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# Comparison of two DNA Extraction Protocols Representing Bacterial Community from Bulk Soil by PCR-DGGE

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***Authors' contributions***

*This work was carried out in collaboration between all authors. All authors designed the study, managed the analysis and performed the statistical analysis. Authors NRM and FVS wrote the first draft of the manuscript and managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** The objective of this work was to investigate the efficiency of DNA extraction from bulk soil using the Britânia® mini mixer combined with phenol extraction to study bacterial communities, from three sampling sites localized in São Gonçalo-RJ, Brazil, comparing it to a commercial kit by the PCR-DGGE technique.
**Study Design:** Molecular fingerprints of bacterial communities in bulk soil from three sampling sites were generated by DGGE after 16S rDNA gene amplification.
**Place and Duration of Study:** Faculdade de Formação de Professores-UERJ and Embrapa Agrobiologia between April 2010 and April 2013.
**Methodology:** Samples of DNA, in triplicate, extracted from bulk soil at three sampling sites localized in São Gonçalo-RJ, Brazil, were obtained by two protocols of DNA extraction. The DNA

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samples were used as template to amplify the 16S rDNA gene using specific primers to α and β-*Proteobacteria* groups. The PCR products were used for a second amplification using the primers F968GC and R1401 for subsequent DGGE analysis. The products from the second amplification were subjected to DGGE and band patterns were statistically analysed.
**Results:** The band profiles obtained from the protocol that used a hand held mini mixer were intense and showed higher similarity among the triplicates from the sampling sites. Although both methods were capable of achieving a representative DNA profile from the soil bacterial community, the band patterns produced by them for the same areas were different.
**Conclusion:** The DGGE profile of specific groups such as α- and β-*Proteobacteria* was a useful tool to compare the two soil DNA extraction protocols and also to compare the community structure of the different sampled areas. The DNA extraction protocol that used the Britânia® mini mixer produced band profiles with higher values of richness, but missed some bacterial targets as the commercial kit did. Both protocols have validity for the study of bacterial communities in bulk soil. The clusters of band profiles obtained via 16S rDNA PCR-DGGE indicated differences in bacterial communities of bulk soil from the three sampling sites for different ecological succession stages localized in São Gonçalo, RJ. The diversity analysis showed that the α-*Proteobacteria* group was predominant in bulk soil from these sites.

*Keywords: Microbial ecology; Proteobacteria; Soil; DNA; DGGE.*

## 1. INTRODUCTION

In the last years, the interest of bacterial community from soil has been growing, with the objective to exploit new genetic resources and to evaluate the impact of anthropogenic practice in the environment. Bacterial diversity is not static, due to their high reproduction capacity combined to their short life cycle [1]. In addition, the microorganisms represent a great potential to biotechnology and are able to give rapid responses to environmental changes [2,3].

The studies with the soil microorganisms had a large spur with the development of various molecular techniques that eliminated the need to grow the microorganisms in traditional culture media [4]. All the methodologies available to assess and compare bacterial communities have advantages and drawbacks [1].

Beyond the studies with the microorganisms present in the soil matrix, the evaluation of microbial communities associated to plant rhizosphere has received vast attention [5,6]. In this functional domain, defined as soil portion influenced by root exudates including the root itself, there is an ample diversity of microorganisms with known ecological importance besides the higher quantity of microorganisms metabolically active in comparison with the soil matrix communities [7]. The extraction of DNA from the bacterial community has been done with soil or root samples of 0.25 g to 1 g using commercial kits [6,8,9,10]. These culture-independent methods exclude the limitations of the traditional bacteria culture because only a portion of community can be cultivated.

The samples of soil and DNA extraction protocols are crucial points for obtaining microbial cells that are representative of a community [11]. Beyond that, the efficiency of cells lyses and the DNA extraction are factors that, also, should be considered during the development of methodology because the outcome of the further molecular analysis depends on it.

Some protocols of DNA extraction, not rarely represent a limitation to the study of bacterial community from the soil, because they require the use of bead beater and/or the use of commercial kits that are expensive for some laboratories. In this way, the representation of the samples and the development of protocols that reduce the cost of reagents and equipments are critic factors, especially in experiment procedure with many treatments. These can turn the laboratorial routines faster and more tangible. A method of soil DNA extraction using a hand held mini mixer that costs 1% of a bead beater and that yielded higher amounts of pure DNA was developed [12], but the PCR products were not analysed by DGGE (Denaturing Gradient Gel Electrophoresis).

The adaptation of a method for DNA extraction from the environment, aiming the reduction of costs should not only focus in the quantity and quality of obtained DNA, but should also consider

the structure and composition of microbial community. It can be done through the DGGE, a fingerprint technique that consists on amplifying of a determined gene sequence from a sample of environmental DNA and the separation of amplicons through a chemical denaturing gradient in polyacrylamide gel, what gives an overview of the most abundant members, evidenced by patterns of bands. The DGGE can also be used to compare the efficiency and reproducibility of different protocols of DNA extraction of microbial community [13], to determine the amplification of different hypervariable regions of rDNA [14], to investigate the preservation of soil samples granting their representation [15], to determine factors that modulate the diversity of bacterial communities [16] and also to evaluate alteration in bacterial community associated to roots of genetically modified plants [17].

The objective of this work was to investigate the efficiency of DNA extraction from bulk soil using the Britânia® mini mixer combined with phenol extraction to study bacterial communities, from three sampling sites localized in São Gonçalo-RJ, Brazil, comparing it to a commercial kit by the PCR-DGGE technique.

## 2. MATERIALS AND METHODS

### 2.1 Sampling

Soil samples were collected from three sampling sites of different ecological succession stages localized in São Gonçalo, RJ, Brazil. The sampling sites consisted of a secondary forest, a reclamation area and a grass area. Soil samples were taken from the surface layer (0-20 cm). Five sub-samples from the distance of 10 m were mixed and it was made in triplicate for each area. The soil samples were then stored at -20ºC. The chemical characteristics of bulk soils from the three sampling sites are described in Table 1.

### 2.2 Extraction of Cells from Soil and DNA Extraction

Extraction of cells from soil samples were done following a modification of the method proposed by [12] that used a hand held mini mixer to induce cell lyses in substitution to a bead beater which is more expensive. Four grams of soil were transferred to a sterilized glass beaker containing 4 mL of 0.12 $mol^{-1}$ phosphate buffer (pH 8.0). The suspension was mixed for five minutes at room temperature and incubated at 4ºC for 30 minutes. After that, 480 µL of 20% Sodium Dodecyl Sulfate (SDS) was added and suspension was kept on ice. The suspension was supplemented with lysozyme (Sigma, Co) at a final concentration of 5 mg $mL^{-1}$. The samples were shaken three times for 90 seconds with Britânia® mini mixer at highest speed, this appliance is easily found in shopping centers and it is not expensive. The modification of the method was done after this step when the soil suspension was divided into aliquots of 1mL inside sterilized plastic micro-tubes using a sterilized plastic Pasteur pipette cut. The reason this was done, was that we only had a micro-centrifuge and we did not have a bead beater. After that the DNA extraction was conducted according to [18]. Each sample was centrifuged at 10.400 RPM for 15 minutes in environmental temperature and the supernatant was transferred to another micro-tube that received equal volume of Tris-Equilibrated Phenol Solution (0.5 M, pH 7.8). The sample was centrifuged again at 10.400 RPM during 15 minutes and the supernatant transferred to clean micro-tubes and another extraction with phenol was done. After that, the supernatant was subjected to two more extractions, but with equal volume of Chloroform: Isoamyl alcohol (24:1). After the fourth extraction, the supernatant was transferred to clean micro-tubes and the DNA was precipitated with equal volume of cold absolute ethanol. The precipitate was washed with 500 µL of 70% ethanol and dried in room temperature. The precipitate was re-suspended in Tris-EDTA (10mM, pH 8.0). The crude DNA preparations were cleaned by Cesium chloride and potassium acetate according to [19]. To certify the efficiency of this protocol, the DNA extraction for all the samples was also done with the UltraClean® Soil DNA Isolation Kit (MO BIO Lab, USA) that is largely applied to studies of bacterial community by the DGGE technique.

### 2.3 Group-Specific PCR of 16S rDNA Fragments

The 16S rDNA fragments were amplified by PCR from soil DNA extracts. Each 50 µL of reaction consisted of 1 µL of DNA (about 10-100 ng), 24µL of water and 25 µL of master mix that consisted of 5 µL of buffer (10 mM), 3 µL of $MgCl_2$ (3.5 mM), 1 µL of dNTP (0.2 µM of each), 1.25 µL of Tween 1%, 0.25 µL Taq DNA polymerase (Invitrogen), 1 µL of each primer (0.2 µM of each) and 12.5 µL of sterilized water. The

primers F203α [20] and R1492 [21] were used to amplify the 16S rDNA of the α-*Proteobacteria* group and the primers F948β [20] and R1492 were used to amplify the 16S rDNA of the β-*Proteobacteria* group. The amplification reaction consisted of initial warming (93ºC; 2 min), 35 cycles composed of: denaturation (93ºC; 1 min), annealing (57ºC; 1 min) and extension (72ºC; 2 min), and each final step of extension (72ºC; 5 min) and then held at 4ºC. Products were checked by electrophoresis in 1% (wt/vol) agarose gels and ethidium bromide staining.

## 2.4 PCR amplification of 16S rDNA fragments for DGGE analysis (PCR-DGGE)

The products of PCR to the α- and β-*Proteobacteria* groups were used as template for a second amplification of 16S rDNA fragments using the universal primers F968GC and R1401 for DGGE analysis [22]. This strategy of doing a second amplification is known as nested-PCR and it helps to increase the number of copies of 16S rDNA from different bacterial species present in bulk soil before using the primers for DGGE analysis [9].

Each 50 µL of reaction consisted of 1 µL of DNA (about 10-100 ng), 24µL of water and 25 µL of master mix that consisted of 5 µL of buffer (10 mM), 3 µL of $MgCl_2$ (3.5 mM), 1 µL of dNTP (0.2 µM of each), 1.25 µL of Tween 1%, 0.25 µL Taq DNA polymerase (Invitrogen), 1 µL of each primer (0.2 µM) and 12.5 µL of sterilized water.

The 16S rDNA of four strains (*Escherichia coli, Vibrio cholera, Salmonella* and *Pseudomonas aeruginosa*) was also amplified to DGGE analysis and their PCR products were mixed to be used as markers to help in the analysis of community fingerprints. To amplify the 16S rDNA of these strains, cell suspensions were done in 1 mL of sterilized water inside sterilized plastic microtubes and they were boiled for five minutes and after that kept on ice, 1µl of these suspensions were mixed to 25 µl of master mix and 24 µl of sterilized water for amplification.

The amplification reaction consisted of initial warming (93ºC; 2 min), 35 cycles composed of: denaturation (93ºC; 1 min), annealing (55ºC; 1 min) and extension (72ºC; 2 min), and each final step of extension (72ºC; 5 min) and then held at 4ºC. Products were checked by electrophoresis in 1% (wt/vol) agarose gels and ethidium bromide staining.

## 2.5 Community Fingerprints by DGGE

PCR products were subjected to DGGE [23] into a denaturing gradient from 50 to 65%, where 100% of denaturation was defined as 7 M of urea in 40% of formamide, prepared with the Bio Rad DCode Multi Mutation Detection System. Volumes of 25 µL of PCR product were added to 10 µL of loading buffer (0.5% blue bromophenol, 40% sacarose, 0.1 mol $L^{-1}$ of EDTA, 5% of SDS), in a final concentration of 1 X. The electrophoresis was carried out in a code (Bio-Rad) system under constant voltage (120V; 60ºC; 16 hours).

A mixture of the PCR products of four bacterial species was applied twice on each DGGE gel as a marker to check the electrophoresis run and to compare fragments migration between gels as in [9].

Following the electrophoresis, the gel was dyed in silver according to [24], air dried and photographed. The similarity of DGGE profiles among the triplicates of each area and between the two protocols of DNA extraction for the three areas studied was done by Cluster analysis using Bionumerics program, the coefficient of Jaccard and unweighted-pair group method (UPGMA) were used to create dendograms describing pattern similarities in bulk soil. The richness, evenness and diversity index were compared as in [10]. Richness (S) refers to the number of bands detected in a soil sample.

**Table 1. Values obtained from chemical characterization of bulk soil samples corresponding to the arithmetic media of triplicates of each area studied. The designation CO was given to a reclamation area, M was to a secondary forest and CI was to an area consisted of grasses**

| | Al | Ca | K | Mg | N | P | pH |
|---|---|---|---|---|---|---|---|
| | ----------(cmol$_c$ dm$^3$)---------- | | | | (%) | (mg/L) | in $H_2O$ |
| **CO** | 1.09 | 0.52 | 78.6 | 0.32 | 0.15 | 1.41 | 4.5 |
| M | 1.15 | 0.52 | 63.33 | 0.37 | 0.15 | 1.80 | 4.77 |
| **CI** | 1.39 | 0.7 | 93.0 | 0.50 | 0.17 | 2.05 | 4.45 |

The diversity is calculated by the diversity index of Shannon (H) to compare the changes in diversity of microbial communities inside all the treatments using the function $H = - \sum Pi \log Pi$, where $Pi = ni/N$, ni is the number of individuals of the specie i in the community, and N is the total number of individuals in the community. The evenness in DGGE (E) is the measure of bands intensity in a soil sample, calculated as $E = H/\ln(S)$.

## 3. RESULTS AND DISCUSSION

### 3.1 DNA extraction and amplification of 16S rDNA

In Figs. 1 and 2, the bands obtained by 16S rDNA amplification from DNA samples received by the use of a hand held mini mixer combined to phenol extraction or by the UltraClean® Soil DNA Isolation Kit is represented as following: a secondary forest (M1, M2, M3), a reclamation area (CO1, CO2, CO3) and an area consisted of grasses (CI1, CI2, CI3). The DNA extraction by a hand held mini mixer and Cesium chloride purification methods of microbial cells from soil resulted in enough and adequate DNA to the next steps of amplification and electrophoresis as the DNA obtained by the commercial kit (Figs. 1 and 2). Others studies demonstrated that different protocols of DNA extraction can yield different amount of DNA extracted from soil [11,12] or from other kinds of samples [25].

Otherwise, the quantity of DNA is not a parameter that by itself can prove that the samples have good representation of microbial community. In this way, the adaptation of a DNA extraction method should also consider the profile of microbial community obtained from the samples, which was done in this work using DGGE analysis to evaluate the efficiency of the protocol that uses a hand held mini mixer to the extraction of cells from bulk soil (Figs. 3 and 4).

### 3.2 Statistical Analysis of Denaturing Gradient Gel Electrophoresis (DGGE) Bands

In Figs. 3 and 4, the band profiles observed were obtained from 16S rDNA amplification of the DNA samples extracted by the use of mini mixer combined with phenol extraction or by the UltraClean® Soil DNA Isolation Kit. The dendrograms were constructed using Jaccard coefficient algorithm and they revealed that the band profiles obtained by the mini mixer protocol were different from those obtained by commercial kit when the α- and β-*Proteobacteria* groups (Figs. 3 and 4), two classes of Gram-negative bacteria from *Proteobacteria* phyla, were studied in the three sampling sites.

High similarity within replicates was observed when [11] used specific primers to the α- and β-*Proteobacteria* groups to study bacterial communities from bulk soil, because it increases the sensibility while reducing the complexity of DGGE patterns. Here, this strategy of using primers to specific groups was adequate to the comparison between the two protocols of DNA extraction.

In the Fig. 3, where band profiles of the α-*Proteobacteria* group were obtained, two main clusters were formed with 40% of similarity. In the Fig. 4, where band profiles of the β-*Proteobacteria* group were obtained, four clusters were formed with 35% of similarity. Comparing these data, the mini mixer protocol grouped the triplicates of sampling sites with higher similarity and also showed more dominant bands than those obtained with commercial kit. Both methods were able to distinguish the bacterial communities of the three sampling sites to the α- and β-*Proteobacteria* groups, and although they are nearby sites and the chemical characteristics of their soils are similar, they have as principal difference their ecological succession stage, which can explain the variations found in the structure of bacterial communities. The monitoring of bacterial communities of soils is equally important as the plant and animal communities are.

The efficiency of soil DNA extraction obtained with a mini mixer (Moulinex, Brazil) was seen before by [12], but they did not amplify their samples to be analysed by PCR-DGGE technique and now the present work comes with additional information about this method because this was compared with a commercial kit that is greatly used to direct DNA extraction from bulk soil to study bacterial communities.

The total number of bands detected in the DGGE gel from the α-*Proteobacteria* group with the two methods was 62 (Fig. 3), and the analysis of the binary matrix showed that 50 of them were obtained with commercial kit and 59 were detected with the hand held mini mixer. The total number of bands detected in the DGGE gel for

the β-*Proteobacteria* group was 47 (Fig. 4), and analysis of binary matrix showed that 42 of those were detected by commercial kit and 37 were obtained by the mini mixer. Depending on the group of *Proteobacteria* studied, one method was able to achieve more bacterial species than the other.

Authors, that used DGGE to compare methods of sampling bacterial communities from soil, have seen that the cultivated and non-cultivated fractions showed exclusive bands in DGGE profiles [17] and they conclude that the protocols were complementary. In the present work, even when we compared only non-cultivated fractions obtained by the two methods, we saw that in the α- and β-*Proteobacteria* groups, there were bands that were not detected by one of the methods and it shows that one protocol was not better than the other, but they are complementary since the two protocols showed exclusive bands in DGGE profiles and it also means that both methods can miss some targets in bacterial communities.

This data confirms that the mini mixer is good enough to recover DNA from bulk soil and is valid to compare the structure of bacterial communities of different areas. It also showed that microbial community from bulk soil changed at different sampling sites, as seen by others methods that used expensive cell disruptor and commercial kit for bulk soil DNA extraction to be analysed by PCR-DGGE technique with the same purpose [9,26-28].

### 3.3 Diversity Analysis of DGGE Banding Patterns

Richness (S) refers to the number of bands detected in a lane of DGGE gel to each bulk soil sample. Here, the arithmetic media of S values obtained for the triplicates of each sampling site was calculated. In the Fig. 5, it could be observed that the α-*Proteobacteria* group is predominant in the three sampling sites and it was better demonstrated by the mini mixer method.

The diversity of species was calculated by the diversity index of Shannon (H) to compare the changes in diversity of microbial communities inside all the treatments using the function $H = -\sum Pi \log Pi$, where $Pi = ni/N$, ni is the number of individuals of the specie i in community, and N is the total number of individuals in community. The Fig. 6 pointed out that the α-*Proteobacteria* group presented higher values of H. Other authors [10] have also seen that the diversity of the α-*Proteobacteria* is slightly higher than other members of *Proteobacteria* phyla in non-rhizosphere soil.

The evenness in DGGE (E) is the measure of the intensity of bands in a soil sample, calculated as $E = H/\ln(S)$. Although the α-*Proteobacteria* group presented higher richness, the Fig. 7 pointed out that the communities of β-*Proteobacteria* presented higher values of evenness for the secondary forest (M) and reclamation areas (CO) when both methods of DNA extraction were used.

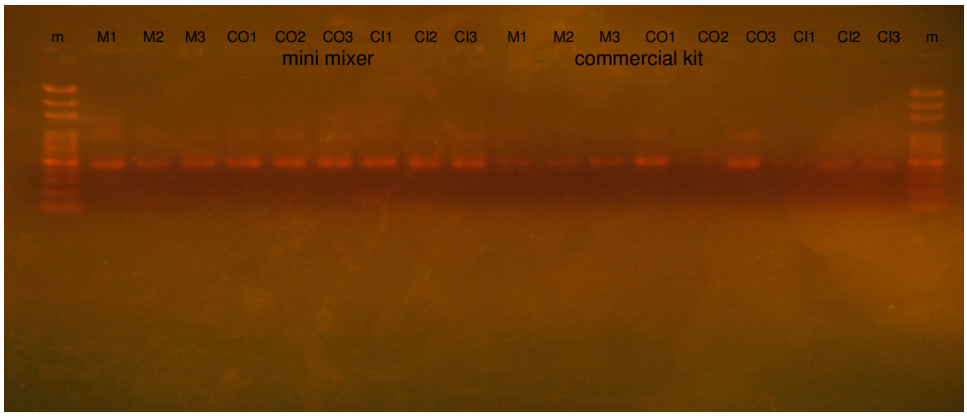


**Fig. 1. Nested-PCR products of 433 pb from 16S rDNA amplified with primers F968GC and R1401 obtained from soil DNA, extracted with a hand held mini mixer or commercial kit and pre-amplified with primers F203 and R1492 to α-*Proteobacteria* group. The sampling sites are a secondary forest (M), a reclamation area (CO) and an area consisted of grasses (CI). The designations 1, 2 and 3 represent the repetitions of samples. The letter "m" on the left and the right sides indicates the 100 bp DNA ladder**

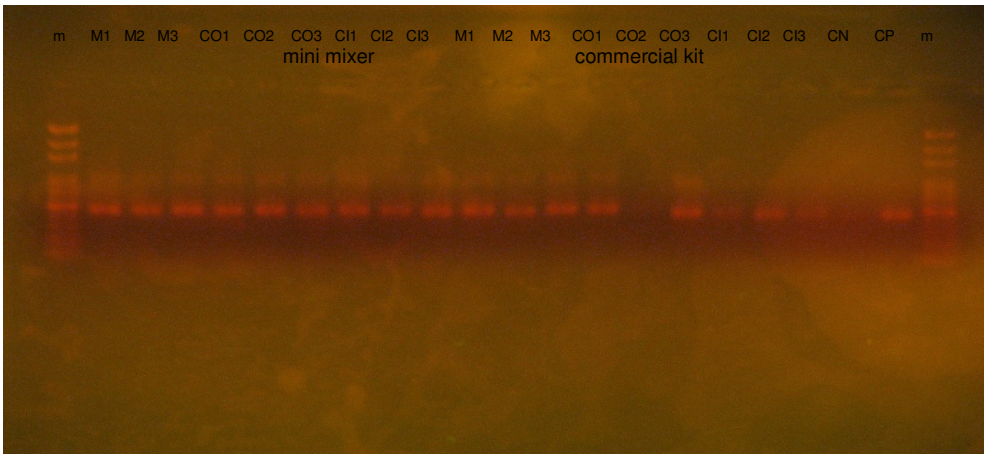


**Fig. 2. Nested-PCR products of 433 pb from 16S rDNA amplified with primers F968GC and R1401 obtained from soil DNA extracted with a hand held mini mixer or with commercial kit and pre-amplified with primers F948 and R1492 to β-*Proteobacteria* group. The designations 1, 2 and 3 represent the repetitions of samples. The sampling sites are a secondary forest (M), a reclamation area (CO) and an area consisted of grasses (CI). The letter "m" on the left and the right sides indicates the 100 bp DNA ladder. CN is negative control and CP is positive control**

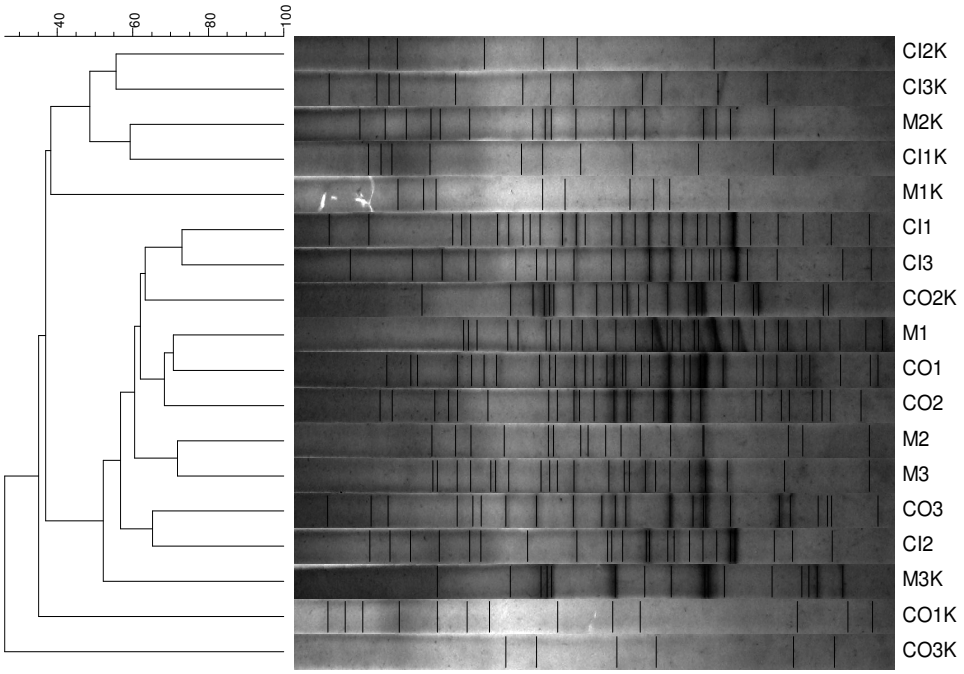


**Fig. 3. DGGE profiles of bacterial communities of a secondary forest (M), a reclamation area (CO) and an area consisted of grasses (CI) obtained by a hand held mini mixer combined with phenol extraction or by a commercial kit followed by amplification of the 16S rDNA fragments with primers F203 and R1492 to the α-*Proteobacteria* group. The designations 1, 2 and 3 represent the repetitions of samples and K is that the DNA was extracted with the commercial kit**

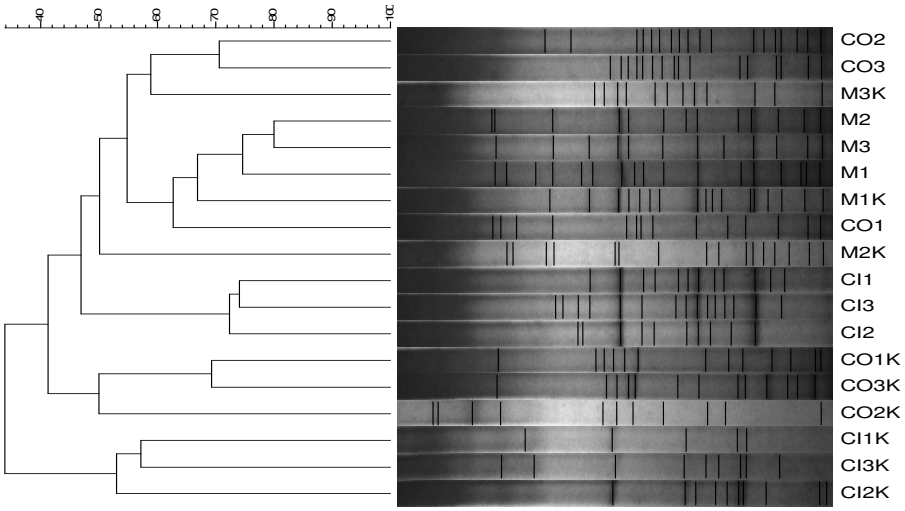


**Fig. 4. DGGE profiles of bacterial communities of a secondary forest (M), a reclamation area (CO) and an area consisted of grasses (CI) obtained by a hand held mini mixer combined with phenol extraction or by a commercial kit followed by amplification of the 16S rDNA fragments with primers F948 and R1492 to the β-*Proteobacteria*. The designations 1, 2 and 3 represent the repetitions of samples and K is that the DNA was extracted with the commercial kit**

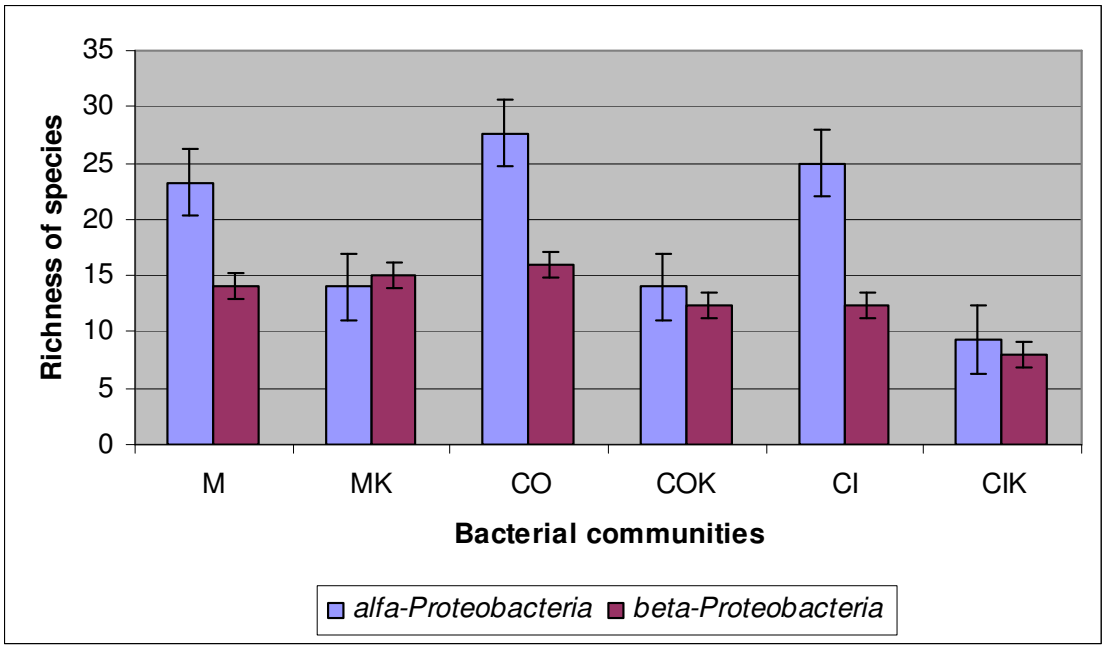


**Fig. 5. Richness of species observed for the communities of the α-*Proteobacteria* and the β-*Proteobacteria* in three sampling sites that are a secondary forest (M), a reclamation area (CO) and an area consisted of grasses (CI). The DNA was obtained by a mini mixer combined with phenol extraction or by a commercial kit (K). The bar represents the standard error of the mean**

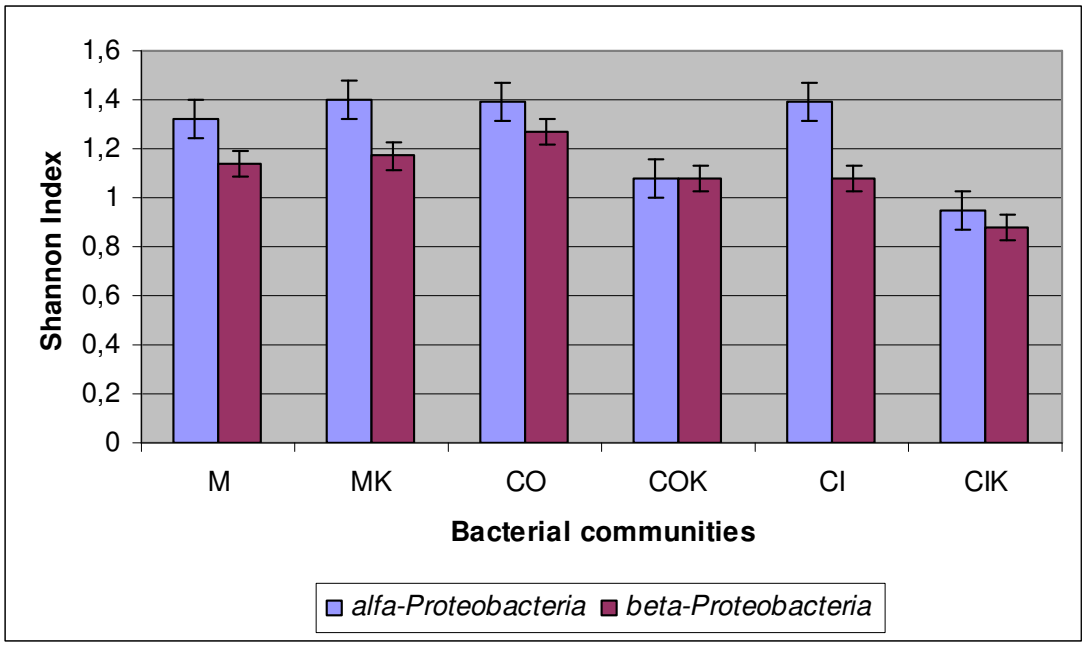


**Fig. 6. Shannon index of diversity obtained for the α-*Proteobacteria* and the β-*Proteobacteria* in three sampling sites that are a secondary forest (M), a reclamation area (CO) and an area consisted of grasses (CI) obtained by a mini mixer combined with phenol extraction or by a commercial kit (K). The bar represents the standard error of the mean. The Shannon index was calculated using the function H= -∑ Pi . LnPi**

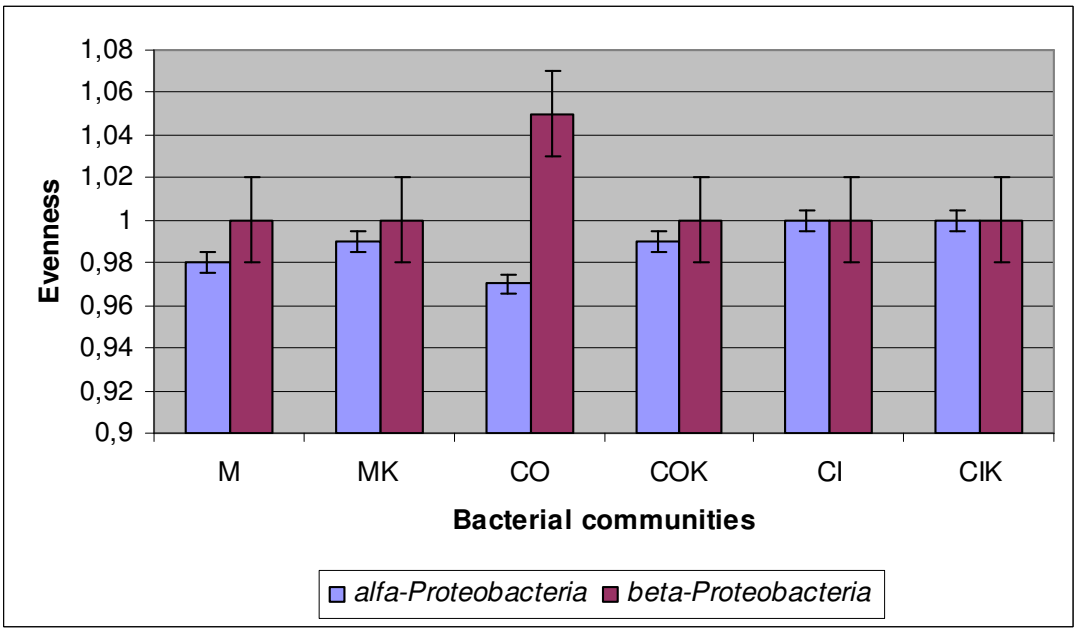


**Fig. 7. Evenness of species of the α-*Proteobacteria* and the β-*Proteobacteria* communities in three sampling sites that are a secondary forest (M), a reclamation area (CO) and an area consisted of grasses (CI) obtained by a mini mixer combined with phenol extraction or by a commercial kit (K). The bar represents the standard error of the mean**

## 4. CONCLUSION

The DGGE profile of specific groups such as α- and β- *Proteobacteria* was a useful tool to compare the two soil DNA extraction protocols and also to compare the community structure of the different sampled areas. The DNA extraction protocol that used the Britânia® mini mixer produced band profiles with higher values of richness, but missed some bacterial targets as the commercial kit did. Both protocols have validity for the study of bacterial communities in bulk soil. The clusters of band profiles obtained via 16S rDNA PCR-DGGE indicated differences in bacterial communities of bulk soil from the three sampling sites for different ecological succession stages localized in São Gonçalo, RJ. The diversity analysis showed that the α-*Proteobacteria* group was predominant in bulk soil from these sites.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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