP.

Table 2.2. Diversity indices for clone libraries based on 16S rRNA gene sequences[a].

	Horseshoe Bend						**Coweeta**		
Index	CT5	NT2	NT5	NF5	OF2	OF5	CU	ST	SM
S[b]	72	80	86	71	56	55	68	53	62
N[c]	93	109	116	99	91	87	114	106	100
Evenness[d]	0.98[e]	0.97	0.96	0.97	0.94	0.95	0.94	0.91	0.94
Richness[f]	0.77	0.73	0.74	0.71	0.61	0.63	0.59	0.49	0.62
Shannon	0.92	0.91	0.90	0.90	0.84	0.86	0.84	0.78	0.84
Chao1	211(393, 146)[g]	276(352,183)	452(933, 262)	201(354,140)	127(243,92)	170(477,96)	158(304,116)	89(206,71)	152(263,109)

[a]Calculations were based on an OTU of less than or equal to a distance of 0.03 using DOTUR.
[b]S defined as the number of OTUs.
[c]N defined as the number of sequences.
[d]Evenness defined as the number of sequences.
[e]Evenness, Richness, and Shannon diversity reported as a ratio of actual value to maximum value such that closer to 1 denotes values approaching maximum.
[f]Richness indicates the species in the sample as it relates to diversity and number of sequences.
[g]Confidence intervals for the Chao1 estimator are shown in parenthesis.

Table 2.3. Ribotypes of the no-tillage and old forest 16S rRNA gene libraries.

Number of individuals	NT[a]	OF[b]
1	118	62
2	17	13
3	2	3
4	1	2
5	3	4
6	1	0
7	3	0
8	1	1
13	1	0
16	0	1
22	0	1
n	225	178

[a]Comparison from both NT2 and NT5
[b]Comparison from both OF2 and OF5

Table 2.4. LIBSHUFF comparisons of Horseshoe Bend samples, including agricultural and forest soils.[a]

X Library	Y Library CT	NT	NF	OF
CT	-[b]	0.643	0.427	0.003
NT	0.013	-	0.159	0.001
NF	0.002	0.012	-	0.106
OF	0.001	0.001	0.307	-

[a]Experimentwise P-value calculated from the Bonferroni correction for all HSB comparisons was 0.012.
[b]No comparison.

Table 2.5. LIBSHUFF comparisons of Coweeta samples.

X Library	Y Library CU	ST	SM
CU	-[b]	0.891	0.096
ST	0.151	-	0.067
SM	0.582	0.094	-

[a]Experimentwise P-value calculated from the Bonferroni correction for all CWT comparisons was 0.34.
[b]No comparison.

Table 2.6. LIBSHUFF comparisons of Coweeta and Horseshoe Bend forest samples[a,b].

HSB	Coweeta	XY	YX
NF	CU	0.074	0.001
	SM	0.002	0.001
	ST	0.004	0.001
OF	CU	0.258	0.62
	SM	0.043	0.13
	ST	0.724	0.233

[a]Experimentwise P-value calculated from the Bonferroni correction for NF to CWT comparisons was 0.006.
[b]Experimentwise P-value calculated from the Bonferroni correction for OF to CWT comparisons was 0.23.

Table 2.7. Distribution of clones in agricultural and forest plots in groupings for the Actinobacteria, α-proteobacteria, and unclassified bacteria.[a] Significance, as determined by the binomial test, is shown by an asterix.

Group	N[b]	Clone name[c]	AG[d]	FOR[e]	Group	N	Clone name	AG	FOR
Actinobacteria					Unclassified				
	5	NT53_F03	5	0		10	CT52_B01	3	7
	5	ST02_A11	2	3		11	CT52_D03*	11	0
	5	CU02_A01	1	4		7	ST02_G08	1	6
Alphaproteobacteria						9	CU03_F10	2	7
	7	ST02_A12	0	7		6	NT53_G01	4	2
	9	NT21_B01*	9	0		10	NF52_A05R*	7	3
	41	NT21_H01	10	31		9	NT21_B10R	4	5
	20	NT51_H12*	13	7		18	CU02_F03	2	16
	43	CT53_B09*	6	37		14	OF22_H02R*	0	14
	13	ST02_E10*	0	13		6	OF51_E04	1	5
Verrucomicrobia						5	NF51_G04	1	4
	10	CU03_C09	1	9		5	ST02_D07	0	5
	22	NT53_D02	8	14		6	SM01_A06	0	6
	10	OF22_E04R	3	7		6	CU01_G03	0	6
Other						17	OF53_D12	4	13
	10	CU01_G01	1	9		6	CU02_H01	1	5
	8	NT51_B07*	8	0		6	NT21_D12R	2	4
	6	CT52_A04	2	4		6	OF22_D02R	1	5
	6	OF53_C11R	0	6		23	ST01_A04*	1	22
	6	OF22_A01R	0	6		19	NF52_F08*	0	19
	6	ST02_F09	0	6		7	OF22_A02R	0	7
	5	SM03_C11	1	4		5	SM01_F02	0	5

[a] Groupings based on less than or equal to a distance of 0.03 using DOTUR.
[b]Number of clones in grouping.
[c]Representative clone for each grouping.
[d]Agricultural clones from CT and NT libraries.
[e]Forest clones from NF, OF, CU, SM and ST.
*Distribution of clones significant (binomial test).

III. COMPARISON OF BACTERIAL COMMUNITIES AND DIVERSITY OF SOIL FROM THREE SITES AT AN EXPERIMENTAL RICE STATION[2]

[2]Upchurch, R.A., Silvana Tarlera, William B. Whitman. *To be submitted* Applied and Environmental Microbiology.

Abstract:

The bacterial communities of a rice-planted pasture rotation system from the Paso de la Laguna experimental rice station in Uruguay were examined by molecular methods. 16S rRNA gene libraries were constructed from the soil of a flooded rice paddy (AG), planted pasture (PAS), and adjacent uncultivated soil (CON). Abundant phyla included the Proteobacteria, Acidobacteria and Firmicutes. While all libraries were composed of similar phylogenetic groups, LIBSHUFF analysis revealed differences across all libraries ($p=0.006$). However, the AG and PAS bacterial communities were subsets of the CON community.

Key words**:** 16S rRNA gene, Rice paddy, Diversity indices

1. Introduction

Prokaryotes carry out most functions in the soil, including nutrient cycling, soil formation, and decomposition (Borneman et al., 1996; Balota et al., 2003). Land management and agricultural practices can have serious effects on microbial communities through direct impacts on physical and chemical properties of the soil (Bossio et al., 1995; Kennedy, 1995; McCaig et al. 2001; Wright and Coleman, 2002; Sohlenius et al., 1982; Greenberg et al., 1998; Ulanova et al., 2000; Buckley and Schmidt, 2001; DeGrood et al., 2005). These effects and their durability on communities have not been well established (Kennedy, 1999; McCaig et al., 2001). With the rise in uncertainty about the sustainability of agricultural systems, researchers have increased measurements of the quality, quantity and effects of farming on prokaryotes in these soils (Bossio et al., 1995). Therefore, farming practices have been studied that increase the sustainability and slow the degradation of soil, such as minimum tillage and crop rotation systems (Balota et al., 2003; Wright and Colemn, 2002).

Flooded rice farming, an especially harsh agricultural practice, creates an oxic/anoxic gradient within in the soil (Liesack et al., 2000). Anaerobic gradients have been well studied in marine systems with the end of the respiratory chain leading to methane production. Rice paddies are known as a major contributor of atmospheric methane (Liesack et al., 2000; Weber et al., 2001; Chin et al., 1999). Though archaea produce the methane, a complex community of both bacteria and archaea are required for this process to take place (Liesack et al., 2000; Bossio et al., 1995).

Paso de la Laguna, an experimental rice station in Montevideo, Uruguay, maintains plots with rice-planted pasture rotations to offset the problems associated with flooding and draining the land (e.g. soil compaction) (Folgarait et al., 2003). Previous studies about the bacteria in rice

paddies revealed a diverse community (Reichardt et al., 1997; Weber et al., 2001). These studies included culture-dependent and culture-independent characterizations from bulk and root-associated soil. Abundant groups were related to the Verrucomicrobia, Actinobacteria, Bacteroidetes, Acidobacteria and especially the Proteobacteria and Firmicutes (Weber et al., 2001; Chin et al., 1999; Hengstmann et al., 1999; Lu et al., 2006; Noll et al., 2005). Bacterial populations along a vertical oxygen gradient in incubated paddy soil show differing organizations such that Proteobacteria dominate in the oxic and clostridia-like bacteria dominate in the anoxic (Ludemann et al., 2000). This gradient forms after only a few hours and remains robust (Noll et al., 2005). The communities, however, go through successional changes based on oxygen depletion and differing bacterial life cycles (i.e. physiology and turnover rates) until the arrangement stabilizes less than a month after flooding (Noll et al., 2005).

To better understand the impact on bacterial populations from rice farming, soil taken from three sites at Paso de la Laguna were used in this study. We compared the communities from a flooded rice paddy, a rotated planted pasture, and the water-logged land bordering the rice plot (control samples).

2. Material and Methods

2.1 *Sample site descriptions*

Flooded rice paddy soil (AG) samples were taken from a 14 ha field at the experimental rice station at Paso de la Laguna, located in the southeastern region of Uruguay. Rice fields were seeded six months prior to sampling with *Oryza sativa* L. (variety INIA Tacuari) followed by the first treatment of urea. All soil analyses were done at the University of Georgia Department of Crop and Soil Science. The soil texture of all plots consisted of a clay loam with a USDA classification of albic natraqualf. The pH ranged from 6-6.28 with an average carbon to nitrogen

ratio of 10.6. The control soil (CON) was adjacent to the AG plot. This plot was water-logged but not used agriculturally. The pH ranged from 6.26-6.36. with an average carbon to nitrogen ratio of 11.9. The pasture soil (PAS), located 30-50 m away from the AG and CON, was used for rice two years prior to this study. Hereford cattle grazed on the plot at the time of sampling. The dominant grasses were ryegrass (*Lolium rigidum* L.), Birdsfoot trefoil (*Lotus corniculatus*), and clover (*Trifolium repens*). The pH ranged from 5.08-5.27 with an average carbon to nitrogen ratio of 10.1.

2.2 *DNA extraction and preparation for 16S rRNA gene libraries*

Soil samples were collected on February 4, 2004. Each plot, AG, CON and PAS, consisted of three subplots about 10 m apart. Five 12 cm cores were taken at each subplot. The first two centimeters were discarded and any large plant matter was removed. All five cores were pooled together for each subplot and shipped on ice to the University of Georgia. They were kept frozen at -20 ^{o}C until the time of extraction.

Total environmental DNA was extracted from 2.5 g of soil using a PowerMax Soil DNA Extraction kit (MoBio Industries). Each reaction consisted of 1 Ready-To-Go bead (Amersham Biosciences), 1 µl of DNA (dilutions determined by PCR analysis), 1 µl of 27F primer (10µmol) [5'-AGA GTT TGA TCM TGG CTC AG-3'], 1 µl of 1492R primer (10µmol) [5'-ACG GYT ACC TTG TTA CGA CTT-3'], and enough dH_2O to bring the total volume to 25 µl. The program started with 1 cycle of 95 ^{o}C for 5 min followed by 15 cycles of 95 ^{o}C for 1 min, 59 ^{o}C for 1 min, and 72 ^{o}C for 2 min with a final cycle of 72 ^{o}C for 4 min on a Mastercycler Gradient (Eppendorf).

The PCR products were immediately cloned using a TOPO TA cloning kit (Invitrogen) using the pCR2.1 vector. Plates were incubated overnight at 37 ^{o}C and then at least 24 hours at

4 ^{o}C. Well-isolated white colonies were picked into 96-well blocks with Luria-Bertani broth, 0.005 % kanamycin (w/v), 0.005 % ampicillin, and 10 % glycerol then grown overnight. Aliquots of cultures from this medium were transferred to sterile 96 well microplates for sequencing. Between 9-12 clones per culture block were screened for inserts using M13F and M13R primers. Both the sequencing and the original culture blocks were stored at –80 ^{o}C.

2.3 *Sequence and alignment viewing and editing*

Sequencing plates were sent to a facility in the Department of Plant Biology at the University of Georgia. The 16S rRNA genes were partially sequenced using the primer 27F. Chromatographs of sequences were viewed and manually edited for quality using CodonCode Aligner, version 1.5 (Codoncode Corporation). Sequences that were less than 410 base pairs were resequenced or not included in further analyses.

Sequence alignments were constructed using Clustal W. Alignments were viewed and manually edited using GeneDoc, version 2.6.002.

2.4 *Library compairsons*

Soil characteristics were compared using ANOVA in the data analysis tools in Excel (Microsoft). Edited alignments were used to calculate distance matrices using the Jukes-Cantor algorithm in the program DNADIST from the Phylip package (Felsenstein, 2004). These matrices were then used as the input file for the program PRELIBSHUFF. This PERL (Practical Extraction and Report Language) program formats a distance matrix into a sample file for use by the program LIBSHUFF version 1.2 (Singleton et al., 2001). The Bonferroni correction was used to correct for experiment-wise error when doing multiple LIBSHUFF comparisons. Two diversity measurements were used to compare clone libraries without regard for their phylogenetic composition. The Shannon diversity index was used as a measure of general

diversity, including richness and evenness (Shannon and Weaver, 1963). The Chao1 estimator was calculated as an alternative to Shannon diversity (Chao, 1984). Both of these calculations were determined at sequence similarity values of 97 % and 99 %. To analyze the distribution of library sets within abundant taxa, DOTUR grouped clones based on a distance of less than or equal to 0.03 (Schloss and Handelsman, 2005). These groups were then analyzed using the binomial test (Siegel, 1956).

2.5 *Taxonomic assessment*

Taxonomic identifications were made using the program RDPquery written by Wade Sheldon and Glen Dyszynski at the University of Georgia (http://simo.marsci.uga.edu/public_db/rdp_query. htm). This program compared clone sequences to type species in the RDP, calculated the percent similarity between individual sequences and the closest-related type species, and used these similarities to make taxonomic assessments (Lasher et al., submitted). The taxonomic ranks were assigned using similarity values for species, genus, family, order, class and phylum as 100 %, 95 %, 92 %, 91 %, 85 % and 80 %, respectively.

3. Results and Discussion

3.1 *Sample sites*

The soils used in this study represented three usages at the time of sampling. The experimental rice station of Paso de la Laguna practices a rotational system where rice cultivation lasts for one to two years in irrigated plots followed by three to five years of improved pasture for cattle grazing. The agriculturally managed plot (AG) was a flooded rice paddy with water-logged, anoxic conditions. The control plot (CON), also flooded, bordered AG but was never used for agriculture. The final plot, a rotation of planted pasture (PAS) experienced cattle grazing for the past two years following rice cultivation. The carbon to

nitrogen ratios were similar across plots. The pH and electrical conductivity (EC) of the soils differed significantly based on ANOVA due to the lower pH and higher EC of the PAS soil (data not shown).

3.2 *Bacterial libraries*

Comparing replicate libraries at the location level found significant differences as determined by LIBSHUFF (Table 3.1). Each replicate library was came from the soil of pooled cores from individual subplots within either the AG, CON or PAS plots with 62-85 clones sequenced for each. For the AG and CON plots, the replicate libraries differed yet different replicates were subsets of another replicate. PAS subplots differed across almost all comparisons. Subplot libraries where then grouped together for further analysis. All libraries contained sequences from bacterial groups that are difficult to lyse (e.g., Actinobacteria), so cell lysis during the extraction was considered complete (More et al., 1994).

Libraries constructed from all three sites contained sequences from similar major phyla (Table 3.2). The RDPagent program assigned the phylogeny of clones through comparison to isolates. The best matches, however, were seen when uncultured bacteria were used for comparison (Figure 3.1). This result was expected because many types of soil prokaryotes have not been cultured.

The most abundant group consisted of the Proteobacteria, a common result found in other libraries (Janssen, 2006; Axelrood et al., 2002; McCaig et al., 1999). A high abundance of Proteobacteria has been seen with other studies investigating soil and rhizosphere-associated bacteria (McCaig et al., 1999; Gremion et al., 2003). Specifically the α- and β-proteobacteria comprised 23 % of the total amount of clones. They also displayed the largest amount of

diversity with more genera in each than other large groups (data not shown). The δ- and γ-proteobacteria also made up nearly 10 % of the data set.

Nearly half the clones included sequences related to the phyla Firmicutes and Acidobacteria. The Firmicutes were the second most abundant phylum with about 25 % of the clones. The Acidobacteria were about 20 % of the clones. Many sequences characterized as Firmicutes, however, grouped with uncultured bacteria of the Acidobacteria when compared to uncultured bacteria (data not shown). A neighbor-joining tree of the clones described as Firmicutes corroborated this finding (data not shown). Other studies found Acidobacteria in soils across the world, but only a few cultured representatives exist in the databases which may be the cause of this inaccuracy (Janssen, 2006; Dunbar et a1, 1999; Dunbar et al., 2002; Furlong et al., 2002).

Both the Actinobacteria and Planctomycetes each contributed more than 5 % of the data set. Actinobacteria, found all over the world and in many different habitats, had more genera and families than the Acidobacteria and other larger taxonomic groups (Janssen, 2006; Basilio, 2003). The Planctomycetes, once regarded as aquatic, are commonly observed in culture-independent surveys of soil bacteria (Liesack et al., 2001). Furthermore, previous studies of rice paddy soil detected both groups (Noll et al., 2005; Hengstmann et al., 1999).

A second set of libraries from the control soil were constructed with the PCR reaction performed with an annealing temperature at 62 ^{o}C (Table 3.3). Overall the taxa found in the CON62 libraries matched that of the CON libraries constructed at 59 ^{o}C except for the phyla Chlorobi, Gemmatimonadetes and the Thermotogae. The Chlorobi was recognized as closely related to the Bacteroidetes and found in many environments (Gupta, 2005). The Gemmatimonadetes lack many isolates, but previous studies demonstrated a widespread

appearance of this phylum (Zhang et al., 2003; Hugenholtz et al.,1998). The Thermotogae are widespread in the environment and are especially known for being strict anaerobic thermophiles (Huber and Stetter, 1999).

Groups of ribotypes were formed using DOTUR average-neighbor program using a distance of 0.03. These groups represented clusters of closely related species. To determine if the distribution of ribotypes were differed between sites, the binomial test was used (Schloss and Handelsman, 2005). As seen in Table 3.4, the first group, composed of Bacilli, contained a significantly larger amount of PAS clones relative to the other plots. The PAS plot also included an enrichment for a single group within the Acidobacteria. This group was related to strain Ellin327, isolated from Australian pasture soil (Sait et al., 2002). The final group showing differences was found in the Betaproteobacteria and consisted of group of clones with low similarity (~92 %) to anything in the RDP. This problem is common with Proteobacteria with only about 19-36 % of proteobacterial clones similar enough to a described organism to be assigned to a genus (Janssen, 2006).

3.3 *Diversity indices*

Diversity measurements of the sites revealed a high level of diversity with stable communities (Table 3.5). The Chao1 calculation estimated the number OTU's (richness) within plots (Hill et al., 2003). These values were similar, as were the richness ratios. Furthermore, the confidence intervals for the Chao1 overlapped, which lent support to the hypothesis of comparable richness.

The Shannon index (H') remained similar across plots, as did the Shannon evenness (E) measurement that was calculated by dividing the H' with H_{max}. These values related to both the richness and abundance of each community; high values signified a community with a large

richness and an even distribution of species imparting stability (Magurran, 2005; Torsvik and Ovreas, 2002). These values conveyed knowledge about differences in species.

3.4 *LIBSHUFF analysis*

Libshuff comparisons significantly differed across all plots (Table 3.6). AG comparisons showed this plot to be different from CON in the Y to X comparisons, but not in the X to Y. In other words, the AG was a subset of CON. The CON encircled the AG, but was not used for rice farming. These results indicated that rice farming significantly changed the bacterial communities between soil from these two plots, but AG still resembled CON. The PAS and AG differed in both directions with neither being a subset of the other. A planted pasture rotation sufficiently altered the community of this field, once used for rice, to yield significant differences between AG and PAS. On the other hand the PAS and CON comparisons showed the PAS communities to be a subset of CON. Taken as a whole, the LIBSHUFF analysis showed that the AG and PAS soil communities were significantly different from one another yet each was more similar to the CON plot which might represent a parent community.

The CON to CON62 comparisons reflected the differences in communities due to PCR bias at different annealing temperatures (Table 3.7). These libraries, though extracted from the same soil, revealed CON62 to be a subset of CON. Upon inspection the major phyla that were present in CON were found in CON62. One group, the Gemmatimonadetes, was removed and LIBSHUFF was repeated. The overall significance did not change, but CON62 did appear more similar to CON where the XY p =0.341 (data not shown). It was clear that a difference in annealing temperature changed the outcome library construction.

References

Axelrood, P.E., M.L. Chow, C.C. Radomski, J.M. McDermott and J. Davies. 2002. Molecular characterization of bacterial diversity from British Columbia forest soils subjected to disturbance. Can J Microbiol 48:655-74.

Balota, E.L., A. Colozzi-Filho, D.S. Andrade and R.P. Dick. 2003. Microbial biomass in soils under different tillage and crop rotation systems. Biology and Fertility of Soils 38:15.

Borneman, J., P. Skrock, K. O'Sullivan, J. Palus, N. Rumjanek, J. Jansen, J. Nienhuis and E. Triplett. 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Evniron. Microbiol. 62: 1935-1943.

Bossio, D.A., and K.M. Scow. 1995. Impact of Carbon and Flooding on the Metabolic Diversity of Microbial Communities in Soils. Appl Environ Microbiol 61:4043-4050.

Buckley, D.H., and T.M. Schmidt. 2003. Diversity and dynamics of microbial communities in soils from agro-ecosystems. Environ Microbiol 5:441-52.

Chao, A., 1984. Estimating the population size for capture-recapture data with unequal catchability. Biometrics 43, 783-791.

Chin, K.J., D. Hahn, U. Hengstmann, W. Liesack and P.H. Janssen. 1999. Characterization and identification of numerically abundant culturable bacteria from the anoxic bulk soil of rice paddy microcosms. Appl Environ Microbiol 65:5042-9.

DeGrood, S.H., V.P. Claassen and K.M. Scow. 2005. Microbial community composition on native and drastically disturbed serpentine soils. Soil Biology and Biochemistry 37:1427.

Dunbar, J., S.M. Barns, L.O. Ticknor and C.R. Kuske. 2002. Empirical and theoretical bacterial diversity in four Arizona soils. Appl Environ Microbiol 68:3035-45.

Dunbar, J., S. Takala, S.M. Barns, J.A. Davis and C.R. Kuske. 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Appl Environ Microbiol 65:1662-9.

Felsenstein, J., 2004. Phylip (Phylogeny Inference Package) version 3.63. Distributed by author. Department of Genome Sciences, University of Washington, Seattle.

Folgarait, P., F. Thomas, T. Desjardins, M. Grimaldi, I. Tayasu, P. Curmi and P. Lavelle. 2003. Soil properties and the macrofauna community in abandoned irrigated rice fields of northeastern Argentina. Biology and Fertility of Soils 38:349.

Furlong, M.A., D.R. Singleton, D.C. Coleman and W.B. Whitman. 2002. Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and the burrows and casts of the earthworm Lumbricus rubellus. Appl Environ Microbiol 68:1265-79.

Greenberg, C.H., and W.H. McNab. 1998. Forest disturbance in hurricane-related downbursts in the Appalachian mountains of North Carolina. For. Ecol. Manag. 104, 179-191.

Gremion, F., A. Chatzinotas and H. Harms. 2003. Comparative 16S rDNA and 16S rRNA sequence analysis indicates that Actinobacteria might be a dominant part of the metabolically active bacteria in heavy metal-contaminated bulk and rhizosphere soil. Environ Microbiol 5:896-907.

Gupta, R.S. 2004. The phylogeny and signature sequences characteristics of Fibrobacteres, Chlorobi and Bacteroidetes. Crit Rev Microbiol 30:123-43.

Hengstmann, U., K. Chin, P. Janssen and W. Liesack. 1999. Comparative phylogenetic assignment of environmental sequences of genes encoding 16S rRNA and numerically abundant culturable bacteria from an anoxic rice paddy soil. Appl. Environ. Microbiol. 65:5050-5058.

Hill, T.C.J., K.A. Walsh, J.A. Harris and B.F. Moffett. 2003. Using ecological diversity measures with bacterial communities. FEMS Microbiology Ecology 43:1.

Huber, R., and K.O. Stetter. 1999. Thermotogales. *In* Encyclopedia of Life Sciences. Nature Publishing Group London, UK.

Janssen, P.H. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl Environ Microbiol 72:1719-28.

Kennedy, A.C. 1999. Bacterial diversity in agroecosystems. Agriculture, Ecosystems & Environment 74:65.

Liesack, W., S. Schnell and N.P. Revsbech. 2000. Microbiology of flooded rice paddies. FEMS Microbiol Rev 24:625-45.

Lu, Y., D. Rosencrantz, W. Liesack and R. Conrad. Structure and activity of bacterial community inhabiting rice roots and the rhizosphere. Environmental Microbiology doi:10.1111/j.1462-2920.2006.01028.x.

McCaig, A.E., L.A. Glover and J.I. Prosser. 1999. Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. Appl Environ Microbiol 65:1721-30.

McCaig, A., L.A. Glover and J. Prosser. 2001. Numerical analysis of grassland bacterial community structure under different land management regimens by using 16S ribosomal DNA sequence data and denaturing gradient gel electrophoresis banding patterns. Appl. Environ. Microbiol. 67, 4554-4559.

Magurran, A.E. 2005. Biological diversity. Curr Biol 15:R116-8.

More, M., J. Herrick, M. Silva, W. Ghiorse and E. Madsen. 1994. Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. Appl Environ Microbiol 60:1572-1580.

Noll, M., D. Matthies, P. Frenzel, M. Derakshani and W. Liesack. 2005. Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. Environ Microbiol 7:382-95.

Reichardt, W., G. Mascarina, B. Padre and J. Doll. 1997. Microbial Communities of Continuously Cropped, Irrigated Rice Fields. Appl Environ Microbiol 63:233-238.

Sait, M., P. Hugenholtz and P.H. Janssen. 2002. Cultivation of globally distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. Environ Microbiol 4:654-66.

Schloss, P.D., and J. Handelsman. 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl Environ Microbiol 71:1501-6.

Siegel, S., 1956. Nonparametric statistics for the behavioral sciences. McGraw-Hill, New York.

Shannon, C.E., and W. Weaver. 1963. The mathematical theory of communication. Univ. Illinois Press, Urbana.

Singleton, D.R., M.A. Furlong, S.L. Rathbun and W.B. Whitman. 2001. Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. Appl Environ Microbiol 67:4374-6.

Sohlenius, B., 1982. Short-term influence of clear-cutting on abundance of soil-microfauna (Nematoda: Rotatoria and Tardigrada) in a Swedish pine forest soil. J. Appl. Ecol. 19, 349-359.

Torsvik, V., and L. Ovreas. 2002. Microbial diversity and function in soil: from genes to ecosystems. Curr Opin Microbiol 5:240-5.

Weber, S., S. Stubner and R. Conrad. 2001. Bacterial populations colonizing and degrading rice straw in anoxic paddy soil. Appl Environ Microbiol 67:1318-27.

Wright, C.J., and D.C. Coleman. 2002. Responses of soil microbial biomass, nematode trophic groups, N-mineralization and litter decomposition to disturbance events in the Southern Appalachians. Soil Biol Biochem. 34, 13-25.

Zhang, H., Y. Sekiguchi, S. Hanada, P. Hugenholtz, H. Kim, Y. Kamagata and K. Nakamura. 2003. Gemmatimonas aurantiaca gen. nov., sp. nov., a gram-negative, aerobic, polyphosphate-accumulating micro-organism, the first cultured representative of the new bacterial phylum Gemmatimonadetes phyl. nov. Int J Syst Evol Microbiol 53:1155-63.

Figure 3.1.

Similarity of the soil clones to sequences in the Ribosomal Database Project (RDP). Cummulative curve of the fraction of clones versus percent similarity. Closed triangles (▲) represent the comparison of the clone sequences to the type sequences in the RDP with >1200 base pair. Closed squares (■) represent the comparison of clone sequences to the entire RDP with lengths from less than to greater than 1200 base pair. The curve is cumulative for the fraction of clones with a minimum percent similarity.

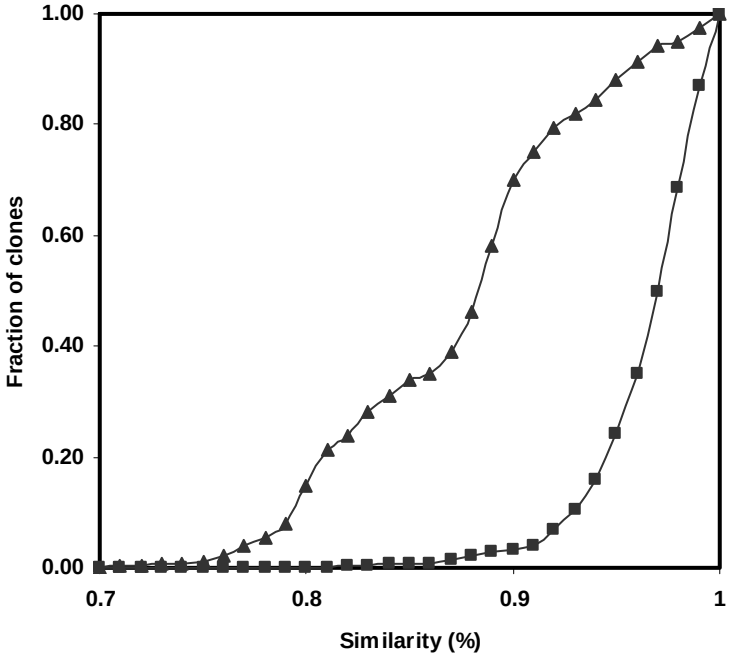

1.00
0.80
0.60
0.40
0.20
0.00
Fraction of clones
0.7
0.8
0.9
1
Similarity (%)

Table 3.1 LIBSHUFF comparisons between replicate libraries for each individual site. Data shown in the XY and YX columns are the p-values[a].

Library	Replicates	XY	YX
AG	1:2	0.464	0.172
	1:3	0.395	0.328
	2:3	0.001	0.062
CON	1:2	0.03	0.671
	1:3	0.041	0.006
	2:3	0.109	0.02
PAS	1:2	0.001	0.001
	1:3	0.002	0.001
	2:3	0.013	0.03

[a]Experimentwise P-value calculated from the Bonferroni correction for AG, CON and PAS comparisons was 0.006, 0.114 and 0.006, respectively.

Table 3.2. Number of phylogenetic assignments of clones in each library

Phylogenetic Group	Number of clones[a]		
	AG	CON	PAS
Acidobacteria	45	63	33
Actinobacteria	10	12	18
Aquificae	1		
Bacteroidetes			
Bacteroidetes			
Flavobacteria	4	2	2
Sphingobacteria		2	2
Chlorobi			
Chloroflexi	1	1	1
Cyanobacteria	1	2	1
Dictyoglomi		1	
Firmicutes	52	72	53
Gemmatimonadetes			
Planctomycetes	10	14	15
Proteobacteria			
Alphaproteobacteria	18	18	24
Betaproteobacteria	27	30	42
Deltaproteobacteria	9	15	17
Gammaproteobacteria	5	11	8
Unclassified[b]	8	17	6
Thermotogae			
TM7	1		
Verrucomicrobia	8	9	3
WS3	1		
Unclassified bacteria[c]	1	2	5
Total (all taxa)	202	271	230

[a]Phylogenetic assignments were done using the Ribosomal Database Project (RDP) with sequence similarity cut-off values of 75 % for phylum and 85 % for class.
[b]Clones which did not share at least 85 % sequence similarity to a type species of Proteobacteria in the RDP.
[c]Clones which did not share at least 75 % sequence similarity to a type species in the RDP.

Table 3.3. Number of phylogenetic assignments of clones in each library

Phylogenetic Group	Number of clones[a]	
	CON	CON62[b]
Acidobacteria	63	44
Actinobacteria	12	8
Bacteroidetes	4	1
Chlorobi		3
Chloroflexi	1	2
Cyanobacteria	2	3
Dictyoglomi	1	
Firmicutes	72	45
Gemmatimonadetes		4
Planctomycetes	14	9
Proteobacteria		
Alphaproteobacteria	18	11
Betaproteobacteria	30	34
Deltaproteobacteria	15	14
Gammaproteobacteria	11	5
Unclassified[c]	17	16
Thermotogae		1
Verrucomicrobia	9	3
Unclassified bacteria[d]	2	4
Total (all taxa)	271	209

[a]Phylogenetic assignments were done using the Ribosomal Database Project (RDP) with sequence similarity cut-off values of 75 % for phylum and 85 % for class.
[b]CON62 refers to the libraries that were constructed using an annealing temperature of 62 ^{o}C with the CON soil.
[c]Clones which did not share at least 85 % sequence similarity to a type species of Proteobacteria in the RDP.
[d]Clones which did not share at least 75 % sequence similarity to a type species in the RDP.

Table 3.4. Distribution of clones in agricultural and forest plots in groupings for the.Firmicutes, Acidobacteria, Alphaproteobacteria and Betaproteobacteria[a] . Significance, as determined by the binomial test, is shown by an asterix on the group N.

Group	AG:CON		N[b]	AG:PAS		N	CON:PAS		N
Firmicutes									
AG1_C05	5	9	14	5	16	*21	9	16	*25
PAS1_C07	3	2	5	3	3	6	2	3	5
CON2_B11	3	2	5	3	2	5	2	2	4
AG2_C08	3	3	6	3	0	3	3	0	3
CON1_D09	0	4	4	0	1	1	4	1	5
Acidobacteria									
AG1_B08	8	13	21	8	2	10	13	2	15
CON3_A05	4	3	7	4	0	4	3	0	3
PAS1_C03	1	0	1	1	6	*7	0	6	*6
AG3_D07	2	5	7	2	0	2	5	0	5
AG1_A08	3	3	6	3	0	3	3	0	3
CON2_B04	1	4	5	1	1	2	4	1	5
PAS3_G05	3	1	4	3	2	5	1	2	3
PAS2_E06	1	2	3	1	3	4	2	3	5
CON1_E08	0	5	5	0	1	1	5	1	6
Alphaproteobacteria									
AG3_A03	0	0	0	2	3	5	2	3	5
Betaproteobacteria									
PAS3_H09	8	5	13	8	7	15	5	7	12
CON1_G03	2	2	4	2	4	6	2	4	6
AG3_C08	1	1	2	1	6	7	1	6	7
AG1_B02	3	1	4	3	2	5	1	2	3
CON2_A06	1	5	6	1	0	1	5	0	*5
PAS1_B05	1	0	1	1	4	5	0	4	4

[a] Groupings based on less than or equal to a distance of 0.01 using DOTUR.
[b]Number of clones in grouping.
*Distribution of clones significant (binomial test).

Table 3.5. Diversity indices for clone libraries based on 16S rRNA gene sequences[a].

Index	AG	CON	CON62[b]	PAS
S[c]	133.00	161.00	121	146.00
N[d]	202.00	271.00	209.00	230.00
Shannon	4.71	4.84	4.56	4.77
Evenness[e]	0.96	0.95	0.95	0.96
Richness	24.87	28.56	22.46	26.85
Chao1[f]	202(137,346)	172(134,249)	140(105,219)	154(123,219)

[a]Calculations were based on an OTU of less than or equal to a distance of 0.03 using DOTUR.
[b]CON62 refers to the libraries that were constructed using an annealing temperature of 62 ^{o}C with the CON soil.
[c]S defined as the number of OTUs or number of groups.
[d]N defined as the number of sequences.
[e]Shannon evenness calculated as H/ln(S).
[f]Confidence intervals for the Chao1 estimator are shown in parenthesis.

Table 3.6. LIBSHUFF comparisons of Uruguay samples[a].

X Library	Y Library		
	AG	CON	PAS
AG	-[b]	0.617	0.001
CON	0.019	-	0.001
PAS	0.01	0.085	-

[a]Experimentwise P-value calculated from the Bonferroni correction for all Uruguay comparisons was 0.006.
[b]No comparison.

Table 3.7. LIBSHUFF comparisons of CON to CON62 samples.

X Library	Y Library	
	CON	CON62
CON	-[a]	0.001
CON62	0.061	-

[a]No comparison.

IV.CONCLUSIONS

The objective of these two studies was to characterize how the compositions of the bacterial communities within soil were impacted by land management using 16S rRNA gene libraries. Over the past decade, the increase in the use of molecular techniques has revealed a vast amount of bacterial diversity within the environment, especially within soil. Little is known, however, about how communities respond to disturbances within their environment. Of the natural and human disturbances of the soil, agriculture exerts one of largest impacts on soil quality and sustainability.

The effects of agriculture on the soil chemical and physical properties, and by extension the bacterial communities, can be long lasting. Previous work has shown that it may take up to 45 years before bacterial communities in successional soil begin to resemble communities from nearby soils unused for cultivation. Furthermore, the tillage regime used influences the impact of cultivation on these properties. Conventional tillage using plowing and disking lowers microbial biomass and respiration rates as compared to less severe methods, such as no-tillage using direct seed drilling. The first study of this project, using agricultural and forest plots from Horseshoe Bend, revealed similar results. The conventional-till communities were significantly different from those of the no-till, as determined by LIBSHUFF analysis. Furthermore, both agricultural communities were significantly different from the nearby old forest and new (abandoned pasture since 1973) forest communities. The old and new forest communities, however, were similar to one another. Both the agricultural communities were subsets of the new forest but not the old forest. This result is especially interesting when considering the LIBSHUFF comparisons of the

new and old forest communities to those of geographically distant (160 km away) never-tilled forest communities of Coweeta. While Coweeta communities appear similar to those of the old forest, they are significantly different from those of the new forest. This suggested that the effects of agriculture could be seen in the comparison of bacterial communities and that the new forest community represented a transitional soil with an intermediate community between agriculture and forest.

Flooded rice farming is a specialized agriculture practice that is especially harsh on the soil with continuous water-logged conditions. The vertical oxygen gradient that forms is an especially strong factor on bacterial communities within these systems. These systems have not been very well characterized in regards to total bacterial groups and diversity. Many countries use either a vegetable or fallow rotation system to offset these problems. The second study done for this project compared the bacterial communities of soils associated with a rice-pasture rotation system of Paso de la Laguna in Uruguay. Three plots were examined. The planted pasture was used for rice farming two years prior to the sampling. Both the rice paddy and control plots were under flooded conditions, but the control soil was never cultivated. LIBSHUFF analysis revealed that all three communities were significantly different from one another. Both the pasture and rice paddy communities, however, were subsets of the control. Therefore, both the flooding and agricultural regiment used influenced the bacterial community composition.