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MOLECULAR CHARACTERIZATION OF BACTERIAL POPULATIONS OF DIFFERENT SOILS

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ABSTRACT

Until recently, few studies were carried out in Brazil about diversity of bacterial soil communities. Aiming to characterize the bacterial population in the soil through 16S rRNA analysis, two types of soil have been analyzed: one of them characterized by intensive use where tomato, beans and corn were cultivated (CS); the other analyzed soil was under forest (FS), unchanged by man; both located in Guaíra, São Paulo State, Brazil. Using specific primers, 16S rRNA genes from metagenomic DNA in both soils were amplified by PCR, amplicons were cloned and 139 clones from two libraries were partially sequenced. The use of 16S rRNA analysis allowed identification of several bacterial populations in the soil belonging to the following phyla: *Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria Verrucomicrobia* in addition to the others that were not classified, beyond *Archaea* domain. Differences between FS and CS libraries were observed in size *phyla*. A larger number of phyla and, consequently, a greater bacterial diversity were found in the under-forest soil. These data were confirmed by the analyses of genetic diversity that have been carried out. The characterization of bacterial communities of soil has made its contribution by providing facts for further studies on the dynamics of bacterial populations in different soil conditions in Brazil.

Key words: metagenome, microbial diversity, 16S rRNA

INTRODUCTION

A large number of physicochemical changes taking place in the soil occur due to agriculture. Such soils suffer physical degradation, such as erosion and compactness, and chemical degradation, which causes nutrient loss. These degradations can sometimes be caused by tillage, careless use of pesticides and fertilizers, and sewage slime, which eventually causes organic matter and biodiversity loss. These environmental changes can modify the microbial population, causing environmental imbalances and affect nutrient recycling in soil (5,12,20).

Several studies have been carried out in order to determine the size of microorganisms' populations in the soil (7,19,29,43,45). Although there are different theories as to the exact number, there is a consensus regarding the fact that the magnitude of the prokaryotic kingdom diversity is very large. Some scientists estimate that a ton of soil could contain 4×10^6 different taxa (7). However, the traditional techniques of isolation and cultivation limit the study of bacteria diversity to those that can be cultivated in laboratory media (33). The use of molecular techniques in microbial ecology has made possible the discovery of new microorganisms previously unknown (29,45). Identification of non-cultivable bacteria that exist in the environment has been based on the determination of 16S rRNA sequences of amplified and cloned genes derived from the soil microbiota's DNA (4,11,25,26.) Sequences of the *rpoB* gene currently have been used with success in studies of microbial

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ecology and phylogeny (32). Few studies on the bacterial community diversity of soil have been carried out in Brazil until now (6,27).

Aiming to study the bacterial populations in the soil through 16S rRNA analysis, two types of soil have been analyzed: one of them characterized by intensive use (center pivot irrigation, intensive use of pesticides and traditional techniques for soil preparation), especially for tomato, beans and corn production; the other analyzed soil was under forest, unchanged by man; both located in Guaíra, São Paulo State, Brazil.

MATERIALS AND METHODS

Soil samples were collected at the Lagoa do Fogão Farm, located in Guaíra city, in the north of São Paulo State. Its geographical coordinates are latitude 20º20' S and longitude 48º23' W, having a 500-meter altitude. The average annual precipitation is 1330mm and the average temperatures range from 17ºC minimum to 30ºC maximum. The cultivated area has been in use for more than ten years. The applied system is the direct cultivation with successive cultures of tomatoes or beans in the winter, from May to June, followed by corn, from August to September, and soy in December. Such agriculture is rather intensive and makes use of all possible resources (fertilizers, pesticides and irrigation) for production during the entire year. In the cultivated area (CS), the soil was classified as "dusky-red latosol". Its physical and chemical characteristics are shown in Table 1.

The under-forest soil (FS), also collected on Lagoa do Fogão Farm, was classified as "Hapludox". Its physical and chemical characteristics are shown in Table 1. This soil is covered by original forest and therefore constitutes a non-disturbed soil, i.e., free from human interferences. The soil collection was made according to the following procedures: twelve simple samples with 400 g each were randomly collected, in a zigzag path over 500 m and within a zero to 20 cm depth, which resulted in a full sample after being put together and homogenized. The final homogenized sample was conserved under refrigeration.

DNA extraction from the soil's microbial population was performed using FastDNA^R Spin Kit for Soil (Bio 101 - catalog #6560-200), according to the manufacturer's instructions. The metagenomic DNA was used in PCR reactions for the amplification of the 16S rRNA gene, by using specific pA primers (5'- AGA GTT TGA TCC TGG CTC AG - 3') and pc5B (5'-TAC CTT GTT ACG ACT T-3'). PCR reaction contained PCR 1X buffer (20mM Tris-HCl (pH 8.4), 50mM KCl), 200 µM of each deoxinucleotide, 1.5 mM $MgCl_2$, 50 pmols of each initiator oligonucleotide, 2.5U Taq DNA polymerase (Invitrogen™, São Paulo, Brazil) and 50 ng metagenomic DNA, in a final volume of 50 µL (25). Ten reactions per soil were performed. Samples were then placed in a thermal cycler for amplification of the gene, and the program described by Kuske (25) was used. The 1.5Kb amplicons were analyzed through electrophoresis and removed from the gel through the addition NaCl 1 M, phenol and chloroform (35).

16S rRNA gene amplicons from both samples were inserted into the pGEM^R- T vector (Promega, Madison, WI, USA -catalog # A3600) according to manufacturer's instructions, and used for the transformation of the *Escherichia coli* DH5α strain. After culturing of the transformed clones, the clones' plasmid DNA was isolated by miniprep (34). The partial sequencing of the 16S rRNA gene was performed making use of 0.4 µL DNA Sequencing-Big Dye Terminator Cycle Sequencing-Ready ABI Prism (Version 3); 3.2 pmols M13/pUC 1211 forward initiator oligonucleotide (5' - GTAAAACGACGGCCAGT - 3'); 100 ng plasmid DNA, 4.6 µL buffer (400 mM Tris-HCl, pH 9; 10 mM $MgCl_2$); and mili-Q (Millipore) H_2O for a 10 mL volume. The reactions were carried out in a thermal cycler as follows: 2 minutes at 96ºC and 40 cycles at 96ºC for 10 seconds, 52ºC for 20 seconds, and 60ºC for 4 minutes. The amplicons were sequenced using a model ABI 3700 capillary sequencer (Applied Biosystems, Foster City, CA, USA).

Each of the unique clone sequences was defined operationally as an operational taxonomic unit (OTU). Sequence analyses were performed with Sequencing Analysis 3.4 and Phred/Phrap/Consed software packages (13), in order to verify sequence quality and to check for possible chimeric origins. Nucleotide sequences were compared with sequences from GenBank using BLAST software (1). The ends of sequences were trimmed with BioEdit v5.0.9 software (14) and subsequently aligned using the software CLUSTALX v.1.81 (41). Two sequence data sets were produced, one for the FS soil, and another for the clones from CS soil samples.

The phylograms were constructed using the Neighbor-Joining algorithm (34) and Jukes Cantor (21) and processed using MEGA software, version 2.1 (23) with a 1,000-repetition

Table 1. Chemical and physical characteristics of cultivated soil (CS) and forest soil (FS).

Parameters	CS	FS
pH	6.2	4.9
Organic matter (g dm^{-3})	41	48
K^+($mmol_c$ dm^{-3})	4.5	1.2
P resin (mg dm^{-3})	63	39
Ca^{2+} ($mmol_c$ dm^{-3})	67	33
Mg^{2+}($mmol_c$ dm^{-3})	50	19
Clay (g kg^{-1})	390	510
Silt (g kg^{-1})	290	250
Fine sand (g kg^{-1})	190	150
Coarse sand (g kg^{-1})	130	90
Textural Class	Argillaceous	Argillaceous

bootstrap. The species richness estimation was performed with the softwares "EstimateS", version 6.0b1 (16), and the nonparametric estimator ACE (Abundance-base Coverage Estimator).

Arlequin software was used to estimate genetic structure among bacterial populations from the two soils and intraspecific genetic diversity. Average pairwise differences were estimated from comparisons within a library of the number of sequence differences between a given clone and all other clones (36). To estimate genetic diversity within the two libraries, some indexes were calculated using a distance method with the Kimura-2-parameter substitution nucleotide model. Nucleotide diversity was estimated from the number of variable positions for aligned sequences in a given library. The 16S rRNA gene sequences deposited in GenBank are shown at the electronic address: http://lbmp.fcav.unesp.br/publications/rodrigo/molecular.htm.

RESULTS

A total of 139 clones belonging to two libraries: intensively cultivated soil (CS) and under-forest soil (FS), were partially sequenced. After sequencing, quality analysis processes and comparison by BLAST, 88 sequences from the FS library (among them, 65 OTUs from different genus) and 50 sequences from the CS library (40 from different genus) presented suitable quality and size for the purpose of the present study. Moreover, the partial sequencing made the comparison of microbial diversity in both soils possible.

After comparison of sequences obtained with the registered sequences in the international bank of genes (GenBank), the similarity rates ranged from 82% to 99%. The majority of the observed sequences had a bacterial origin, except for one sequence belonging to the A*rchaea* domain. All the sequences were registered at GenBank (table showed at the electronic address http://lbmp.fcav.unesp.br/publications/rodrigo/molecular.htm). Furthermore, taxonomically unclassified bacteria made up 2.3% of the FS library and 8% of the CS library.

Different sequences with more than 100 bases having 20 or higher Phred quality (13) were aligned and afterwards used for the construction of phylograms in order to phylogenetic assign the taxonomically unclassified sequences. This has also allowed us to distinguish the bacterial populations belonging to cultivated and under-forest soil, as well as to assess the frequency of different microorganisms in such soils, as shown in Figure 1 and Figure 2.

In the CS library, 40 different representatives classified into 5 phyla were found, whereas only 4 sequences could not be classified within any phyla, as shown in Figure 1. On the other hand, in the FS library, which had 65 different representatives, all were gathered in 6 phyla, except for two clones (2.3%) that could not be classified (Figure 2). Less than a quarter of the assessed clones could not be classified in a known phylum of the bacteria domain. Sequences that could not be allocated to a known phylum are represented by the letters NC in the phylograms. Through analysis of different sequences from both soils we observed that within the 105 different sequences (40 CS and 65 FS) (Table 2), only 8 appeared in both soils.

The phylum presenting the largest number of different bacteria in the FS library was *Acidobacteria* (43.3%). The *Proteobacteria* phylum (36%) had the largest number of bacteria in the CS library.

The phylum *Verrucomicrobia*, which represents 19.3% of the total clones (17 clones, with 15 different clones), was the third largest phylum in terms of diversity in the FS library.

Representatives of phylum *Firmicutes* were more abundant in the CS library, representing 34% of the total clones (17 clones, 12 of them different), as is shown in table 2. In the FS library, the diversity of this phylum is extremely small, 4.5%.

Table 2. Distribution of the cloned 16S rRNA gene sequences, grouped by *phyla* from cultivated soil (CS) and forest soil (FS).

Domain Bacteria PHYLA	CS Number of distinct clones	CS Total Number of clones observed	%	FS Number of distinct clones	FS Total Number of clones observed	%
Acidobacteria	0	0	0	19	39	44.3
Actinobacteria	4	5	10	4	4	4.5
Bacteroidetes	3	3	6	5	5	5.7
Firmicutes	12	17	34	4	4	4.5
Proteobacteria	14	18	36	16	17	19.3
Verrucomicrobia	3	3	6	15	17	19.3
Unclassified bacteria (NC)	4	4	8	2	2	2.3
Total	40	50	100	65	88	100.0

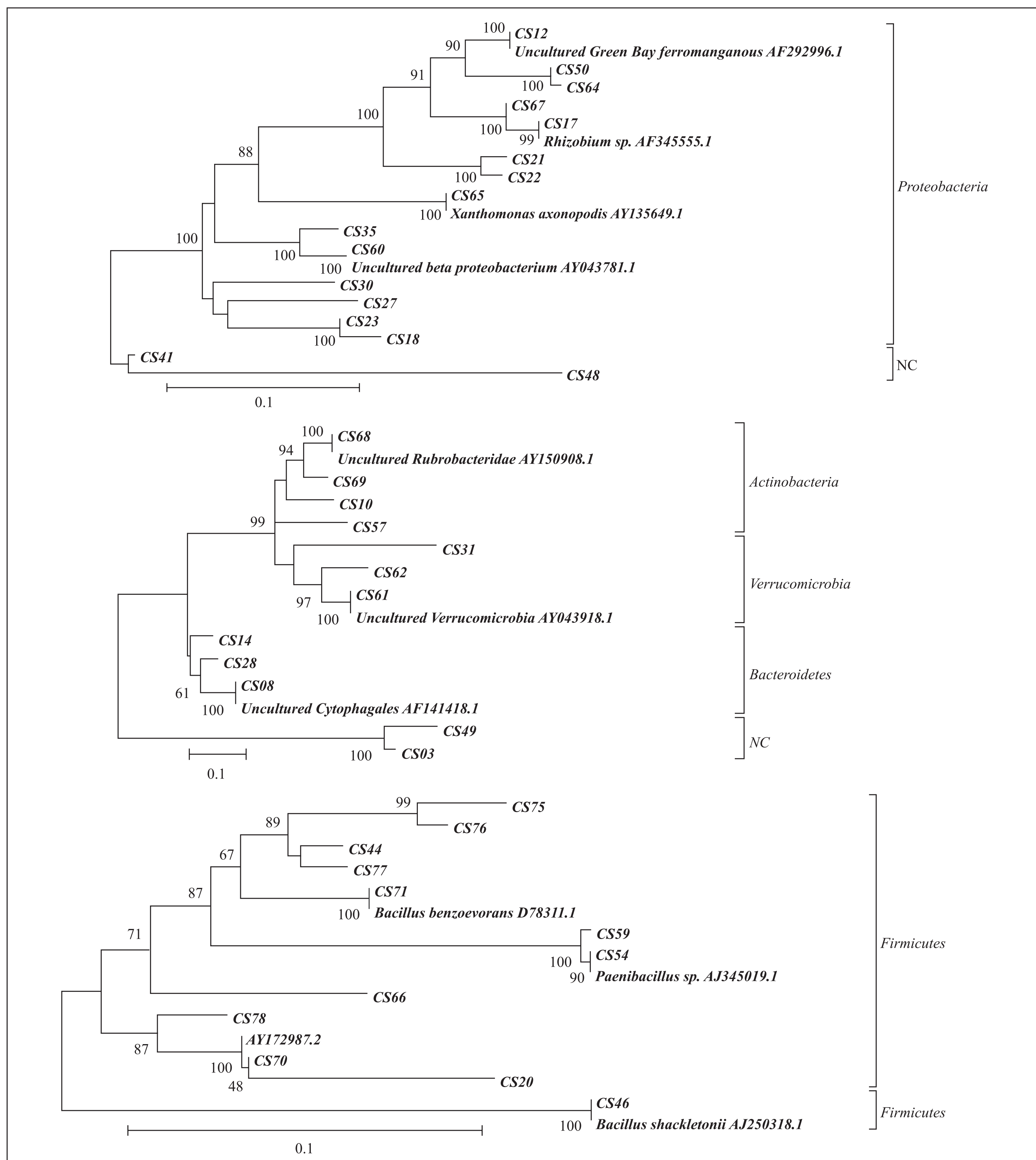

Figure 1. Dendrogram of partial sequence from 16S rDNA from CS (Cultivated soil), grouped by phylum. These dendrograms were constructed from a matrix of Jukes-Cantor distances by the neighbor-joining method. The numbers at the nodes of the tree indicate bootstrap values for each node out of 100 bootstrap resamplings (values below 50 are not shown). The scale bar represents 0.1 substitutions per base position. The key NC represents clones that could not be grouped with any other phyla.

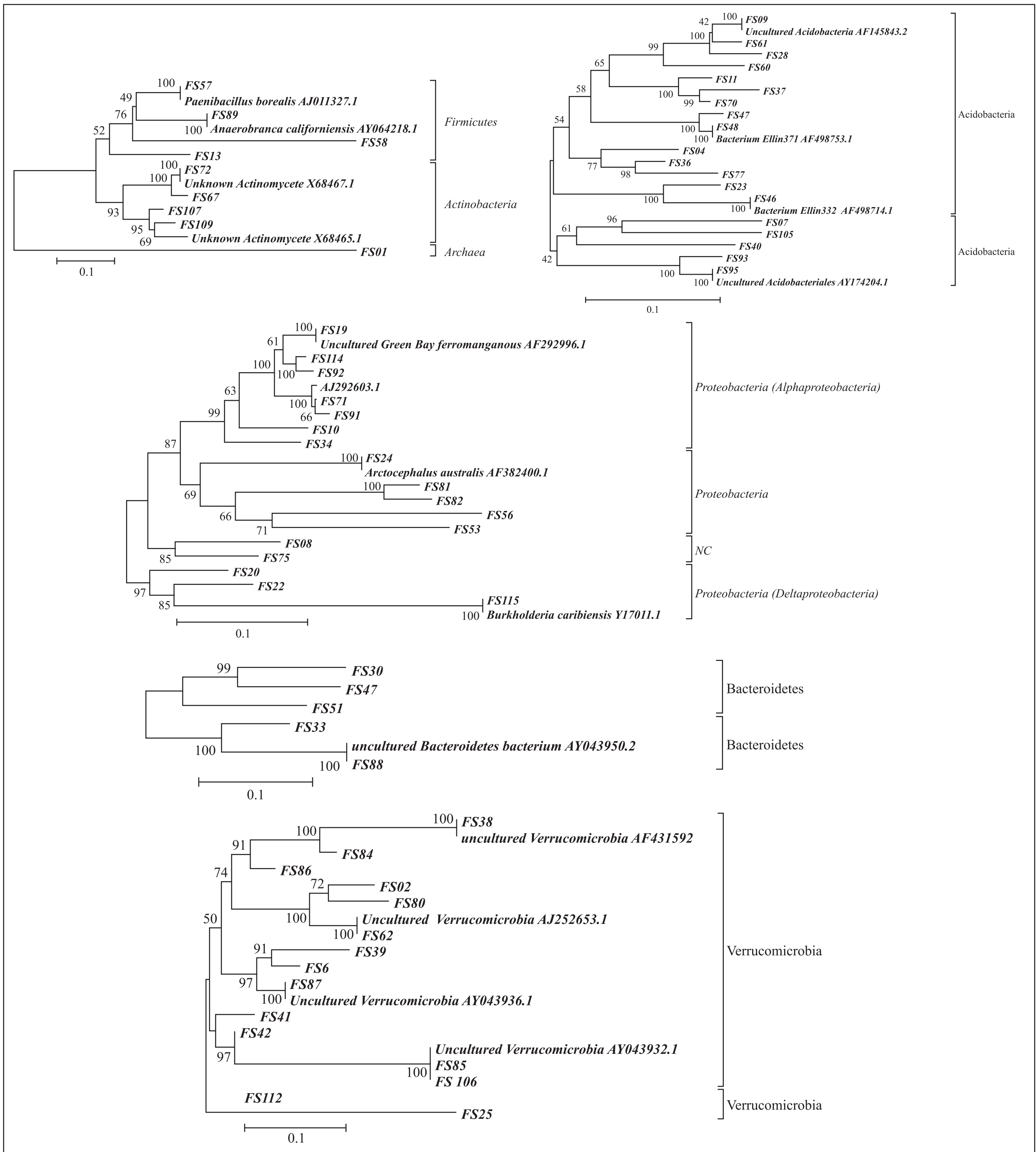

Figure 2. Dendrogram of partial sequence from 16S rDNA from library FS (Forest soil), grouped by phylum. These dendrograms were constructed from a matrix of Jukes-Cantor distances by the neighbor-joining method. The numbers at the nodes of the tree indicate bootstrap values for each node out of 100 bootstrap resamplings (values below 50 are not shown). The scale bar represents 0.1 substitutions per base position. The key NC represents clones that could not be grouped with any other phyla.

The second *taxon* with the most representatives in the FS library (Table 2) is the phylum *Proteobacteria*, representing 19.3% of the total clones (17 clones, 16 different OTUs). In the CS library, this phylum represents 36% of the total clones (18 clones), with 14 different sequences, and is the phylum with most representatives.

The bacteria belonging to the *Actinobacteria* phylum can also be described as gram-positive with high levels of G+C. They are found in the FS library, representing 4.5% of the total clones, with 4 different OTUs. There is a slightly larger number in the CS library: 10% of the total clones, with 4 different OTUs.

The *Bacteroidetes* phylum also has a small number of representatives in both soils: 6% of the total clones, and 3 different sequences in the CS library. In library FS, this quantity rose to 5.7% of the total clones, 5 different sequences and one physiologically different phylum, including aerobic and anaerobic bacteria. Among the genders of this phylum, the most frequently reported in soils is *Flexibacter*.

Although the oligonucleotide used in the present study is specific for bacteria, it was possible to detect the isolated presence of an *Archaea* in library FS. Representing only 1% of the total clones, the *Archaea* domain was present in only one representative.

Analysis of the genetic differentiation (Fst 0.013, $P \leq 0.05$) between the two libraries shows that the greatest source of variation is intra-specific, that is, the individuals belonging to the same library present a greater average genetic diversity among them (98.65) than when compared to individuals from the other library (1.35).

The FS library presented different bacterial phyla from the CS library. Diversity indexes were calculated for each library and indicate the presence of genetic diversity among the individuals that constitute each library. The CS library has a smaller number of copies of genes and polymorphic loci than FS library, and also a smaller genetic diversity, reflected by the average difference between pairs (pairwise differences) (Table 3). The FS library, on the other hand, has a greater index of nucleotide diversity, polymorphic loci and average difference between pairs than library CS, which is a consequence of the genetic diversity that exists within it. The Ace diversity index confirms the results above (Table 3).

Table 3. Intraspecific population analysis from cultivated soil (CS) and forest soil (FS).

Index	Library CS	Library FS
Nucleotide diversity	0.297616 +/- 0.143504	0.355038 +/- 0.169744
Average pairwise differences	135.117798 +/- 58.745682	161.146693 +/- 69.612515
Ace	291,03	337,15

DISCUSSION

Studies approaching the estimates of bacterial diversity in soil report different quantities of bacteria that cannot be classified within a known phylum (2,44). Among all taxons presented in this characterization, that which suffered the largest changes was the phylum *Acidobacteria*. This could possibly be caused by soil pH, which was higher in cultivated soil (CS) (6.2 em $CaCl_2$, Table 1) than in under-forest soil (FS) (4.9 em $CaCl_2$, Table 1), due to limestone application. This fact reinforces the observations by Hirashi *et al.* (17) regarding the better development of these bacteria in acidic environments.

The bacteria belonging to the phylum *Acidobacteria* stood out the most in the FS library, representing 19 different clones, amounting to a total of 39 (44.3% frequency) (Table 2). This phylum, proposed in 1995 (Hirashi *et al.*, 1995), is not well known yet, and environmental studies report the occurrence of this phylum's sequences in several ecosystems. Organisms belonging to this phylum were found in volcanic soils, cultivable soils, forests and areas under pinus, besides swamp, river and thermal source sediments, suggesting that these microorganisms are important ecological components. This can be observed in the present study and others (9,18,39).

As proposed in 1997 (15), the phylum *Verrucomicrobia* has widespread representatives in the environment, especially in the soil, as does the phylum *Acidobacteria*. Members of this phylum have been observed in several environments such as soil (3), although little is known about their function. In the CS library, the number of phylum *Verrucomicrobia* representatives was unexpectedly low as is shown in table 2, representing only 6% of the total soil clones, of which 3 were different.

Representatives of phylum *Firmicutes* were more present in CS. A possible explanation is that some representatives of this phylum, such as bacteria of the genus *Bacillus* and *Clostridium*, use as survival strategy a rapid growth when there are nutrients in abundant quantities, which is known as strategy r (2). These strategists are not good competitors, having rapid multiplication as their only adaptation advantage in relation to competitors. They prevail only if there is a great quantity of available nutrients in low-competition areas. Consequently, there are huge variations in their populations. Strategists r are usually found in unstable environments suffering transitions (2). Cultivated soils are continuously under anthropic action, which causes constant changes in the microbial community and favors strategists r. By analyzing the diversity of *Firmicutes* in library CS, we observed that all sequences belonged to the class *Bacilli*. Among them were *Paenibacillus sp,* known for fixing nitrogen, and *Bacillus benzoevorans,* which appears in cultivable soils around the world (40).

On the other hand, in the library FS, the diversity of such phylum is extremely small, representing 2.9% of

the total clones, with three sequences being different: one from the *Bacilli* class, the other two from the *Clostridia* class. Representatives of both classes are able to fix nitrogen and said to foster the growth of certain species of plants (24). Moreover, the data on abundance (Table 2) confirms what has been said above about these organisms' survival strategy and its ineffectiveness when there is strong competition for soil nutrients.

The phylum *Proteobacteria* is the largest and most varied group of cultivated bacteria, presenting a great morphologic and metabolic diversity. Because of such characteristics, it occurs in several different environments, accounting for its occurrence in an under-forest soil. Other studies have also observed its presence in many different environments (8,18,37,39). However, it occurs more frequently in cultivated soils, as shown in Table 2. It is possible that the most significant presence of bacteria belonging to the *Proteobacteria* phylum in cultivated soil is linked to the fact that some representatives of the *Alphaproteobacteria* class develop more readily in fertilized soils (30).

The study of microbial diversity through analysis of libraries of 16S rRNA clones from two pasture soils, with different fertilizer application, has shown that in both areas *Alphaproteobacteria* class representatives were the most frequent. The high growth rates of bacteria belonging to the *Alphaproteobacteria* class in soils with an abundance of available nutrients suggest that they are "strategists r" (39). The fact that the quantity of such bacteria is highest in cultivated soils (CS) can be confirmed in Table 2, which is in accordance with the aforementioned studies.

Another point that should be observed is that the order *Rhizobiales* belongs to the class *Alphaproteobacteria*, and many representatives of this order are nitrogen-fixers. Beans were grown in the intensely cultivated soil (CS), which might account for the increased number of this order's representatives. It is also important to point out that the other observed representatives, such as *Herbaspirillum rubrisubalbicans, Bradyrhizobium sp* and *Rhizobium sp*, play an important role in plant development, either by producing IAA or fixing nitrogen (43).

The members of the *Actinobacteria* and *Bacteroidetes* phyla are apparently the same size in CS and FS soils. Similar results were obtained when under-forest soil and vegetables soil were compared. Representatives of the phylum were also present in the under-forest soil in a slightly larger quantity (42). Representatives of the *Actinobacteria* phylum produce slow-growth antibiotics that are commonly found in pasture soils (3,9) and cultivated soils (4,28,31), nearly always with small populations. An exception is a soil cultivated with wheat in Holland. Analyses of the 16S rRNA sequences resulted in more than 70% of the clones belonging to this phylum (39). In the present study, a small population – slightly larger in the under-forest soil – was observed in the soils. Previous studies report that the *Actinobacteria* phylum usually forms small populations, compared to other phyla (9). Moreover, its frequency is not affected in cultivated soils (9,28). Some cultures such as eucalyptus, however, may foster the development of this bacterial community, as observed by Silveira (38). Observed in small populations, the *Bacteroidetes* phylum has already been reported in arid soil from the United States (25), in cultivated soils, under-forest soils and pasture soils (3,4,8).

The *Archaea* domain lives in extreme conditions and is frequently found in the soil. However, it is not possible to claim that other member of the *Archaea* domain are not present, since the "primer" used is not specific for them, therefore this phylum was not used for the computation in Table 2 and Table 3. The occurrence of the *Archaea* domain was also reported both in pasture soils and soils covered by Amazon native forest (3).

The greater nucleotide diversity index in FS (Table 3) indicates the possibility of the same position in the DNA sequence having one or more nucleotide variations. This is confirmed by the average difference index in the base pairs that appears in FS (Table 3), which is calculated by the comparison of differences among the nucleotides that share the same position in a clone's sequences in relation to all other clones in a library (36). The great intra-specific diversity indicates that the diversity in library FS may correspond to a greater microbial diversity in soil of this area. The greater genetic diversity, calculated through DNA sequence analysis, is confirmed by the larger number of phyla found in this soil. One hypothesis to explain this fact is that the FS soil, covered by forest vegetation, has not yet suffered anthropic action. As a result, bacterial populations have a longer period of time to adapt to the environment and thus present more synergism.

This bacterial characterization of gene 16S rRNA allowed the identification of several bacterial populations in the soil belonging to the following phyla: *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Verrucomicrobia* and *Archaea*, in addition to others that were not classified, beyond *Archaea* domain. Differences between FS and CS libraries were observed in the size of the *phyla*. A larger number of phyla and, consequently, a greater bacterial diversity were found in the under-forest soil. These data were confirmed by analyses of genetic diversity that have been carried out previously. The present study has made its contribution by providing data for further studies on the dynamics of bacterial populations in different soil conditions, as well as by improving current understanding populations of microorganisms that exist in Brazilian soils.

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Resumo

Caracterização molecular de populações bacterianas de diferentes solos

Até o momento poucos estudos foram realizados no Brasil a respeito da diversidade de comunidades bacterianas no solo. Com o objetivo de caracterizar as populações bacterianas presentes no solo através da análise do gene 16S rRNA, foram analisados dois solos: um caracterizado pelo uso intensivo, principalmente para a produção de tomate, feijão e milho (CS); e outro sob floresta (FS), não modificado pelo homem, ambos do município de Guaíra, no estado de São Paulo, Brasil. Usando oligonucleotídeos específicos, de genes 16S rRNA do DNA metagenomico de ambos os solos foram amplificados por PCR, amplicons foram clonados e 139 clones de duas bibliotecas foram seqüenciados. O uso da técnica de 16S rRNA, gerou a identificação de diferentes populações de bactérias de solo pertencentes aos filos *Acidobacteria Actinobacteria Bacteroidetes Firmicutes Proteobacteria Verrucomicrobia, Archaea*, além das não classificadas. Diferenças entre as bibliotecas FS e CS foram observadas no tamanho dos filos. Um grande número de filos e, consequentemente, uma grande diversidade bacteriana foi observada no solo sob floresta. Estes dados foram confirmados pela análise de diversidade genética realizada. A caracterização de comunidades do solo apresentada neste trabalho contribuiu fornecendo dados para estudos posteriores sobre a dinâmica das populações bacterianas em solos de diferentes condições no Brasil.

Palavras-chaves: metagenoma, diversidade microbiana, 16S rRNA

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