Bacterial diversity of soil under eucalyptus assessed by 16S rDNA sequencing analysis

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Abstract – Studies on the impact of *Eucalyptus* spp. on Brazilian soils have focused on soil chemical properties and isolating interesting microbial organisms. Few studies have focused on microbial diversity and ecology in Brazil due to limited coverage of traditional cultivation and isolation methods. Molecular microbial ecology methods based on PCR amplified 16S rDNA have enriched the knowledge of soils microbial biodiversity. The objective of this work was to compare and estimate the bacterial diversity of sympatric communities within soils from two areas, a native forest (NFA) and an eucalyptus arboretum (EAA). PCR primers, whose target soil metagenomic 16S rDNA were used to amplify soil DNA, were cloned using pGEM-T and sequenced to determine bacterial diversity. From the NFA soil 134 clones were analyzed, while 116 clones were analyzed from the EAA soil samples. The sequences were compared with those online at the GenBank. Phylogenetic analyses revealed differences between the soil types and high diversity in both communities. Soil from the *Eucalyptus* spp. arboretum was found to have a greater bacterial diversity than the soil investigated from the native forest area.

Index terms: genetic diversity, metagenomic, microbial communities.

Diversidade bacteriana de solo sob eucaliptos obtida por seqüenciamento do 16S rDNA

Resumo – Estudos sobre impacto do *Eucalyptus* spp. em solos brasileiros têm focalizado propriedades químicas do solo e isolamento de microrganismos de interesse. No Brasil há pouco enfoque em ecologia e diversidade microbiana, devido às limitações dos métodos tradicionais de cultivo e isolamento. A utilização de métodos moleculares no estudo da ecologia microbiana baseados na amplificação por PCR do 16S rDNA têm enriquecido o conhecimento da biodiversidade microbiana dos solos. O objetivo deste trabalho foi comparar e estimar a diversidade bacteriana de comunidades simpátricas em solos de duas áreas: uma floresta nativa (NFA) e outra adjacente com arboreto de eucaliptos (EAA). Oligonucleotídeos iniciadores foram utilizados para amplificar o 16S rDNA metagenômico do solo, o qual foi clonado usando pGEM-T e seqüenciado para determinar a diversidade bacteriana. Foram analisados 134 clones do solo NFA e 116 clones do solo EAA. As seqüências foram comparadas às depositadas no GenBank. Análises filogenéticas revelaram diferenças entre os tipos de solos e alta diversidade em ambas comunidades. No solo de arboreto de *Eucalyptus* spp. foi encontrada maior diversidade bacteriana em comparação com o solo da área de floresta nativa.

Termos para indexação: diversidade genética, metagenômica, comunidades microbianas.

Introduction

The microbial diversity of soil is huge (Ward, 2002), the domain Bacteria includes 23 phyla, and there are only 6.250 known species (Garrity et al., 2005). Estimates point that just one to 10% of microorganisms can be isolated by common culture methods (Borneman et al., 1996; Kuske et al., 1997). Considering only the cultivated microorganisms of microhabitat, a great amount of interesting knowledge about some biological interactions can be lost (Atlas & Bartha, 1998).

Soil cannot be considered an isolated ambient, because direct interactions exist among the microorganisms, water, air, plants and animals (Drozdowicz, 1997). To control and keep soil's quality, it is important to maintain the diversity of microorganisms, because they carry out important functions, such as to promote toxins removal and participate in the biogeochemical cycles (Borneman et al., 1996). The type and presence of plants as well as soil management procedures are factors that can be decisive in the composition of many soil microbial communities, however, there are insufficient reports on the influence of the plants species type on the microbial communities (Nüsslein & Tiedje, 1999).

The genus *Eucalyptus* belongs to the family Myrtacea and Brazil is the greatest producer of this monoculture worldwide. Industrial production, including paper and cellulose, are among the most important for the Brazilian economy (Andrade, 2001). Crop rotation of this plant for industrial purposes can diminish nutrients in rich soils, however, the use of this monoculture in degraded acid soils is a method which returns soil organic matter to soils and is recommended under certain condition (Lima, 1996).

Most studies on eucalyptus focus on the impact this tree has on the chemical properties of the soil, nutrient content, ions exchange capacity and microbial activity (Lima, 1996), as an example microorganisms involved in sulfur transformations (Pinto & Nahas, 2002) and natural phosphates solubilization (Silva Filho et al., 2002). Several papers describe major impacts of the use of this monoculture on the microbial communities (Lima, 1996) as well as the possibility of ecological changes on soils cultivated under eucalyptus (Fonseca, 1984).

Given the fact that molecular techniques unveil much more of the enormous environmental microbial diversity than cultural techniques, there is a need to correlate the biodiversity with the soil chemistry and ecosystem function (Kuske et al., 1997; Sandaa et al., 1998).

Studying biodiversity based on an extraction of all DNA from a given environment, air, soil, water, can be called a metagenomic approach (Rondon et al., 2000) and bacterial diversity can be assessed sequencing 16S rRNA genes. This methodology constitutes a powerful approach to explore soil microbial diversity, which includes the uncultivable microorganisms (Hentschel et al., 2002). 16S rDNA segments can be considered excellent molecular markers since they harbor characteristics conserved in all bacterial genomes during evolution (Atlas & Bartha, 1998). First metagenomic approaches using this methodology in Brazil were reported by Borneman & Triplett (1997), who analyzed the bacterial diversity of soil from several Amazon environments.

The objective of this work was to investigate the genetic and phylogenetic diversity, as well as to estimate the number of bacterial species in soils of two Brazilian areas: a native forest (NFA) and an *Eucalyptus* spp. arboretum (EAA). This was done by analyzing 16S rDNA cloned from the metagenomic DNA of each soil type. This strategy represents a way to take into consideration the uncultivable microorganisms, gathering data related to the expressed genes which are still unknown.

Material and Methods

In May 2002, soil samples were extracted from two areas situated at 21º14'S, 48º17' W, approximately 500 m apart one from another, near Faculdade de Ciências Agrárias e Veterinárias (FCAV/Unesp). Two soil samples were used, one collected from a native forest area (NFA), classified as tropical broadleaf semideciduous forest, typical eutrofic Rhodic Hapludox moderate A, kaolinitic-oxidic, eutroferric, very clayey texture (Embrapa, 1999), and the other soil sample collected from an *Eucalyptus* spp. arboretum (EAA), cultivaded since 1969, classified as typical eutrofic Rhodic Hapludox, moderate A, kaolinitic hypofferic, medium texture (Embrapa, 1999), and not subjected to any agricultural practice. Sampling strategy was a random zigzagged manner, 20 different spots (0–20 cm of soil depth). The 20 samples of each location were then homogeneized, resulting in a composit sample for each soil. An aliquot of each sample was set apart with the objective to follow chemical and granulometric analysis (Table 1), and another one for metagenomic DNA extraction.

Total metagenomic DNA was extracted from soil using the FastDNA Spin Kit for Soil (Bio 101 – catalog #6560-200), according to the manufacturer instructions, using 1 g of soil from each area. Metagenomic DNA was used in PCR reactions as the template to amplify the 16S rRNA gene, using specific primers pA 5'- AGA GTT TGA TCC TGG CTC AG - 3' and pc5B 5'-TAC CTT GTT ACG ACT T-3" (Kuske et al., 1997). The PCR reaction was carried out with 1X buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl], 200 µM of each deoxynucleotide, 1,5 mM $MgCl_2$, 50 pmols of each

primer, 2.5U Taq DNA polymerase (Invitrogen, São Paulo, Brazil) and 50 ng metagenomic DNA, comprising a final volume of 50 µL (Kuske et al., 1997). Ten reactions were carried out per soil. Samples were placed in a thermal cycler to amplify the gene, adopting the program described by Kuske et al. (1997). The amplicons with 1.5 kb were analyzed by electrophoresis and were evaluated from the gel by adding 1M NaCl, phenol and chloroform (Sambrook et al., 1989).

The 16S rRNA gene amplicons obtained were inserted in pGEM T vector (Promega, Madison, WI, USA – catalog # A3600), according to manufacturer instructions, and used to transform the *Escherichia coli* DH5α lines. After cultivating the transformed clones, plasmids were isolated by miniprep (Sambrook et al., 1989). The partial sequencing of the gene 16S rRNA of each clone was done using 0.4 µL DNA Sequencing-Big Dye Terminator Cycle Sequencing-Ready ABI Prism (Version 3); 3.2 pmols M13/pUC1211 of the oligonucleotide primer (5' - GTAAAACGACGGCCAGT - 3'); 100 ng of plasmidial DNA, 4.6 µL of buffer (400 mM Tris-HCl, pH 9; 10 mM $MgCl_2$); and H_2O mili-Q (Millipore) to a 10 µL volume. Reactions were incubated in a thermal cycler for two minutes at 96ºC and subjected to 40 cycles of 96ºC for ten seconds, 52ºC for 20 seconds, and 60ºC for four minutes. Amplicons were sequenced by a capillary sequencer, model ABI 3700 (Applied Biosystems, Foster City, CA, USA).

The quality of generated sequences was verified through the software Sequencing Analysis 3.4 (Applied Biosystems, Foster City, CA, EUA), which produced electropherograms. These electropherograms were then analyzed by the programs Phred (Ewing et al., 1998), Phrap and Consed (Gordon et al., 1998).

Table 1. Chemical and granulometric parameters from the native forest (NFA) and from the eucalyptus arboretum (EAA).

Parameters	NFA	EAA
pH ($CaCl_2$)	6.2	5.5
Organic matter (g dm^{-3})	75	61
P (mg dm^{-3})	63	17
K ($nmol_c$ dm^{-3})	2.9	4.8
Ca ($nmol_c$ dm^{-3})	410	49
H+Al ($nmol_c$ dm^{-3})	15	28
Mg ($nmol_c$ dm^{-3})	80	40
Clay (g kg^{-1})	430	440
Silt (g kg^{-1})	256	120
Fine sand (g kg^{-1})	130	180
Coarse sand (g kg^{-1})	184	260
Textural classification	Clayey	Clayey

Sequence selection was made through the program Contgen, with a minimum requirement level of 400 bases and Phred quality over 20. Sequences were subjected to nucleotide similarity comparison, and the sequences were placed in the GenBank data bank, accessed through National Center for Biotechnology Information (NCBI). The program used for comparison was Basic local alignment search tools (Blast) (Altschul et al., 1997).

Clones with frequency higher than one were selected through electropherogram analysis, by the Blast scoring result, so that the phylogram presented only one representative of each organism reported by the NCBI. Each distinct sequence obtained was defined as Operational Taxonomic Unit (OTU).

The distinct sequences were verified through a pre-alignment performed by the program BioEdit v5.0.9 (Hall, 1999), and subsequently aligned by the program CLUSTALX v.1.81 (Thompson et al., 1997) and subjected to phylogenetic group analysis. The phylograms were constructed using the Neighbor-Joining algorithm (Saitou & Nei, 1987), with the model of nucleotide replacement of Kimura 2-P (Kimura, 1980), using the software MEGA, version 2.1 (Kumar et al., 2001) with 1,000 bootstrap replicate.

Distance matrices for both soils were generated from sequences aligned by BioEdit (Hall, 1999), through the program DNADIST [PHYLIP] (Felsentein, 1989) using the nucleotide replacement model Jukes and Cantor (Jukes & Cantor, 1969). These distance matrices were used for genetic diversity analysis and library comparisons by the program Libshuff (Singleton et al., 2001). The program Libshuff was used to estimate heterologous coverage curves to determine how much of a library was represented in another one.

Aligned sequences of the gene 16S rRNA were used to estimate the genetic difference (F_{st}) between the libraries of both areas, as well as average pairwise differences and nucleotide diversity through the program Arlequin (Schneider et al., 2000).

The estimated number of the species in each area was reached with the help of the program estimates version 6.0b1 (Hill et al., 2003) through the nonparametric estimator ACE (Abundance-base Coverage Estimator), using the sampled frequency of each clone from the libraries.

Results and Discussion

The results from the Blast program revealed that 72% of the sequences were novel and from bacteria that have not been cultivated yet. This was expected since bacterial diversity in the soil is very high and still largely unknown. A soil study in the Amazon region (Borneman & Triplett, 1997), using the same molecular technique, also found that a large number of their sequences were most similar to bacteria that have not yet been cultivated. This result confirms the heterogeneous composition of the soils, and shows that cloning the gene 16S rRNA is an important tool to identify novel bacterial sequences in natural ecosystems, which in turn may help lead to their isolation for biotechnology purposes.

Out of 62 OTU found in NFA, 51 clustered in eight different phyla, and 11 of them could not be classified to main bacterial phyla, and represent currently unknown bacteria. In EAA soil, among 61 OTU sequenced, 56 were clustered in 7 phyla, and 5 OTU are not still classified (Figures 1, 2 and 3).

Analyzing clones distribution in the different phyla, the majority of the NFA sequences, 44.78%, fell within the *Acidobacteria* phylum (Table 2). It was expected to find a larger percentage of this phylum in EAA, given that the soil of that area presented lower pH (Table 1). Instead, a more homogeneous distribution of phyla was observed, with 25% of the sequences within the *Acidobacteria* clade (Table 2). The phylum *Acidobacteria* was proposed by Hirashi et al. (1995) based on 16S rDNA sequences, and despite sequences from this group recovered globally, this group is relatively unknown (Nüsslein & Tiedje, 1999). Currently there are three isolates catalogued as part of the phylum *Acidobacteria* (Quaiser et al., 2003) . However, molecular studies indicate that this phylum may be found in almost every habitat in the world, in different ecosystems, at an average of 30 to 50% of bacterial population (Quaiser et al., 2003).

Distinct frequencies of some phyla in each area were observed. Microorganisms that belonged to the phyla *Bacteroidetes* and *Planctomycetes* were found in larger quantity in NFA, and sequences classified as *Actinobacteria*, *Firmicutes* and *Verrucomicrobia* are in larger quantities in the EAA soil. The population increase of actinomycetes in EAA in relation to NFA (Table 2) was observed. There is high economical interest in this phylum due to its capability of producing many antibiotics, exerting control over other bacterial groups. Soil bacteria from the eucalyptus soil may play an important role in the soil under eucalyptus (Canhos & Vazoller, 1997)

A predominance of the phyla *Actinobacteria* was observed in the soil under eucalyptus. The presence of organic nutrients exuded by roots attracts chemoorganotrophic microorganisms that can exploit them (Cardoso & Freitas, 1992). Exudates are selective, and only a limited number of microbial species prevail in a determined soil root environment. It is interesting to hypothesize that eucalyptus may have indirectly selected for the bacterial diversity found in this soil.

The increase of actinomycetes may also be attributed to an instability caused by eucalyptus monoculture, because of its advantages over most other bacterial groups and their spore production, which are important resistance structures of induced microorganisms when the environment suffers an impact. The perception of this group's increase under this cultivation, in relation to the area under native forest, reveals new perspectives for future studies searching for biotechnological products, or metabolic products, especially not yet cultivable actinomycetes.

Similar results were achieved by Pereira et al. (1999), who evaluated the effect of environmental conditions on bacterial communities, using traditional cultivation techniques in two areas of the Brazilian Cerrado, one of them a natural ecosystem (native vegetation) and the other a soybean field. Results showed an increase of actinomycetes, probably due to environmental changes caused by soybean cultivation. When instability takes place in a natural ecosystem, this particular phylum appears to develop an important role in the bacterial community, occupying a greater ecological niche, where it was not the majority, possibly aiming to correct the environment in the long run. A hypothesis of an increased role for actinomycetes has to be balanced by the inherent short comings involved in both culture techniques and using PCR techniques to quantitatively assess changes in microbial communities (Rondon et al., 2000).

The results obtained in this study for the phyla *Verrucomicrobia* showed a population increase from 2.24% in NFA to 19.83% in EAA (Table 2). Different results were found by Lee et al. (1996). In a study carried out in fields of Australia and the USA, they observed that approximately 1 to 10% of the bacteria belonged to this phylum. Eucalyptus farming may be one soil factor that selects for increased representation of this phylum in soil.

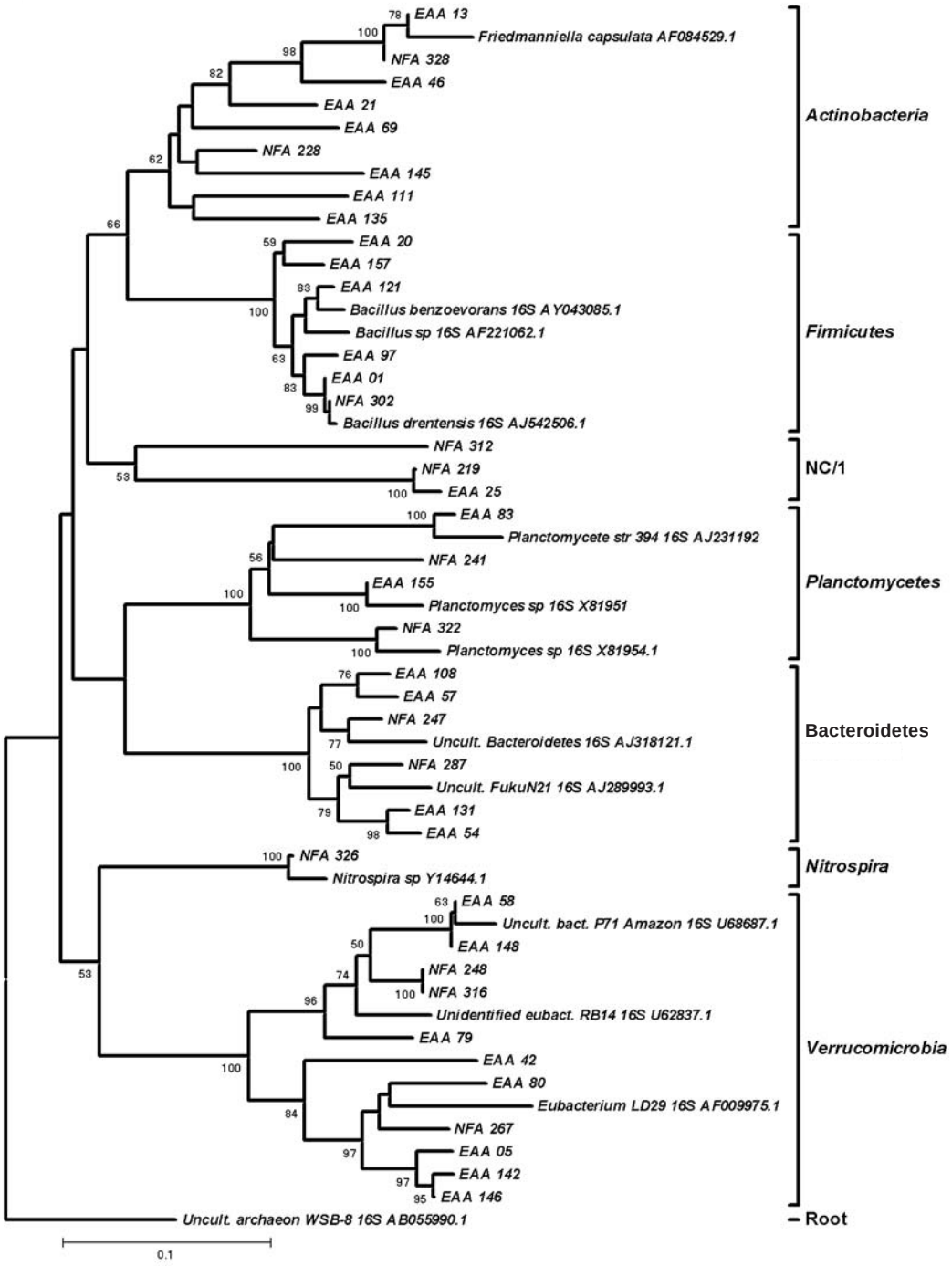

Figure 1. Phylogenetic dendrogram showing clones belonging to *Verrucomicrobia*, *Planctomycetes*, *Nitrospira*, *Actinobacteria* and *Firmicutes*, obtained from partial sequences of 16S rRNA gene from native forest soil (NFA) and eucalyptus arboretum area (EAA). The distance matrix and construction were performed using Kimura 2P algorithm and the Neighbor-Joining method, with a 1,000-repetition bootstrap above 50%. The 16S rDNA sequence of Uncultured Archaeon (no. GenBank AB 055990) was used as outgroup. All clones are numbered and sequences in the database are indicated by the GenBank access number.

Lindström et al. (2004) investigated the factors that affect the bacterioplankton composition in different depths in a lake in Sweden with different phosphorus (P) treatments, and concluded that the high availability of this element favored this group's population. In contrast, the soil under eucalyptus (EAA), which showed lower phosphorus content than the soil under forest, had a population increase of this phylum (Table 1).

Only one sequence was classified as belonging to the phylum *Nitrospira*, and this particular microorganism was only found in the NFA soil. The percentage of sequences of *Proteobacteria* in NFA and EAA was found to be in similar proportions: 17.16 and 22.40%, respectively (Table 2). The OTU of this phylum are distributed in four different classes in both soils: *α-Proteobacteria, β-Proteobacteria, δ-Proteobacteria* and *γ-Proteobacteria* (Figure 2). Different studies performed in Scottish (McCaig et al., 1999) and Brazilian soils (Pereira, 2003) showed that this phylum prevailed when compared to the other phyla. These authors suggest that *Proteobacteria* phyla is favored in soils that have been used to agricultural practices, which however has not occurred in soils in which eucalyptus cultivars were grown for a long time.

The analysis of the bacterial population diversity in the studied soils by non parametrical estimative analysis (ACE) showed a higher bacterial diversity in the area under eucalyptus, with 139 OTU, while this index in the area under forest was 117 OTU. Greater bacterial diversity in soil under eucalyptus than in a decidous tropical forest was reported. Similar results were observed in the work performed by Fonseca (1984). This author made chemical, physical and microbiological analysis in three

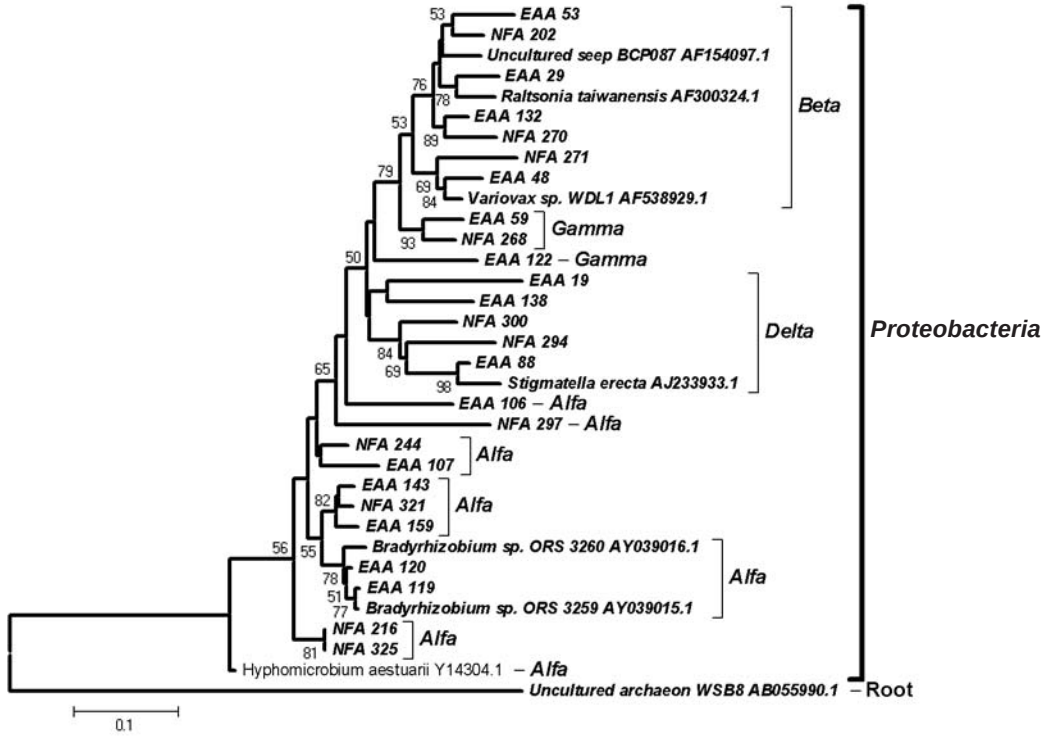

Figure 2. Phylogenetic dendrogram showing clones belonging to *Proteobacteria*, obtained from partial sequences of 16S rRNA gene from native forest soil (NFA) and eucalyptus arboretum area (EAA). The distance matrix and construction were performed using Kimura 2P algorithm and the Neighbor-Joining method, with a 1,000-repetition bootstrap above 50%. The 16S rDNA sequence of Uncultured Archaeon (no. GenBank AB 055990) was used as outgroup. All clones are numbered and sequences in the database are indicated by the GenBank access number.

different areas of the Vale do Rio Doce region of Brazil: one under natural forest, another with pasture, and the third an area of reforestation with three different species of *Eucalyptus*. Using microbial cultivation techniques with selective means, this author showed the presence of 2.5 times more microorganisms in 10 g of the soil of the reforested area than in all the other soils.

From the molecular and statistical results showed in this work, besides the reports of Fonseca (1984), the impact of both reforestation and the cultivation of eucalyptus for a long period from one site and from one composite soil sample, contrary to what was thought (Lima, 1996), does not appear to result in a bacterial diversity decrease compared with a single composite sample from an adjacent forest soil site.

The genetic diversity analysis of the heterologous 16S rDNA clones for the libraries NFA (A) and EAA (B) resulted in significant values ($p \leq 0.001$), 1.208 to NFA versus EAA and 0.993 to EAA versus NFA in the representativeness analysis. These analyses suggest

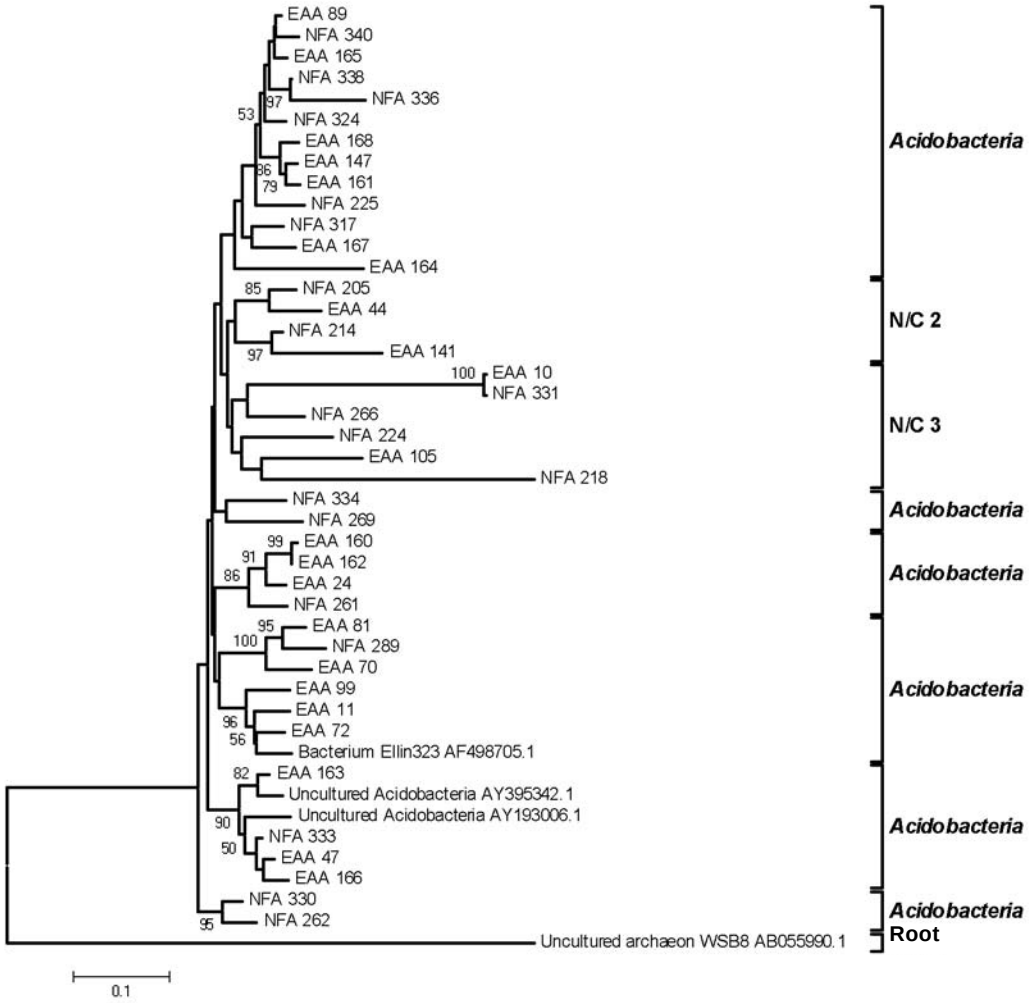

Figure 3. Phylogenetic dendrogram showing clones belonging to *Acidobacteria*, obtained from partial sequences of 16S rRNA gene from native forest soil (NFA) and eucalyptus arboretum area (EAA). The distance matrix and construction were performed using Kimura 2P algorithm and the Neighbor-Joining method, with a 1,000-repetition bootstrap above 50%. The 16S rDNA sequence of Uncultured Archaeon (no. GenBank AB 055990) was used as outgroup. All clones are numbered and sequences in the database are indicated by the GenBank access number.

that the studied areas are distinct, but there is a high level of similarity between the two soils. Similar results were obtained by Dunfield & King (2004), who used the same method to study the bacterial community of four soils from different places in Hawaii, USA, through the coxL gene. These authors observed that most bacterial phyla are found in every soil.

Besides, the genetic differentiation index between both bacterial communities (NFA and EAA), calculated by F_{st}, was significant (F_{st} = 0.024 for $p<0.05$), which suggests that even though the value is relatively low, both communities are structurally different and probably undergo different selective pressures. On the other hand, an interesting factor was the existence of a larger genetic diversity in each soil, individually, than between EAA and NFA communities. The results of the inter-population genetic variation, calculated by the test AMOVA, showed an average of 97.60, which means that there is a great variety of organisms within the same community, even though most clones are present in both soil samples.

The software Arlequim was used to analyze 444 base pairs for each cloned sequence from both soils, from which was calculated the average pairwise differences. The means were 88.17±38.15 and 100.60±43.52 for NFA and EAA, respectively. The numbers of polymorphic nucleotides in soils were 328 and 353 for NFA and EAA, respectively. The nucleotide diversity means were 0.20±0.09 and 0.23± 0.11 for NFA and EAA, respectively. These values indicate higher genetic diversity of the gene 16S rRNA in EAA in comparison to NFA.

Chemical EAA soil analyses indicate that some elements were found in lower concentrations in relation to NFA (Table 1). However, in this area, there are nutrients necessary for bacterial survival in the first 20 cm of depth without compromising the microbial diversity. NFA soil was considered to be a soil under climax vegetation, with no agricultural practice and theoretically a stable bacterial community adapted and in dynamic equilibrium with that environment. An environment of higher synergism and a lower diversity, compared to the soil under eucalyptus and in which the ecosystem suffered an alteration, increasing its population dynamics and, consequently, the bacterial diversity. Soils are always complex unraveling that complexity is always a challenge. As well as root exudates, eucalyptus leaf litter, because of their nature, has an acidifying effect on the soil (Dakora & Philips, 2002).

These results are snapshot of two adjacent soils under different vegetation and reveal different bacterial communities and those differences are probably plant related. Whether those differences are related to large scale to chemical changes, like pH, or to smaller scale changes to subtleties in plant exudates, remains to be revealed.

Table 2. Distribution of the cloned 16S rDNA sequences from native forest (NFA) and *Eucalyptus* arboretum (EAA).

Domain : Bacteria Phylum	NFA Number of distinct clones[1]	NFA Number of clones observed	%	EAA Number of distinct clones[1]	EAA Number of clones observed	%
Acidobacteria	23	60	44.78	14	29	25.00
Actinobacteria	4	7	5.22	7	17	14.65
Bacteroidetes	3	15	11.19	4	4	3.45
Firmicutes	2	2	1.50	5	7	6.05
Nitrospira	1	1	0.75	0	0	0.00
Planctomycetes	2	3	2.24	2	2	1.72
Proteobacteria	13	23	17.16	16	26	22.40
Verrucomicrobia	3	3	2.24	8	23	19.83
No classified	11	20	14.92	5	8	6.90
Total	62	134	100.00	61	116	100.00

[1]The 16S rRNA gene sequences were deposited at GenBank under acession numbers: AY631041 to AY631045, AY635574 to AY635578, AY640035 to AY640043, AY640045, AY640049, AY642762, AY697512 to AY 697539, AY697548, AY697554, AY697574, AY697575, AY697625 to AY697628, AY697630 to AY697639, AY697641 to AY697652, AY697654 to AY697667, AY697704, AY697720, and AY697725 to AY697728.

Conclusions

1. The sequencing of the gene 16S rRNA is an effective way to discover novel bacterial biodiversity and to compare bacterial diversity in natural and eucalyptus-reforested ecosystems.

2. Soil from the *Eucalyptus* spp. arboretum has greater bacterial diversity than soil from the native forest area.

3. Soils under eucalyptus plantation may be important areas for the bioprospection of actnomycetes for biotechnological purposes.

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