Molecular markers for detection of species from the soil microbiota of the brazilian cerrado

Marcadores moleculares para detecção de espécies da microbiota do solo do cerrado brasileiro

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ABSTRACT
Native microbiota in the Cerrado biome is still underexplored and may prove applicable to biotechnology. Given the Cerrado’s current devastation status, knowing its native microbiota is of paramount importance and may contribute to the existing knowledge gaps. Molecular markers are specific or sensitive elements designed to characterize the microbiota and detect species as they anneal and initiate DNA amplification in specific gene regions. One of the difficulties in developing new biomarkers is the low amount of species-specific genomic sequences available in the databases. Thus, this study aimed to design and test four primer sequences to assist in the rapid identification of soil microorganisms in four parks of Cuiabá. The primers were designed from sequences specific to the bacterial species and those available in the database and were tested for the following soil microorganism species: Acinetobacter soli, Pseudomonas species (sp.), Lysinibacillus varians, and Rhodanobacter thiooxydans. Our results showed that these molecular biomarkers were efficient, with 91.66% positive PCR detection for Acinetobacter soli and Pseudomonas sp. In conclusion, these
designed molecular biomarkers will be suitable for a rapid and precise characterization of these species in environmental samples. Furthermore, our designed markers may help determine species in regions that are under ecological threat.

**Keywords:** Bacteria, Applied Biotechnology, Oligonucleotides, Genes.

**RESUMO**
A microbiota nativa no bioma Cerrado ainda é subexplorada e pode se mostrar aplicável à biotecnologia. Dada a atual situação de devastação do Cerrado, conhecer sua microbiota nativa é de suma importância e pode contribuir para as lacunas de conhecimento existentes. Os marcadores moleculares são elementos específicos ou sensíveis projetados para caracterizar a microbiota e detectar espécies à medida que se recozem e iniciam a amplificação do DNA em regiões genéticas específicas. Uma das dificuldades no desenvolvimento de novos biomarcadores é a baixa quantidade de sequências genômicas específicas de espécies disponíveis nos bancos de dados. Assim, este estudo objetivou projetar e testar quatro seqüências primer para auxiliar na rápida identificação de microorganismos do solo em quatro parques de Cuiabá. Os primers foram desenhados a partir de sequências específicas para as espécies bacterianas e aquelas disponíveis no banco de dados e foram testados para as seguintes espécies de microorganismos do solo: Acinetobacter soli, Pseudomonas species (sp.), Lysinibacillus varians, e Rhodanobacter thiooxydans. Nossos resultados mostraram que estes biomarcadores moleculares foram eficientes, com 91,66% de PCR positivos para Acinetobacter soli e Pseudomonas sp. Em conclusão, estes biomarcadores moleculares projetados serão adequados para uma caracterização rápida e precisa destas espécies em amostras ambientais. Além disso, nossos marcadores projetados podem ajudar a determinar espécies em regiões que estão sob ameaça ecológica.

**Palavras-chave:** Bactérias, Biotecnologia Aplicada, Oligonucleotídeos, Genes.

**1 INTRODUCTION**

Soil life is substantially represented by existing organisms and the activities they perform. Preserving this microbial resource is essential to maintain human quality of life through ecosystem services that ensure sustainable soil health (Wall *et al*., 2019).

The morphological identification of bacteria is significant, but the molecular characterization of strains provides precision and aids in discovering potential biotechnological enzymes (Reddy *et al*., 2018). These enzymes may present the potential to be used in soil bioremediation by endophytic bacteria (Santos *et al*., 2018).

The development of molecular approaches to characterize soil bacteria brings a new perspective in the characterization of the vast diversity of microbial communities (Kowalska *et al*., 2019). Isolating and identifying microorganisms in soil fragments is critical for obtaining genetically stable strains (Adnan and Tan, 2007). Many natural products with added economic value are derived from soil microorganisms of economic, social, and environmental importance (Daniel 2004). Furthermore, metagenomic studies of microorganisms can identify their vast diversity, facilitating comparisons between
microbiomes, and providing knowledge that may be applied to several fields of the industry and research (Fierer, 2017).

A study conducted in Central Park, USA, showed that microbial biodiversity in a single urban fragment resembles several fragments worldwide (Ramírez et al., 2014). This finding highlights the importance of the analysis of soil preservation and health to maintain the balance in microbial communities.

The Cerrado biome is a biodiversity hotspot and the second largest biome in South America (Myers et al., 2000). Several reports show the importance of maintaining the native vegetation Cerrado. The diversity of microorganisms found in native forest soils can be up to ten times greater than species richness in pasture soils (Quirino et al., 2009). The microbiome diversity is justified by the wide variety of animals and plants (Ferreira et al., 2017), despite anthropic activities, which cause constant disturbance to the ecosystem and reduce biodiversity (Gainsbury and Colli, 2019).

The study of the Cerrado microorganism diversity may unravel the effect of disturbance on the ecosystem (Shade et al., 2012) and contribute to understanding the soil microbial community (Schenberg, 2010). The analysis of physical, chemical, and microbial attributes can determine which changes occur in soil properties (Bulluck et al., 2002). In addition, microbial abundance is a critical factor in attesting the health of urban fragments and can be related directly to the degree of ecosystem disturbance (Bao et al., 2019).

The bottleneck in soil microbiology studies is the discrepancy between the existing population of cultivable and non-cultivable bacteria (Fierer 2017). This discrepancy is clarified by meta-genomic studies, which link soil genetics with function-related microorganism processes (Jansson and Hofmockel, 2018).

The multifunctionality of terrestrial microorganisms (i.e., different communities performing different functions) makes them biochemically versatile (Delgado et al., 2016). However, the microorganism variety involved in the decomposition process allows different species to break complex biochemical structures (Coleman et al., 2004).

Molecular biomarkers are complex elements employed in a polymerase chain reaction (PCR) test to characterize the molecular features of non-cultivable soil bacteria (Pereira et al., 2006). These markers have been relevant due to their high specificity and sensitivity, are essential and can help protective in to environment (Saiki et al., 2006; SILVA et al., 2020). These markers are capable of accurate and reliable results, as long as their design contains a minimum of 20 primer-dimers and possess an ideal annealing temperature for the developed oligonucleotide sequence (Kowalska et al., 2019; Bustin and Huggett 2017).
Analysis using species-specific primers can optimize time, especially for pathogens requiring immediate characterization (Pastro et al., 2018). Therefore, in this study, four species-specific primers were designed and tested to assist in the Cerrado soil microbiome's rapid characterization.

2 MATERIAL AND METHODOLOGY

The soil microbiome studies were conducted in four urban parks of Cuiabá, Mato Grosso (Figure 1): (a) Tia Nair Park, (b) Water Park (Parque das Águas), (c) Mãe Bonifácia State Park, and (d) Massairo Okamura Park, in accordance to license and scientific research permits 200/2017, 285640/2017, and 396175/2017, granted by the state and municipal agencies (SEMA-MT and Cuiabá City Hall, respectively).

Figure 1: Mapping of sampled points four urban parks of Cuiabá, Mato Grosso. Parque Tia Nair (a), Parque das Águas (b), Parque Mãe Bonifácia (c) and Parque Massairo Okamura (d). Source: Search Results. Observed at the collect point: Exotic plants(vegetation) were more than native plants, both and more native than exotic plants.

Two soil samples were collected from three locations per park during the dry season in early September 2017 and the rainy season in late October 2017. The total DNA extraction was conducted by modifying pre-established protocols for the specific sample type (Aljanabi and Martinez, 1997; Junqueira and Silveira, 2010; Sambrook et al., 1989). The 16S ribosomal DNA (rDNA) amplification gene fragments utilized 968 Forward 5’-AAC GCG AAG AAC CTT AC-3’ and 1401 Reverse 5’-CGG TGT GTA CAA GAC CC-3’ primer oligonucleotides (Nubel et al., 1996). The PCR mix consisted of 12.5µl OneTaq hot start 2x Master Mix with Standard Buffer (Biolabs), 10µl of MilliQ Water, 0.5µl of each primer, 1µl BSA (Bovine Serum Albumin), and 1.5µl of sample DNA to make up 25µl of the
final volume. The amplification cycles occurred at temperatures of 95°C for 5'; 95°C for 45'; 55°C for 45'', 72°C for 1'; 72°C for 7'; and 4°C.

The most common organisms identified for the primer design, based on both the 16S rDNA sequencing analysis and comparing available GenBank database sequences (https://www.ncbi.nlm.nih.gov/genbank/), are shown in Table 1. After designing the primer, these sequences were edited and subjected to structural analysis using the software OligoAnalyzer 3.1. The primers were aligned with the sequences using the BLAST tool in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

| Species                          | Type | Sequences (5' – 3')a | Base pairs (pb) | Orientation | GeneBank Access |
|---------------------------------|------|----------------------|-----------------|-------------|----------------|
| Acinetobacter soli              | AciF | GACGATCTGTAGCGGGTCTG | 318             | Forward     | KJ806489       |
|                                 | AciR | AAGAGCCTCCTCCTCGITTA |                 | Reverse     |                |
| Pseudomonas species (sp.)       | PsedF| ATTAAGTTGACCGGCTGGGG | 910             | Forward     | JQ861800       |
|                                 | PsedR| ATCACACCCTGGTAACCCTC |               | Reverse     |                |
| Lysinibacillus varians          | LyvaF| AGGCAACGATGCGTAACCC  | 229             | Forward     | JQ861800       |
|                                 | LyvaR| CTGGCAGGATGTTAGCGGTG |               | Reverse     |                |
| Rhodanobacter thiooxydans       | RhotF| ATCGAGACCGAGACGATGC  | 1049            | Forward     | AB741464.1     |
|                                 | RhotR| TCCAATCGGTCGTTCCAG   |                 | Reverse     |                |

Biomarkers were tested and evaluated through PCR for their amplification specificity. Amplifications using species-specific primers followed the same steps as described for 16S rDNA primers. Post amplification, the products were tested on 1.5% electrophoresis gel, stained with 1 µl Blue Juice (10x), and 1 µl GelRed® (Biotium), and compared to a 100 kb DNA ladder (KASVI). The dendrogram was developed using an online MEGA 7 program with a p-distance analysis.

3 RESULTS AND DISCUSSION

A total of 24 sequenced 16S rDNA gene samples had satisfactory results in obtaining the main species of occurrence in the analyzed soils. These species belong to the Gammaproteobacteria class, except Lysinibacillus varians, which belong to the Firmicutes class, and both species are related phylogenetically (Figure 2). The genera and bacterial species with the highest genetic identity degree were Acinetobacter soli, Pseudomonas species (sp.), Lysinibacillus varians, and Rhodanobacter thiooxydans.
Figure 2: Dendrogram of species through 16S gene analysis. These species belong to the Gammaproteobacteria class, except Lysinibacillus varians, which belong to the Firmicutes class and are phylogenetically related as evidenced in the dendrogram.

The annealing of primers *Pseudomonas* sp. and *Acinetobacter soli* was satisfactory for 91.66% of the samples. Primers for *Lysinibacillus varians* and *Rhodanobacter thiooxydans* species did not anneal despite multiple tests and protocol modifications. Additionally, the genus *Pseudomonas* sp. were tested with control PCR using a pure *P. aeruginosa* strain (CBAM 0679).

According to previous studies (Kowalska et al., 2019 and Fierer 2017), with the advent of molecular biology, rapid identification of large populations of bacteria is now possible. Four bacterial primers were designed for species in urban park soils. These designed molecular markers allowed the rapid identification of bacterial species in the Cerrado soils.

We also found that *Acinetobacter soli* had full amplification in the 24 samples among the Aci primer sequences (GenBank: BBNM0100001.1). This molecular biomarker was the most efficient among those developed with a 100% amplification at all of the four urban parks of Cuiabá-MT. The presence of *Acinetobacter soli* bacteria in these soils is an indicator of environmental balance, as this genus usually develops in forest soils. This genus may also have critical biotechnological applications for the degradation of petroleum and its derivatives (Kim et al., 2008). They found that bacteria capable of degrading ammonia in native forest soils are more genetically diverse than the soil microbiota disturbed by conventional planting (Peixoto et al., 2006). Some bacterial strains use new biochemical strategies to degrade one of the most commercialized and environmentally harmful synthetic polymers: polyethylene. This polymer is resistant to microbiota attacks due to its chemical composition (Peixoto et al., 2017).

The strains of *Pseudomonas* sp. (GenBank: Y927414.1) are useful for sulfide gas bio-oxidation, even at high concentrations (Xu et al., 2016). Its strains can metabolize through oxidation and hydrolysis, the endosulfan compound, a highly toxic and environmentally persistent organochlorine pesticide (Zaffar et al., 2018). The oligonucleotide for this species obtained an effectiveness of 83.33% of amplification, indicating that its usefulness for being easily detected in soil samples.

The primer Lyva (*Lysinibacillus varians*, GenBank: KX011876.1) was not effective for amplification despite multiple bioinformatic program tests and PCR analysis. However, this marker
would identify biodegrading bacteria from persistent environmental contaminants such as Bromate Diphenyl Ether (BDE), which has low volatility and solubility in aqueous media (Zhu et al., 2014). The toxicity of this compound class has been related to immunological, hepatotoxic, neurotoxic, and even endocrine changes (Annunciação et al., 2018). The large-scale genomic sequencing may guarantee the availability of new oligonucleotide sequences.

The oligonucleotides designed for Rhot (Rhodanobacter thiooxydans, GenBank: QBUW01000007.1) did not amplify in any of the PCR analysis. Importantly, these species can metabolize thiosulfate, which is common in the drug industry and in the industry for paper bleaching (Bui et al., 2010).

The absence of the Rhot and Lyva primer annealing via PCR for the tested and analyzed gene regions may be attributed to the sequence designs being to China. They may present distinct nucleotide alterations, because of evolution, for the Cerrado microbiome. There are several reasons for non-nucleotide binding in the primer annealing, such as a mismatch in complementary bases and positional base pairing (Long et al., 2013). Furthermore, the sequence for amplification in Rhodanobacter thiooxydans is extensive, which may also have been a reason for the absence of positive results for this species.

We did not find pure strains for the remaining species in other microorganism databases. Therefore, we were unable to test controls for all samples. However, a 30% positive amplification of random sample sequences confirmed the effectiveness of primers for Acinetobacter soli and Pseudomonas sp.

In addition to optimizing research time, PCR-based detection methods are more productive and effective and contribute to the knowledge of microorganism diversity in ecosystems (Zhang et al., 2019). In conclusion, given the Cerrado soil microbiome’s significance, it is essential to encourage pure strains selection and novel primer designs. Rapid and reliable detection of these microorganisms is still a challenge due to their scarce genomic data.

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