# Biodiversity of Prokaryotes from High-Elevation Grassland Organosols of the Parana State, Brazil

Etto, R.M.[1]; Galvão, C.W.[2]; Galvão, F.[3]; Curcio, G.R.[4]; Rachwal, M.F.G.[4]; Pedrosa, F.O.[1]; Cruz, L.M.[1]; Steffens, M.B.R.[1]

[1]Dep. de Bioquímica e Biologia Molecular, UFPR, PR, Brazil; [2]Dep. de Biologia Estrutural, Molecular e Genética, UEPG, PR, Brazil; [3]Dep. de Ciências Florestais, UFPR, PR, Brazil; [4]EMBRAPA Florestas, PR, Brazil.

The High-Elevation Grasslands of the Atlantic Mountain Range, and the First, Second and Third Plateau cover 14% of the Parana State area and contain hydromorphic soils consisting primarily of organic matter, called organosols. In this work, the Atlantic Mountain Range and Second Plateau organosols, with large geographical distance, distinct geological history and similar ecological roles, were selected for prokaryotic diversity investigation. Total DNA of the both soils was extracted and used as template with specific oligonucletides primers for the amplification of *nifH,* a marker gene for diazotrophs and 16S rRNA, for Bacteria. Analysis of RFLP profiles of *nifH* and 16S rRNA amplicons revealed that the Atlantic organosol had higher total bacterial diversity and lower diazotrophic diversity than the Second Plateu organosol. 16S rRNA gene libraries from both organosols were constructed and sequenced. Analysis with the RDPII database showed the predominance of the Acidobacteria phylum in both environments (≈ 45%). Moreover, in the Atlantic Mountain Range's library the following phyla was also found: Proteobacteria (21%), Bacteriodetes (11%), Firmicutes (6%), Actinobacteria (2%) and unclassified (15%). In the Second Plateau's library, the diversity was restricted to Proteobacteria (15%), Choroflexi (1%) and unclassified (39%). The highest diversity in the Atlantic Mountain Range is consistent with the profile obtained in RFLP analysis. These results show that despite the floristic similarities, the bacterial communities of the analyzed organosols present structural differences.

*Key words:* biodiversity of prokaryotes; high-elevation grassland organosols; *nifH*; 16S rRNA.
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# Biodiversity of Prokaryotes from High-Elevation Grassland Organosols of the Parana State, Brazil

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[1]Depto. de Bioquímica e Biologia Molecular, UFPR, PR, Brazil; [2]Depto. de Biologia Estrutural, Molecular e Genética, UEPG, PR, Brazil; [3]Depto. de Ciências Florestais, UFPR, PR, Brazil; [4]EMBRAPA Florestas, PR, Brazil.

## ABSTRACT

The High-Elevation Grasslands of the Atlantic Mountain Range, and the First, Second and Third Plateaus cover 14% of the Paraná State area and contain hydromorphic soils consisting primarily of organic matter, called organosols. In this work, the Atlantic Mountain Range and Second Plateau organosols, with large geographical distance, distinct geological history and similar ecological roles, were selected for prokaryotic diversity investigation. Total DNA of the both soils was extracted and used as templates with specific primers for the amplification of *nifH*, a marker gene for diazotrophs and 16S rDNA, for bacteria. Cluster analysis of RFLP profiles of *nifH* and 16S rDNA amplicons revealed that the 20 cm deep sample collected in the Second Plateau (CG20) and the 50 cm deep (CG50) collected in the same region are closer to each other than to the 20 cm deep sample collected in the Atlantic Mountain Range (SM20). 16S rDNA gene libraries of the three samples were constructed and 328 clones were sequenced. The comparison of the clone sequences with the RDP II database of 16S rDNA indicated predominance of Acidobacteria, followed by the Proteobacteria phylum in the threes samples. Community analysis using SONS indicated a 5 $OTU_{0.03}$ overlap among the libraries. S-Libshuff analysis yielded no significant differences between the CG20 and CG50 communities ($P = 0.2678$), which is consistent with the cluster analysis of RFLP profiles. These results showed that, despite the edaphic factor and floristic similarity, the bacterial communities of the sample SM20 and CG20 are structurally different. Moreover, the validation of the clusters by library sequence analysis showed that the molecular marker RFLP is a rapid and efficient tool for studies of prokaryotic community structures.

## GENERAL OBJECTIVES

Investigate the bacterial and diazotrophic diversity of distinct horizon organosols from the High-Elevation Grassland of the Parana State.

## SPECIFIC OBJECTIVES

- Extraction of the total DNA of organosols collected 20 cm (SM20) deep in the Atlantic Mountain Range, and collected 20 cm (CG20) and 50 cm (CG50) deep in the Second Plateau;
- Comparison of the bacterial and diazotrophic community structures found in SM20, CG20 and CG50 by RFLP;
- Comparison of the bacterial diversity found in SM20, CG20 and CG50 by 16S rDNA library sequencing.

## MATERIAL AND METHODS

**DNA EXTRACTION AND AMPLIFICATION.** DNA was extracted using the Ultraclean Soil DNA kit (MO BIO Laboratories, Carlsbad, CA). The PCR conditions and the specific primer sequences for the amplification of 16S rDNA, and *nif*H were established based on LANE (1991) and ROSCH et al. (2002).

**RFLP ANALYSIS.** 500ng of the amplified 16S rDNA or *nifH* from the collected samples was digested with *Hinf*I, *or Hae*III restriction enzymes at 37°C for 5 h. Digested products were separated by native polyacrylamide gel electrophoresis (12%) and visualized after ethidium bromide staining under UV light. The similarity dendogram was estimated through a binary matrix of present and absent DNA bands using the computer program NTSYSpc (Numerical Taxonomy of multivariate Systems) version 2.1 (Applied Biostatistics Inc). The dendograms were built by the algorithm UPGMA.

**16S rDNA LIBRARY.** 16S rDNA PCR products of 1.5 kb were cloned into the p-GEM-T vector (Promega) and transformed into *E. coli* DH5 $\alpha$ cells, previously treated with 100 mM ice-cold CaCl2. Several transformants were randomly picked, and the plasmids were extracted by alkaline lysis (SAMBROOK et al., 1989). 576 clones were sequenced in a *MegaBACE 1000* DNA automated sequencer with the primer 27F.

**SEQUENCE ANALYSIS.** Nucleotide sequences (reads) were trimmed for base quality. Sequences were aligned with clustalW 1.8 and a distance matrix calculated by the kimura method using DNAdist (both programs running under BIOEDIT 7.08 package). A total of 328 sequences spanning the V2-V3 region of 16S rDNA were obtained from SM20 (131), CG20 (103), and CG50 (94) samples.

**OTU DEFINITION and TAXONOMIC AFFILIATION.** Sequences of gene libraries were clustered into OTUs using the program DOTUR (SCHLOSS and HANDELSMAN, 2005) and compared against Ribosomal Database Project II database using the SEQUENCEMATCH program for taxonomic affiliation.

**LIBRARY COMPARISONS.** Comparisons were performed using the program SONS, which implements nonparametric estimators (SCHLOSS and HANDELSMAN, 2006) and S-LIBSHUFF which uses the Cramer von-Mises statistic. S-LIBSHUFF calculates *P* values by using a random-permutation proc h the statistical significance of overlapping co al., 2004).

## RESULTS

### RFLP ANALYSES OF 16S rDNA SHOWED THAT THE CG50 AND CG20 BACTERIAL COMMUNITIES ARE STRUCTURALLY CLOSER TO EACH OTHER THAN SM20

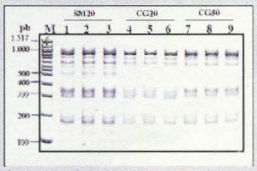



Figure 1 - 16S rDNA fingerprinting of SM20, CG20, and CG50 bacterial communities of organosols obtained after digestion with *Hinf*I restriction enzyme. The three independent assays are represented. M: 100bp DNA marker.

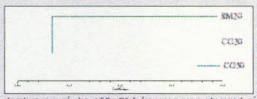



Figure 2 - Similarity dendrogram of the 16S rDNA fingerprinting obtained after digestion with the restriction enzymes *Hae*III and *Hinf*I, generated by program NTSYS pc 2.1, using Jaccard's similarity coefficient and the UPGMA algorithm.

### RFLP ANALYSES OF *nifH* SHOWED THAT THE CG50 AND CG20 DIAZOTROPHIC COMMUNITIES ARE STRUCTURALLY CLOSER TO EACH OTHER THAN SM20

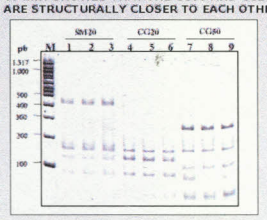



Figure 3 - *nifH* fingerprinting of SM20, CG20 and CG50 bacterial communities of organosols obtained after digestion with the *Hae*III restriction enzyme. The three independent assays are represented. M: 100bp DNA marker.

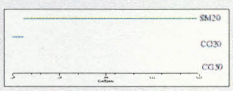



Figura 4 - Similarity dendrogram of *nifH* fingerprinting obtained after digestion with the restriction enzymes *Hae*III and *Hinf*I, generated by program NTSYS pc 2.1, using Jaccard's similarity coefficient and the UPGMA algorithm.

### THE THREE LIBRARIES OF 16S rDNA GENE SHOWED A HIGH RICHNESS WITH DOMINANCE OF THE ACIDOBACTERIA CLASS

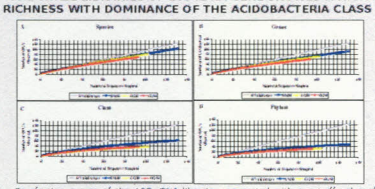



Figura 5 - Rarefaction curves of the 16S rDNA libraries, generated with a cut-off value of 0.03 for species definition (A), 0.09 for genus (B), 0.15 for class (C) and 0.2 phylum (D). Data were generated with the DOTUR program (SCHLOSS and HANDELSMAN, 2005; 2006).

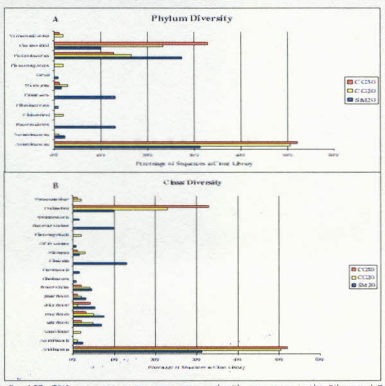
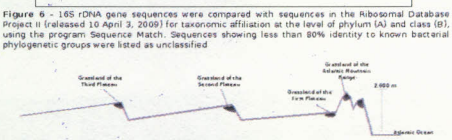
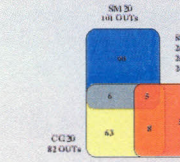



Figure 6 - 16S rDNA gene sequences were compared with sequences in the Ribosomal Database Project II (released 10 April 3, 2009) for taxonomic affiliation at the level of phylum (A) and class (B), using the program Sequence Match. Sequences showing less than 80% identity to known bacterial phylogenetic groups were listed as unclassified.




### COMPARISONS OF BACTERIAL COMMUNITY MEMBERSHIPS SHOWED THAT THE 16S rDNA LIBRARIES CG20 AND CG50 ARE STRUCTURALLY CLOSER TO EACH OTHER THAN SM20




| | Shared Chao1 | $J_{abund}$ | $J_{abund}$ | $\theta$ | $\theta_{-se}$ |
|---|---|---|---|---|---|
| SM20-CG20 | 55.5 | 0.1935 | 0.07776 | 0.159 | 0.05863 |
| SM20-CG50 | 33 | 0.1684 | 0.1671 | 0.037 | 0.02433 |
| CG20-CG50 | 192.9 | 0.3877 | 0.246 | 0.102 | 0.04021 |

Figure 7 - Venn diagram comparing the $OTU_{0.03}$ memberships found in the SM20 ($n = 131$), CG20 ($n = 103$) and CG50 ($n = 94$) libraries. Five OTUs overlapped in three samples, representing 32 sequences of the total 328. Shared-Chao1 richness , $J_{abund}$ and $\theta$ estimates of $OTU_{0.03}$ community structure and membership along with standard errors (SE) are reported in the accompanying table.

### COMPARISON OF THE 16S rDNA LIBRARIES SHOWED NO STATISTICAL DIFFERENCE BETWEEN SAMPLES CG20 AND CG50

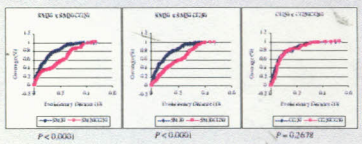



Figure 8 - A partial plot of the comparisons between 16S rRNA gene libraries generated by the S-LIBSHUFF program. *P* values are reported below.

## CONCLUSIONS

- The geographical distance between High-Elevation Grasslands seems to influence the structural differences of bacterial and diazotrophic communities, more than does the sampled horizon depth;
- The three analyzed samples of High-Elevation Grassland organosols showed predominance of the Acidobacteria phylum, followed by Proteobacteria;
- The validation of the clusters by library sequence analysis showed that the molecular marker RFLP is a rapid and efficient tool for studies of prokaryotic community structures.

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