Robust DNA protocols for tropical soils

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ABSTRACT

Studies in the Amazon are being intensified to evaluate the alterations in the microbial communities of soils and sediments in the face of increasing deforestation and land-use changes in the region. However, since these environments present highly heterogeneous physicochemical properties, including contaminants that hinder nucleic acids isolation and downstream techniques, the development of best molecular practices is crucial. This work aimed to optimize standard protocols for DNA extraction and gene quantification by quantitative real-time PCR (qPCR) based on natural and anthropogenic soils and sediments (primary forest, pasture, Amazonian Dark Earth, and várzea, a seasonally flooded area) of the Eastern Amazon. Our modified extraction protocol increased the fluorometric DNA concentration by 48%, reaching twice the original amount for most of the pasture and várzea samples, and the 260/280 purity ratio by 15% to values between 1.8 to 2.0, considered ideal for DNA. The addition of bovine serum albumin in the qPCR reaction improved the quantification of the 16S rRNA genes of Archaea and Bacteria and its precision among technical replicates, as well as allowed their detection in previously non-amplifiable samples. It is concluded that the changes made in the protocols improved the parameters of the DNA samples and their amplification, thus increasing the reliability of microbial communities’ analysis and its ecological interpretations.

1. Introduction

Studies on the microbial communities in Amazonian soils and sediments have been intensified to understand the impacts of deforestation and land-use change on their taxonomic and functional diversity (e.g., Rodrigues et al., 2013; Paula et al., 2014; Lammel et al., 2015; Mendes et al., 2015; Navarrete et al., 2015a; Meyer et al., 2017). However, these environments are complex in their physicochemical properties and contain potential organic and inorganic contaminants to nucleic acids isolation that cannot be completely removed by most extraction methods, remaining in the DNA samples and hindering downstream techniques (Moreira, 1998). Thus, the development of best practices is imperative to overcome these issues in molecular studies.

The extraction of DNA from environmental samples can be performed using direct or indirect (which involves an initial cell extraction step) approaches (Gabor et al., 2003) and requires lysis through physical, chemical, and/or enzymatic methods to disrupt the cell walls and membranes of the microorganisms and release their nucleic acids into the medium; followed by the removal of cell fragments, DNA capture and purification from contaminants (Roose-Amsaleg et al., 2001). Numerous studies have compared the most important extraction methods for soils and sediments (e.g., Stach et al., 2001; Carrigg et al., 2007; Inceoğlu et al., 2001; Terrat et al., 2012; Leite et al., 2014; Devi et al., 2015), aiming to obtain DNA samples of high concentration and purity and consequently generate low-biased representations of their microbial communities (Robe et al., 2003). These include commercial kits, listed by Dhaliwal (2013), which are often used due to their practicality and optimized features for several environments.

Following extraction, several contaminants such as clay minerals, debris, proteins, humic substances, phenolic compounds, salts, and metal ions can still be present in environmental DNA samples (Wilson, 1997; Griffiths et al., 2000; Schrader et al., 2012; Leite et al., 2014; Narayan et al., 2016). Thus, an additional purification step is often required but can be expensive, time-consuming, and lead to DNA loss (Kreader, 1996). Alternatively, additives can be used in PCR-based methods to relieve their inhibition, including in quantitative real-time PCR (qPCR) reactions (Schriewer et al., 2011; Albers et al., 2013; Sidstedt et al., 2015). The most used PCR additive is the bovine serum albumin (BSA), a transport protein that can bind to lipids and organic molecules, thus being able to reduce several types of inhibition (Hedman and Rådström, 2013).

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Up to date, there are no comprehensive DNA protocols optimized for Amazonian soils. Therefore, this work aimed to improve standard protocols for DNA extraction and qPCR based on natural and anthropogenic soils and sediments (primary forest, pasture, Amazonian Dark Earth, and varzea, a seasonally flooded area) of the Eastern Amazon through minor laboratory modifications, aiming to increase the concentration and quality of the extracted DNA and reduce the inhibition of the qPCR reactions using BSA.

2. Material and methods

2.1. Soil sampling and characterization

The sampling sites are located at the Tapajós National Forest and its surroundings, in the Pará State, Brazil, Eastern Amazon. The forest has a tropical monsoon climate (Am Köppen) and consists mostly of nutrient-poor oxisols and ultisols (Silver et al., 2006; IBAMA, 2004). This region is covered by primary, logged, and secondary forests as well as lands converted to pasture and agricultural fields, including manioc, rice, beans, corn, soybean, sugarcane, coffee, black pepper, and fruit crops (O’Antona et al., 2006; Steward, 2007). It also harbors Amazonian Dark Earths, soils resulting from human activities mainly between 2,500 to 500 BP (Neves et al., 2003) that cover about 10% of the Amazon (Mann, 2002) and are characterized by high levels of stable carbon (such as charcoal and humic substances), organic matter, and nutrients (Mann, 2002; Kämpf et al., 2003; Lehmann et al., 2003; Novotny et al., 2007; Glaser and Birk, 2012); and lowlands that periodically receive water and sediments from their adjacent rivers, which constitute 13% of the territory (Nascimento and Homma, 1984).

The sampling was conducted in May and October 2016 at four sites: (1) primary forest (PF, 3°17′44.4″S 54°57′46.7″W); (2) pasture (PA, 3°18′46.7″S 54°54′33.4″W); (3) Amazonian Dark Earth (ADE, 2°50′36.1″S 54°58′32.6″W); (4) and varzea (VA, 2°22′44.8″S 54°44′21.1″W), a type of seasonally flooded area (floodplain). At each site, three soil samples from 0 to 10 cm depth were collected. Following their transportation to the laboratory, aliquots were sent to the Department of Soil Science of the Luiz de Queiroz College of Agriculture (ESALQ/USP) for the determination of the following physicochemical properties: pH determined in 0.01 M calcium chloride (CaCl₂); soil organic matter (SOM) determined by colorimetry; nitrogen (N) determined by the Kjeldahl method; phosphorus (P) extracted with ion exchange resin and determined by colorimetry; potassium (K) determined by atomic emission spectrophotometry; calcium (Ca) and magnesium (Mg) extracted with ion exchange resin and determined by atomic absorption spectrophotometry; exchangeable aluminum (Al) extracted with 1 M potassium chloride (KCl) and determined by the colorimetric method; potential acidity (H + Al) determined with the SMP buffer; boron (B) extracted with hot water and determined by colorimetry; copper (Cu), iron (Fe), manganese (Mn), and zinc (Zn) extracted with DTPA and determined by atomic absorption spectrophotometry. Sum of bases (SB), cation-exchange capacity (CEC), base saturation (V), and aluminum saturation (m) calculations were made based on these previous results. The contents of sand, silt, and clay were determined by the hydrometer method and classified according to the United States Department of Agriculture (USDA) classification (2018).

2.2. DNA extraction and quantification

The soil DNA extraction was performed using the DNeasy PowerLyzer PowerSoil Kit (Qiagen), a widely used commercial kit for soils due to its mechanical-chemical-methods for cell lysis, patented inhibitor reagent to remove organic and inorganic contaminants (comprising humic acids, cell debris, and proteins), and silica membranes for DNA capture and cleaning (MO BIO Laboratories, 2016). The total DNA of each sample was extracted by two methods: the manufacturer’s and an optimized protocol, in which after adding the solution C1, the samples were vortexed for 15 min at maximum speed, and centrifuged for 3 min at 10,000 x g. Besides, all the incubations of the modified protocol after adding the solutions C2 and C3 were made at minus 20 °C (-20 °C) instead of 4 °C. The DNA samples were evaluated on a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) and a Qubit fluorometer Q232857 using the dsDNA BR Assay Kit (Thermo Fisher Scientific).

2.3. Quantitative real-time PCR of the 16S rRNA genes of Archaea and Bacteria

The 16S rDNA genes were quantified by quantitative real-time PCR through the standard-curve method using the primer pairs Arch1S9F (Ovreás et al., 1997) and Arch1S9R (Stahl and Amann, 1991) for Archaea, and 926F and 1062R (De Gregorio et al., 2011) for Bacteria. For both genes, the qPCR of each DNA sample extracted with the optimized protocol was performed in a 10-μL reaction on a StepOnePlus instrument (Thermo Fisher Scientific) in triplicate for each of the tested treatments: (1) no bovine serum albumin and (2) 0.1 μL of BSA (20 mg mL⁻¹) (Thermo Fisher Scientific), resulting in a final concentration of 200 μL⁻¹. Each 10-μL reaction mixture included 5 μL of SYBR Green ROX qPCR Master Mix (Thermo Fisher Scientific), 1 μL of each primer (5 pmol), 1 μL of DNA (10 ng), and ultra-pure H₂O to complete 10 μL. The amplification conditions for Archaea consisted of 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 50 s followed by a melting curve of 95 °C for 15 s, 57 °C for 1 min and 95 °C for 15 s; and for Bacteria, 95 °C for 10 min, 40 cycles of 95 °C for 45 s, 60 °C for 15 s and 72 °C for 20 s followed by a melting curve of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. The results were analyzed using StepOne Software v2.3 (Thermo Fisher Scientific), exported as spreadsheets, and converted into the number of gene copies per ng of DNA.

2.4. Statistical analysis

The soil physicochemical properties were analyzed by analysis of variance (ANOVA) followed by the Tukey’s post-hoc test using the agricolae package 1.2–8 (de Mendiburu, 2017) in R studio 1.0.153 (RStudio Team, 2016) and also subjected to a non-metric multidimensional scaling (NMDS) based on the Gower’s distance using the vegan package 2.5 (Oksanen et al., 2018). The NMDS plot was generated using the ggplot2 package 3.0.0 (Wickham, 2016). The results from the NanoDrop and Qubit quantification of the DNA samples and qPCR of the 16S rRNA genes of Archaea and Bacteria were aligned rank-transformed and analyzed by a two-way mixed-design ANOVA using the ARTool package 0.10.5 (Kay and Wobbrock, 2018). Post-hoc tests (Holm-adjusted) were carried out using the lmeans package 2.27–62 (Lenth, 2016).

3. Results and discussion

3.1. Soil physicochemical properties

The chosen sites, which represent different Amazonian environments in this study, exhibited contrasting physicochemical properties. The sampled soils and sediments were classified as sandy clay loam (PF), clay (PA and ADE), and sandy loam (VA), according to the USDA textural classification (2018). The pH of all samples was found to be acidic, ranging from 3.5 to 5.1 (Figure 1 and Table 1). Using ANOVA followed by Tukey’s post-hoc test at a 0.05 level of significance, the PF site had the lowest pH and, together with VA, the highest values of Al and m. In opposition to most of the Amazonian soils, considered weathered, highly acidic, and low-fertile, ADEs are known to present large amounts of charcoal and humic substances as well as more organic matter and nutrients than their surroundings, including N, P, Ca, Mg, S, Mn, and Cu (Mann, 2002; Kämpf et al., 2003; Lehmann et al., 2003; Novotny et al., 2007; Glaser and Birk, 2012). In our study, this site presented elevated...
macronutrient levels, with the highest values of Ca, Mg, SB, CEC, and V among the studied soils. ADE also had higher contents of SOM and N than PF and VA, and P compared to PF. The VA site, which receives sediments from both Tapajos and Amazon rivers in the rainy season, showed the highest levels of Cu and Mn. This site also showed higher contents of K and Zn than PF and ADE.

The soil environment results from multiple interacting factors, including texture, pH, and nutrient content (Robe et al., 2003). A number of studies have revealed the importance of physicochemical properties on the structure and functioning of tropical soil microbial communities (Lammel et al., 2018; Merloti et al., 2019; Pedrinho et al., 2019), although access to this diversity can be challenging as soil characteristics (including clay and organic matter contents) influence the efficiency of microbial DNA recovery (Roose-Amsaleg et al., 2001). In this sense, the results of the physicochemical analysis demonstrated that even nearby areas within the Amazon biome provide different environments for soil microbial communities; and therefore, it is imperative to establish a unique DNA extraction protocol that takes into account all these heterogeneous properties.

### 3.2. DNA concentration and purity

The choice of the DNA extraction method is a crucial step in molecular studies, which can affect the detection of microbial communities’ structure and composition (Inceoglu et al., 2010; Terrat et al., 2012; Hallmaier-Wacker et al., 2018). The DNeasy PowerLyzer PowerSoil Kit

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**Table 1.** Mean and standard deviation of the soil chemical properties of the primary forest, pasture, Amazonian Dark Earth, and várzea sites.

| Properties     | Units    | Primary forest | Pasture | Amazonian Dark Earth | Várzea |
|----------------|----------|----------------|---------|----------------------|--------|
| pH             |          | 3.53 ± 0.06 c  | 4.37 ± 0.29 ab | 4.83 ± 0.23 a       | 4.07 ± 0.06 b |
| SOM g dm⁻³     |          | 32.67 ± 3.51   | 40.03 ± 19.66 ab | 70.67 ± 13.05 a     | 7.33 ± 1.15 c |
| N mg kg⁻¹      |          | 1,822.33 ± 77.42 b | 2,837.33 ± 1,277.18 ab | 3,894.33 ± 610.26 a | 1,348.67 ± 271.14 b |
| P mg dm⁻³      |          | 6.00 ± 1.00 b  | 7.67 ± 1.62 ab  | 15.67 ± 3.51 a      | 14.67 ± 4.04 ab |
| S mg dm⁻³      |          | 6.00 ± 2.00 a  | 4.67 ± 1.15 a   | 14.00 ± 2.00 a      | 13.00 ± 12.29 a |
| K mmol dm⁻³    |          | 0.37 ± 0.06 c  | 1.63 ± 0.85 ab  | 0.67 ± 0.15 bc      | 1.97 ± 0.06 a  |
| Ca mmol dm⁻³   |          | 3.00 ± 0.00 b  | 16.00 ± 13.86 b | 130.67 ± 6.66 a     | 5.67 ± 1.53 b  |
| Mg mmol dm⁻³   |          | 1.67 ± 0.58 b  | 6.00 ± 4.36 b   | 18.00 ± 3.00 a      | 5.33 ± 1.53 b  |
| Al mmol dm⁻³   |          | 12.33 ± 0.58 a | 4.67 ± 3.21 b   | 1.00 ± 0.00 b       | 16.00 ± 1.73 a |
| H + Al mmol dm⁻³|         | 82.00 ± 5.20 a | 56.00 ± 3.46 ab | 33.33 ± 11.72 b     | 75.33 ± 19.63 a |
| SB mmol dm⁻³   |          | 4.70 ± 1.21 b  | 23.63 ± 17.90 b | 149.33 ± 3.64 a     | 12.97 ± 3.05 b |
| CEC mmol dm⁻³  |          | 86.70 ± 3.98 b | 79.63 ± 14.44 b | 182.67 ± 8.09 a     | 88.30 ± 22.31 b |
| V %            |          | 5.33 ± 1.15 c  | 27.67 ± 15.89 b | 82.00 ± 5.29 a      | 14.67 ± 2.08 bc |
| m %            |          | 72.67 ± 6.35 a | 22.67 ± 18.01 b | 1.00 ± 0.00 b       | 55.67 ± 3.79 a |
| B mg dm⁻³      |          | 0.41 ± 0.03 a  | 0.41 ± 0.04 a   | 0.22 ± 0.08 b       | 0.23 ± 0.02 b  |
| Cu mg dm⁻³     |          | 0.23 ± 0.06 b  | 0.53 ± 0.32 b   | 0.37 ± 0.21 b       | 3.53 ± 1.38 a  |
| Fe mg dm⁻³     |          | 99.67 ± 43.68 a| 87.67 ± 17.01 a | 67.00 ± 31.19 a     | 118.67 ± 9.29 a |
| Mn mg dm⁻³     |          | 3.53 ± 2.92 b  | 5.47 ± 6.62 b   | 15.93 ± 8.24 b      | 115.10 ± 62.73 a|
| Zn mg dm⁻³     |          | 0.47 ± 0.15 b  | 1.33 ± 1.29 ab  | 0.23 ± 0.06 b       | 3.03 ± 1.19 a  |

Different letters indicate significant differences among sites according to the Tukey’s post-hoc test (p < 0.05). SOM, soil organic matter; H + Al, potential acidity; SB, sum of bases; CEC, cation-exchange capacity; V, base saturation; m, aluminum saturation.
were made regarding the duration and temperature of its steps. Biomass abundance (Narayan et al., 2016), no work has been done so far to optimize DNA protocol respecting its unique composition and varied according to the land-use since a significant interaction (F3,8 = 10.462, p < 0.012) using the optimized protocol, reaching almost twice the original amount for most of the PA and VA samples (Table 2), also noticed as thin bands on agarose gel electrophoresis (data not shown). This improvement was not so clearly observed in the NanoDrop data; however, spectrophotometric methods are considered less specific and accurate in comparison with the Qubit system (O’Neill et al., 2011). The results from both quantification methods varied according to the studied site (Qubit: F3,8 = 6.617, p = 0.015; NanoDrop: F3,8 = 6.161, p = 0.018), with post-hoc tests (Holm-adjusted) indicating a significant difference (p < 0.05) between ADE and VA. The 260/280 ratio increased (F1,8 = 7.817, p = 0.006) using the optimized protocol, reaching almost twice the original amount for most of the PA and VA samples (Table 2), also noticed as thin bands on agarose gel electrophoresis (data not shown). Humic acids are the most common contaminant co-extracted with DNA (Yeates et al., 1998), which can phenolic groups that can denature biological molecules by bonding to amides or oxidize to form a quinone that covalently bonds to proteins and DNA (Young et al., 1993). In addition, humic acids can inhibit DNA polymerase and chelate magnesium ions, an essential cofactor for its activity (Tsai and Olson, 1992a, 1992b; Sidstedt et al., 2015). Different molecular mechanisms associated with their effect on SYBR Green assays have also been proposed (Zipper et al., 2003). Humic acids are not easily removed from DNA extracts by purification methods (Lakay et al., 2007; Sagova-Marceckova et al., 2008), but the impacts of these contaminants on qPCR amplification can be relieved using BSA, a widely used additive for environmental samples that contain potential inhibitors, in concentrations ranging from 40 to 400 ng μL⁻¹ (Schriewer et al., 2011). In our samples (all samples were previously adjusted so that each amplification reaction for both genes contained 10 ng of DNA), the BSA addition increased (F1,8 = 54.966, p < 0.001) the quantification of the 16S rRNA gene of Bacteria (Table 3). For the archaeal 16S rRNA gene, a significant effect of the treatment (F1,8 = 80.062, p < 0.001), studied site (F3,8 = 6.411, p = 0.016) and their interaction (F3,8 = 9.148, p = 0.006) was indicated. Post-hoc analysis (Holm-adjusted) showed that the difference in the archaeal abundance between the BSA treatment and control from the ADE site was significantly different (p < 0.05) compared to the differences found in PF and PA sites.

Besides highly increasing gene abundance, the BSA addition allowed the detection of both genes in non-amplifiable DNA samples (without the additive) and improved the precision of the quantification among technical replicates, ensuring the replicability of the results. Although the

| Site               | Protocol | Qubit Concentration | NanoDrop Concentration | 260/280 | 260/230 |
|--------------------|----------|---------------------|------------------------|--------|--------|
| Primary forest     | Original | 9.94 ± 2.37         | 15.13 ± 3.01           | 1.63 ± 0.07 | 1.55 ± 0.45 |
|                    | Modified | 11.00 ± 1.39        | 16.87 ± 1.78           | 1.88 ± 0.09 | 1.14 ± 0.19 |
| Pasture            | Original | 10.57 ± 6.91        | 17.32 ± 14.01          | 1.69 ± 0.10 | 1.86 ± 0.17 |
|                    | Modified | 19.40 ± 14.38       | 16.56 ± 7.34           | 1.96 ± 0.12 | 1.18 ± 0.46 |
| Amazonian Dark Earth | Original | 33.57 ± 5.86       | 28.81 ± 3.80           | 1.77 ± 0.01 | 1.87 ± 0.05 |
|                    | Modified | 33.77 ± 5.29        | 29.56 ± 4.95           | 1.90 ± 0.03 | 1.67 ± 0.16 |
| Várzea            | Original | 5.81 ± 1.28         | 10.12 ± 1.19           | 1.62 ± 0.05 | 1.22 ± 0.26 |
|                    | Modified | 10.63 ± 1.87        | 9.49 ± 2.27            | 1.95 ± 0.09 | 0.99 ± 0.07 |

*p < 0.05; **, p < 0.01; ***, p < 0.001.
effect of BSA on relieving the influence of inhibitors can vary according to the DNA polymerase used in the qPCR reaction (Albers et al., 2013), considering the conditions applied to this study, this additive was essential for the gene quantification from samples complex in inhibitors, such as the soils and sediments of the Amazon. Higher BSA concentrations (400 ng μL⁻¹) were also tested and showed to decrease the quantification for most samples in comparison to our BSA treatment, but the results varied according to the site and target gene (data not shown).

4. Conclusion

We conclude that our optimized extraction protocol increased the concentration and purity of the DNA samples, as well as the BSA addition in the qPCR reaction allowed better gene amplification and quantification, thus increasing the reliability of the molecular data and the inferences to be drawn from them regarding the microbial communities in soils and sediments of the Amazon.

Declarations

Author contribution statement

Andressa Monteiro Venturini, Fernanda Mancini Nakamura, Júlia Brandão Gontijo: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Aline Giovanna da França: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.
Caio Augusto Yoshiura: Analyzed and interpreted the data; Wrote the paper.
Jéssica Adrielle Mandro: Conceived and designed the experiments; Performed the experiments.
Siu Mui Tsai: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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Table 3. Mean and standard deviation of the qPCR quantification (copies ng⁻¹ DNA) of the 16S rRNA genes of Archaea and Bacteria for the primary forest, pasture, Amazonian Dark Earth, and várzea sites using no (control) and 200 ng μL⁻¹ of BSA; followed by the results (degrees of freedom, F-values, and p-values) of the two-way mixed-design ANOVA of the aligned rank-transformed data.

| Site                  | Treatment       | 16S rRNA of Archaea | 16S rRNA of Bacteria |
|-----------------------|-----------------|---------------------|---------------------|
| Primary forest        | Control         | 9.48E± 0.02 1.11E±03| 1.44E±0.04 2.41E±04 |
|                       | BSA             | 1.51E± 0.35E±03     | 4.98E±0.14 1.40E±05 |
| Pasture               | Control         | 3.52E±0.13 5.8E±03  | 3.99E±0.04 7.41E±03 |
|                       | BSA             | 2.43E±0.10 1.01E±04 | 6.46E±0.35 3.55E±05 |
| Amazonian Dark Earth  | Control         | 5.38E±0.02 7.7E±02  | 4.70E±0.03 8.14E±03 |
|                       | BSA             | 4.51E±0.04 8.84E±03 | 2.10E±0.05 1.84E±05 |
| Várzea               | Control         | 6.71E±0.02 6.04E±02 | 1.26E±0.04 1.09E±04 |
|                       | BSA             | 2.65E±0.04 7.06E±03 | 2.17E±0.05 1.58E±05 |

Site: 3 * p < 0.05; **, p < 0.01; ***, p < 0.001.
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