T-RFLP analysis of soil bacterial structure from Cerrado within the Sete Cidades National Park, Brazil

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The aim of this study was to compare the structure of soil bacterial communities across a gradient of three different types of Cerrado within Sete Cidades National Park, northeast Brazil. We evaluated the preserved Cerrado sites across a gradient ranging from ‘campo graminioide’ (CG; open grassland), ‘Cerrado strictu sensu’ (CSS; savannah), and ‘Cerrado’ (CD; closed formation). Bacterial diversity, as estimated by T-RF richness and alpha and beta diversity showed different patterns across the gradient of the Cerrado. For the restriction enzyme Hha I and Msp I, the highest T-RF richness and beta diversity were found at CSS. The distribution and abundance of various fragments sizes varied between different sites. The non-metric multidimensional scaling analysis obtained with the restriction enzyme Hha I separated the sites according to bacterial community; whereas the analysis based on the restriction enzyme Msp I did not clearly separate the evaluated sites. The results show that bacterial structure does not follow the same pattern as found in the others regions with Cerrado vegetation.

Keywords: T-RFLP; soil microorganisms; bacterial community; tropical soil

Introduction

The Brazilian Cerrado being the second largest Brazilian biome, after the Amazon rainforest, is an ecosystem containing high biodiversity.[1] The ecosystem extends across almost the entire country and is one of the most biodiverse savannahs in the world, containing approximately 40% of endemic species.[2] The Cerrado is a complex formation varying from open grassland (‘campo graminioide’) to a closed formation (‘cerradão’), with intermediary savannah (‘cerrado stricte censu’).[3]

In the northeast of Brazil, the Cerrado may be found in the states of Piauí and Maranhão with an area of 20 million ha.[4] Particularly in the Piauí state, the Brazilian government has established the Sete Cidades National Park, which covers 6221 ha and aims to study and protect biodiversity.[5] In this park, a Long-Term Ecological Program (PELD) was initiated to study the plant diversity within the Sete Cidades National Park, and it identified variable plant diversity across a gradient of the Cerrado, from ‘campo graminioide’ to ‘cerradão’.[6, 7] However, studies involving the soil microbial structure from Cerrado within the Sete Cidades National Park are non-existent.

The lack of knowledge regarding the soil microorganisms in preserved Cerrado ecosystem may be a cause of concern because soil organisms are involved in vital ecosystem functions.[8] Molecular profiling approaches have been widely used to assess the microbial ecology in different ecosystems.[9-11] More specifically, terminal restriction fragment length polymorphism (T-RFLP) is a rapid, highly reproducible, and robust molecular tool for the study of bacterial community structure.[12] This technique has been successfully applied for the evaluation of bacterial structure within several sites, such as the soils of preserved [13] or disturbed sites [11] and forest soils.[14] Therefore, we used this approach to assess the bacterial structure across a gradient of the Cerrado within the Sete Cidades National Park.

The soil bacterial communities are influenced by several factors, such as soil physiochemical properties, climate conditions, and plant diversity, which drive their distribution in the environment.[15] Specifically, plant diversity directly influences the bacterial structure, through carbon sources in plant litter, and indirectly, through their rizospheric effects.[16] As PELD identified shifts in plant diversity and physicochemical properties in the different types of Cerrado within the Sete Cidades National Park, we hypothesized that the soil bacterial community structure may similarly differ across this gradient. Therefore, we aimed to compare the structure of soil bacterial communities, using T-RFLP, from a Cerrado within the Sete Cidades National Park, and identify the variables, which influence the bacterial structure.

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Material and methods

The study was conducted within Sete Cidades National Park (PNSC) (04°02′–08′S and 41°40′–45′W), located in the northeastern state of Piauí. The park covers an area of 6221 ha. The climate is sub-humid moist with great deficiency of water, bedroom and megathermal small annual thermal amplitude. There are two distinct seasons (wet and dry) during the year, with higher annual average temperatures at 25 °C. The area has an annual average rainfall of 1558 mm distributed in February–April.

We evaluated preserved sites (with 1000 m² each one) under Cerrado, belonging to the long-term ecological program (PELD-CNPq) from Brazilian government, across a gradient ranging from ‘campo graminoide’ (CG; open grassland), ‘Cerrado strictu sensu’ (CSS; savannah), and ‘Cerradão’ (CD; closed formation) (Figure 1; Table 1). Basically, CG is covered by a continuous grass stratum which does not exist in CD; while CD is covered by woody stratum with varying density of shrubs and low trees which is absent in CG. Intermediately, CSS is covered by grass, shrubs, low trees and woody stratum.[17]

Each site was divided in six transects (replication) where soil samples were collected at 0–20 cm depth (three points per transect which were mixed to obtain a composite sample per transect) in March (wet season), 2014. All soil samples were immediately stored in sealed plastic bags and transported in an ice box to the laboratory. A portion of the soil samples was stored in bags and kept at −20 °C for DNA analysis and another portion was air-dried, sieved through a 2-mm screen and homogenized for chemical analyses.

Soil chemical properties were determined and measured using standard laboratory protocols. Soil pH was determined in a 1:2.5 soil/water extract. Available P and exchangeable K⁺ were extracted using Mehlich-1 extraction method and determined by colorimetry and photometry, respectively (Table 2). Total organic C (TOC) was determined by the wet combustion method using a mixture of potassium dichromate and sulfuric acid under heating.[18]

Soil DNA was extracted from 0.5 g (total humid weight) of soil using the Power Soil DNA Isolation Kit (MoBIO Laboratories, Carlsbad, CA, USA), according to the manufacturer’s instructions. The DNA extraction was performed in triplicate for each soil sample. The quality and relative quantity of the extracted DNA was determined using a Thermo Scientific NanoDrop 2000. For the T-RFLP analysis, the bacterial 16S rRNA gene was amplified with the primers 27F (5′ end labeled with FAM fluorescent dye) and 1492R. The PCR mixture (25 μL) contained 10 ng of template DNA, 2.5 μL of 10× reaction buffer (Invitrogen, Carlsbad, CA), 3.0 mM MgCl₂, 0.2 mM of each dNTP (Eppendorf, Germany), 0.25 mM of each forward (f) and reverse (r) primer (Integrated DNA Technologies, Coralville, IA, USA) and 1.0 U of Platinum Taq DNA Polymerase (Invitrogen). Cycling was performed as follows: 3 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 59 °C for 45 s, 72 °C for 1 min, and a final extension step at 72 °C for 15 min, using a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems). Triplicate PCR products for soil samples were analyzed by gel electrophoresis and purified with the Qiagen PCR purification kit (Qiagen, Valencia, CA, USA). PCR products (60 ng) were digested with the enzymes Msp I and Hha I (Invitrogen, USA) in 20 μL separate reactions, at 37 °C for 3 h. The DNA was precipitated using isopropanol. The DNA pellets were resuspended in 9.8 μL deionized formamide and 0.2 μL of GeneScan-500 ROX internal size standard (Applied Biosystems, USA) and denatured at 94 °C for 5 min and immediately transferred onto ice. Fragments were analyzed in an ABI 3100 automated sequencer (Applied Biosystems) following manufacturer’s instructions.

The size and intensity of each T-RF was estimated using Peak Scanner Software v1.0 (Applied Biosystems). The peaks represent fragments of different sizes, and the peak areas represent the relative proportion of these fragments. The number of peaks in each electropherogram was interpreted as the operational taxonomic units (OTUs) richness in the community.

The richness, evenness, and diversity of bacterial communities were calculated. Richness (S) is the number of unique taxa present in each sample (here represented by number of OTUs), while evenness describes the equitability with which T-RF in the sample are distributed among taxa.[19] Evenness (J) was calculated as $E = H' / H_{\text{max}}$, where $H_{\text{max}} = \log_{2}(S)$. The Shannon index ($H'$) is a measure of diversity that combines information about richness and evenness and ranges between 0 and $\log(S)$. The Shannon index is computed as: $H' = - \sum(p_i)(\ln p_i)$; where $p_i$ are the relative abundance of T-RFs.

The data were evaluated for normality and subjected to analysis of variance to detect significant differences among the areas studied; when a significant $p$-value was detected, the means were compared using the least significant difference test ($p < 0.05$). Non-metric multidimensional scaling (NMDS) plot of Principal Coordinates Analysis was generated by Euclidean distance matrices. To analyze potential differences between sites in bacterial community composition and associations with environmental variables, the distance-based redundancy analysis was performed after inspecting the length of gradient. Analyzes were implemented in CANOCO for Windows 5.03 (Microcomputer Power, Ithaca, USA).
Soil bacterial diversity, as estimated by T-RF richness, and alpha and beta diversities (Shannon and Whitaker indices, respectively), showed variable patterns across the gradient and the restriction enzymes used (Table 3). For restriction enzyme Hha I and Msp I, the highest T-RF richness and beta diversity was found at CSS. From all OTUs identified for the enzyme Hha I, 30, 54, and 23 were unique for CG, CSS, and CD, respectively. For the enzyme Msp I, the OTUs 28, 34, and 22 were unique for CG, CSS, and CD, respectively (Figure 2).

The distribution and abundance of various fragment sizes according to the range of standard used are presented in Figure 3. The data show differences in bacterial community composition among the evaluated sites. In both the restriction enzymes, the fragment lengths 67 and 105 bps showed greater abundance in all evaluated sites. However, the abundance of fragment lengths varied according to the restriction enzymes used and sites.
For enzyme $Hha$ I, the fragments lengths 73 and 202 bps showed greater abundance in CG and CSS, whereas in CD, fragment lengths of 90 and 111 bps were more abundant. For the enzyme $Msp$ I, the fragment lengths 73 and 75 bps showed greater abundance in CG and CSS, whereas the fragment length of 112 bps was abundant in CD.

The NMDS analysis based on T-RFLP profiles obtained with the restriction enzyme $Hha$ I separated the sites according to bacterial community, whereas the analysis based on the restriction enzyme $Msp$ I did not clearly separate the evaluated sites (Figure 4). For the enzyme $Hha$ I, which separated the sites according to the bacterial community, the redundancy analysis (RDA) showed that differences in the soil bacterial community structure were related to TOC and altitude, whereas for the enzyme $Msp$ I, RDA showed differences in the soil bacterial community structure related to longitude, Mg content, and plant richness (Figure 5).

Table 1. Main characteristics of the evaluated sites: ‘campo graminoide’ (CG), ‘cerrado strictu sensu’ (CSS) and ‘cerrado’ (CD).

| Characteristic | CG          | CSS         | CD          |
|---------------|-------------|-------------|-------------|
| Longitude     | $41^\circ 42'10"$W | $41^\circ 42'11"$W | $41^\circ 42'13"$W |
| Latitude      | $04^\circ 07'21"$S | $04^\circ 07'22"$S | $04^\circ 07'28"$S |
| Altitude (m)  | 215         | 221         | 238         |
| Soil temperature (°C) | 29         | 27          | 26          |
| Vegetation    | a           | b           | c           |
| Clay (g kg$^{-1}$) | 210.2      | 235.8       | 251.4       |
| Silt (g kg$^{-1}$) | 100.7      | 121.3       | 104.5       |
| Sand (g kg$^{-1}$) | 689.1      | 642.9       | 644.1       |

*aAndropogon fastigiatus; Aristida longifolia; Eragrostis maypurensis.*

*bAndropogon fastigiatus; Aristida longifolia; Terminalia fagifolia; Magonia pubescens; Hymenaea courbaril; Plathymenia reticulata; Qualea grandiflora; Combretum mellifluum; Lippia origanoides; Anacardium occidentale; Simarouba versicolor; Vatairea macrocarpa.*

*cAspidosperma discolor; Parkia platycephala; Terminalia fagifolia; Piptadenia moniliformis; Plathymenia reticulata; Anacardium occidentale; Copaifera coriacea; Thiloa glucocarpa; Casearia grandiflora.*

Table 2. Average of soil chemical at different types of savannah: ‘campo graminoide’ (CG), ‘cerrado strictu sensu’ (CSS) and ‘cerrado’ (CD).

| Site | TOC (g kg$^{-1}$) | pH | P (mg kg$^{-1}$) | K (cmolc kg$^{-1}$) | Al (cmolc kg$^{-1}$) | Ca (cmolc kg$^{-1}$) | Mg (cmolc kg$^{-1}$) | Na (cmolc kg$^{-1}$) |
|------|-----------------|----|----------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| CG   | 4.3$^{b}$       | 4.9$^{a}$ | 3.5$^{a}$ | 1.4$^{a}$ | 0.23$^{a}$ | 0.10$^{a}$ | 0.02$^{b}$ | 0.48$^{a}$ |
| CSS  | 8.2$^{a}$       | 4.7$^{a}$ | 4.6$^{a}$ | 1.8$^{a}$ | 0.36$^{a}$ | 0.33$^{a}$ | 0.11$^{b}$ | 0.49$^{a}$ |
| CD   | 9.1$^{a}$       | 4.6$^{a}$ | 4.8$^{a}$ | 1.6$^{a}$ | 0.39$^{a}$ | 0.26$^{a}$ | 0.08$^{a}$ | 0.39$^{a}$ |

Table 3. Richness and diversity estimation of T-RFs derived from 16S rRNA based on enzymes $Msp$ I and $Hha$ I data, at different types of savannah: ‘campo graminoide’ (CG), ‘cerrado strictu sensu’ (CSS) and ‘cerrado’ (CD).

| Site | Richness* | $H'$ | $S'$ | Whitaker** | Richness | $H'$ | $S'$ | Whitaker |
|------|-----------|------|------|------------|----------|------|------|----------|
|      |           |      |      |            |          |      |      |          |
| CG   | 119$^{b}$ | 4.16$^{a}$ | 0.53 | 0.77$^{b}$ | 157$^{b}$ | 4.31$^{a}$ | 0.46 | 0.98$^{b}$ |
| CSS  | 124$^{a}$ | 4.01$^{a}$ | 0.52 | 1.00$^{a}$ | 162$^{a}$ | 4.32$^{a}$ | 0.48 | 1.05$^{a}$ |
| CD   | 116$^{b}$ | 4.14$^{b}$ | 0.48 | 0.85$^{b}$ | 158$^{b}$ | 4.14$^{b}$ | 0.42 | 0.94$^{b}$ |

*Chao richness; $H'$ – Shannon index; $S'$ – evenness.

**Whitaker diversity.

Figure 2. Venn diagram of the operational taxonomic units (OTUs) based on the 16S rRNA gene with enzyme restriction: (a) $Hha$ I enzyme and (b) $Msp$ I enzyme.

Notes: ‘campo graminoide’ (CG), ‘cerrado strictu sensu’ (CSS) and ‘cerrado’ (CD).

Figure 4. Reaction-based restriction enzyme 16S rRNA gene analysis. (a) $Hha$ I enzyme and (b) $Msp$ I enzyme. Notes: ‘campo graminoide’ (CG), ‘cerrado strictu sensu’ (CSS) and ‘cerrado’ (CD).
Discussion

There was a shift in the structure of soil bacteria as indicated by the variation of T-RF richness and diversity indices. Different patterns of bacterial structure were evident according to the restriction enzymes used. The highest bacterial richness found in CSS, associated with the restriction enzymes may be related to higher plant diversity found in this site [20]. The same authors evaluated the richness and diversity of plants in a gradient of the Cerrado within the Sete Cidades National Park and
found high richness and diversity in CSS as compared with CG and CD. This higher plant diversity in CSS provides diverse carbon inputs and rhizospheric effects that increase the density and diversity of bacteria.  

The alpha diversity did not vary across the gradient, which may indicate no variation in bacterial diversity within each evaluated site. According to Magurran, the magnitude of the alpha diversity is related to the richness of species per unit area and the distribution of the number of individuals per species. On the other hand, beta diversity measures the rate of change in species from one location to another. This index ranges from 0.0, when two samples do not show any difference in species composition, to 2.0, indicating a maximum difference. Our results from the current study showed that there was a shift in bacterial diversity with the restriction enzymes across a gradient of the Cerrado showing a maximum index of 1.0 associated with CSS.

The bacterial structure showed a variable pattern according to the restriction enzyme used, where the enzyme *Hha* I appeared to be more effective in identifying different bacterial diversity in preserved sites, as also observed by Navarrete et al. in native preserved vegetation of the Amazon rainforest. On the other hand, the enzyme *Msp* I was not able to separate bacterial structure across a gradient of the preserved Cerrado within the Sete Cidades National Park. According to Navarrete et al., the enzyme *Msp* I appears to be more effective in identifying differences in bacterial diversity from disturbed sites.

We identified exclusive OTUs across the gradient of the Cerrado, indicating that there are distinct soil bacterial communities at each site. This result demonstrates that shifts in the size and composition of the microbial communities occur not only during the land conversion from native forest to agricultural land but also along a gradient of preserved sites. This indicates that different microbial communities may appear or prevail across a gradient of native ecosystems.

The pattern of T-RFs is a composite of DNA fragments with unique lengths and may reflect the composition of one or more dominant organisms in the community. Therefore, different sizes of T-RFs indicate differences in the sequences of 16S rRNA genes and phylogenetically distinct species or populations of organisms. Our results from the current study indicated a variation in the abundance of T-RFs among the sites, and suggest a shift in bacterial structure across the gradient of the Cerrado. In addition, some prominent T-RFs were at relatively higher proportions than others, representing the dominant microbial groups in the bacterial communities.

The NMDS analysis confirmed that enzyme *Hha* I was able to identify bacterial diversity across a gradient of the preserved Cerrado where the sites were grouped separately, suggesting variable bacterial communities among the evaluated sites. Our results disagree with those of Araujo et al. who evaluated bacterial diversity in the Brazilian Cerrado and found that bacterial community composition is similar among CD and CSS. Therefore, our results may support the early hypothesis proposed by Castro et al. who suggested the existence of three hot spots of biodiversity in the Brazilian Cerrado (the southeastern, central plateau, and
northeastern regions) where the pattern of distribution of plant species between the regions varies. Therefore, the pattern of bacterial diversity of the Cerrado within the northeastern region, as represented by the Sete Cidades National Park, may confirm to the same hypothesis and may therefore be different to bacterial diversity patterns in the southeast region [28] and the central plateau.[27] The results of the current study present an important finding regarding the bacterial diversity in the Brazilian Cerrado as the results may suggest the existence of a hot spot of biodiversity in the Brazilian Cerrado.

The RDA analysis showed that altitude and TOC, for the restriction enzyme Hha I, and longitude, Mg, and plant richness, for the enzyme Msp I, influence bacterial diversity in the preserved Cerrado. Altitude is an important factor that indirectly influences bacterial diversity by directly affecting the temperature, oxygen availability, and vegetation change.[29] TOC significantly influences bacterial richness and diversity as organic carbon is an important source of energy and nutrients for soil microorganisms.[30] On the other hand, plant richness contributes to bacterial diversity through enhanced nutrient flow [31] and changes in microbial abundance.[32]

**Conclusion**

The analysis of T-RFLP clearly separated the structure of bacterial communities across a gradient of the Cerrado within the Sete Cidades National Park. The results of bacterial profiling allowed the observation that the bacterial structure does not follow the same pattern found in other regions associated with the Brazilian Cerrado, such as the southeastern and central plateau regions, and it suggests that the Cerrado in the northeastern region may be a hot spot of bacterial diversity. Further studies should be conducted to identify microbial groups and prospective genes with biotechnological potential.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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