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# Comparison of protocols for DNA extraction from fecal samples of capuchin monkeys, *Sapajus nigritus*, and marmosets, *Callithrix* sp.

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**ABSTRACT.** Fecal sampling is a non-invasive methodology for obtaining DNA for genetic studies in wild animals. The capuchin monkey, *Sapajus nigritus* and the invasive marmosets in Southeast Brazil, *Callithrix jacchus*, *C. penicillata,* and their hybrids, have been among the targets of these studies. The commercial kits for fecal DNA extraction from feces, still demand a high cost, and to circumvent this situation, homemade protocols can be used. Our objective was to test the protocols of Finger (2015) and Doyle & Doyle (1987) to extract fecal DNA from *S. nigritus* and *Callithrix* spp., evaluating their efficiency for PCR and the influence of collection time and storage method. Thirteen fecal samples were collected in Rio de Janeiro in different locations and periods. Three experiments were designed to test the protocols. In test 1, with samples collected in 2015, 2018, and 2019 preserved in 70% ethanol, no DNA was obtained with any protocol. The Finger protocol was also not successful in tests 2 and 3, with samples collected in 2023. The Doyle & Doyle protocol was efficient in tests 2 and 3, with extractions on the day of collection and up to a month of storage at -20°C. *Callithrix* fecal DNA generated an amplicon in the first PCR, whereas for *S. nigritus*, only in the second PCR, after dilution of the DNA and probable reduction of inhibitors. The Doyle & Doyle (1987) protocol was efficient for extracting fecal DNA of the species studied from fresh feces or from those stored for up to 30 days at -20°C.

**Keywords:** Feces; non-invasive method; non-human-primates.

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## Introduction

The capuchin monkey, *Sapajus nigritus* (Goldfuss, 1809), is endemic to the Atlantic Forest, ranging from Southeastern to Southern Brazil, extending to Northeast Argentina (Lynch Alfaro et al., 2012). The conservation status of this species is ‘Near Threatened’, primarily due to habitat loss caused by forest fragmentation (International Union for Conservation of Nature [IUCN], 2021). The genus *Callithrix* comprises six species endemics to Brazil, which inhabit different regions. *Callithrix aurita* (Geoffroy, 1812) is native to the Atlantic Forest in the Southeastern region, specifically in the states of Minas Gerais, Rio de Janeiro, and São Paulo (Loureiro, Guimarães-Lopes, Rodrigues & Massara, 2023). The conservation status of this species is ‘Endangered’ due to habitat loss, competition, and hybridization with invasive species, such as *C. penicillata* and *C. jacchus* ([IUCN], 2021), originally from the Cerrado and the Caatinga, respectively. These species were introduced in the Atlantic Forest in the Southeastern region through human activities (Malukiewicz, 2019), and, as a result, interbreed, producing fertile hybrids. All of these hybrids can mate with *C. aurita* (Nogueira et al., 2022), resulting in the loss of the native species’ gene pool (Malukiewicz, 2019).

The DNA of all these species is widely used for studies in many fields of knowledge, such as ecology, behavior, taxonomy, and genetics. For example, in assessing the loss of genetic variability and to investigate parasitic load through molecular techniques (Quirós et al., 2018; Solórzano-Garcia & Léon, 2018; Coimbra et al., 2020; Hilário et al., 2022; Cáceres et al., 2022). Obtaining DNA from feces is a non-invasive sampling methodology, avoiding potential harm caused during captures (Wayne & Morin, 2004; Lefort et al., 2022; Sappington, 2019). Currently, commercial kits are preferentially used for DNA extraction from fecal samples (Zhang et al., 2006; Arguello-Sánchez et a., 2018; Detwiler, 2019; Mallot et al., 2018). However, despite their

efficiency, the high cost of these kits can render their use impractical (Santos et al., 2018). There are several homemade protocols for obtaining DNA from feces, and the choice of the most suitable method should consider the type of sample used, the concentration of the molecule to be extracted, and the species under study (Nonohay & Hepp, 2017). These protocols provide a lower-cost alternative, employing techniques such as phenol-chloroform, guanidine thiocyanate/silica, and cetyltrimethylammonium bromide/phenol: chloroform:isoamyl alcohol (Zhang et al., 2006).

The quality of DNA obtained from fecal samples is often inferior in terms of purity compared to DNA extracted from blood samples, for instance. This is especially true for herbivorous species due to the presence of polysaccharides, which are known inhibitors of the Polymerase Chain Reaction (PCR) (Zhang et al., 2006), and phenolic compounds, which are oxidative and, when present, give a dark coloration to the DNA (Schenk et al., 2023). No single homemade protocol appears to be effective for extracting DNA from all species, making testing and adjustments necessary depending on the species under study (Vallet et al., 2008).

Capuchin monkeys are omnivorous, exhibiting dietary flexibility with a large proportion of plant consumption, approximately 65%. It has been estimated that fruits are the most consumed items, followed by leaves, seeds, and flowers (Rasec-Silva et al., 2023). Marmosets, on the other hand, are considered exudate-insectivores, with about 83% of their diet consisting of plants, mostly fruits, followed by gums and flowers (Pinheiro & Pontes, 2015). Consequently, DNA obtained from the feces of these primates may contain contaminants that inhibit genetic analyses.

The storage duration of fecal samples can influence the success of DNA extraction and subsequent amplification (Zhang et al., 2006; Hale et al., 2015), with higher PCR success rates observed when fresh feces are used (Zhang et al., 2006).

Among homemade protocols, the Finger's protocol (2015) was developed to extract DNA from human feces, as well as omnivorous primates, and is aimed at diagnosing intestinal parasites. This protocol was designed to remove PCR inhibitors present in feces. Meanwhile, the Doyle and Doyle (1987) protocol is used to extract DNA from plant species using the detergent Cetyltrimethylammonium Bromide (CTAB). This reagent not only lyses cells to release DNA but also separates polysaccharides, which are abundant in plants and are known to inhibit DNA amplification by PCR (Schenk et al., 2023).

Our objective was to compare the efficiency of two homemade protocols, Finger (2015) and Doyle & Doyle (1987), for extracting DNA from the feces of *Sapajus nigritus* and *Callithrix* spp., as well as the success of PCR amplification using the extracted DNA. Additionally, three tests were conducted to evaluate the influence of collection time and the storage method of fecal samples. Our hypothesis is that DNA extraction and PCR amplification success will be higher in fecal samples with shorter storage times.

## Material and methods

Collection and Storage of Fecal Samples. Ten fecal samples from *Sapajus nigritus* and three from *Callithrix* spp. were collected from five different locations in the state of Rio de Janeiro: Marambaia Island (MI), Tijuca National Park (TNP), Itatiaia National Park (INP), the campus of the *Universidade Federal Rural do Rio de Janeiro* (UFRRJ) and the Wildlife Animal Screening Center (CETAS) from Seropédica.

Of the ten capuchin monkey samples, seven were collected in 2015, 2018, and 2019, stored in 70% ethanol under refrigeration, and three in 2023, stored at -20°C. Among the three marmoset samples, one was collected in 2019, stored in 70% ethanol under refrigeration, and two in 2023, stored at -20°C (Table 1). All samples were stored at the Animal Genetics Laboratory of the Institute of Biological and Health Sciences (ICBS) UFRRJ.

The procedures for obtaining fecal samples were approved by the Animal Use Ethics Committee (CEUA) of ICBS/UFRRJ (013/2018) and authorized by SISBIO/ICMBio (57417-6).

DNA Extraction. Three tests were conducted considering the collection time and storage method of fecal samples from *S. nigritus* and *Callithrix* spp. to evaluate the efficiency of two homemade protocols: Finger (2015) and Doyle & Doyle (1987). Test 1: DNA was extracted in 2022 and 2023 from seven capuchin monkey fecal samples collected in 2015 (three samples), 2018 (three samples), and 2019 (one sample), as well as one marmoset fecal sample collected in 2019. These samples were stored in 70% ethanol under refrigeration until extraction. Test 2: DNA was extracted immediately after collection from three fresh capuchin fecal samples and two marmoset fecal samples collected in 2023. Test 3: The protocols were tested on the same samples of Test 2, one month after collection. The samples were stored at -20°C without the addition of 70% ethanol (Table 1). The extraction results were visualized on 0.8% agarose gel stained with GelRed.

Protocol Finger (2015). Each test used 500 mg of feces per sample, transferred to a 2 mL plastic tube. The sample was washed with 1 mL of distilled and autoclaved water, homogenized by agitation, and centrifuged at 2000 rpm for 2 minutes. The supernatant was discarded, and the washing process was repeated three times. For cell lysis, the precipitate was resuspended in 700 µL of Rose Buffer (10 mM Tris pH 8.0, 300 mM EDTA pH 8.0, 1% Sodium Dodecyl Sulfate/SDS, and 2% polyvinylpolypyrrolidone/PVPP). After vortex homogenization, the samples were incubated at 60°C for 20 minutes with intermittent agitation and cooled to room temperature (22°C). The samples were centrifuged for 5 minutes at 13000 rpm. To the supernatant, 154 µL of a 10% cholestyramine and 27% NaCl solution was added, followed by incubation at room temperature for 5 minutes and centrifugation for 5 minutes at 13000 rpm. For precipitation, 500 µL of the supernatant was transferred to a 1.5 mL tube, and 50 µL of 3M sodium acetate (pH 5.3) and 1 mL of 100% ethanol (pre-chilled at -20°C) were added. The mixture was gently inverted, incubated on ice for 15 minutes, and centrifuged at 13000 rpm for 15 minutes. The precipitate was washed with 500 µL of 70% ethanol (4°C), centrifuged for 2 minutes at 13000 rpm, air-dried, and resuspended in 100 µL of Tris-EDTA Buffer (10 mM Tris pH 8.0, 300 mM EDTA pH 8.0) before incubation at 37°C for dilution.

Protocol Doyle & Doyle (1987). This method used 500 µL of CTAB buffer added to 100 mg of feces in a 1.5 mL tube, vortexed, and incubated in a water bath at 65°C for 1 hour. After cooling to room temperature, 500 µL of chloroform:isoamyl alcohol (24:1) was added, vortexed, and centrifuged for 10 minutes at 5000 rpm. The supernatant was transferred to a new tube with 250 µL of cold isopropanol, inverted, and chilled for 30 minutes. It was then centrifuged for 20 minutes at 12000 rpm. The precipitate was washed twice with 1 mL of cold 75% ethanol, dried, and resuspended in approximately 30 µL of TE 1X buffer for complete dilution at 37°C before quantification.

DNA Quantification. The extracted DNA was quantified using 0.8% agarose gel and compared to a lambda (λ) DNA marker of 50 ng $\mu L^{-1}$ and 25 ng $\mu L^{-1}$. Each gel well received 3 µL of DNA with loading buffer containing bromophenol blue and GelRed. DNA bands were visualized under a transilluminator.

Polymerase Chain Reaction (PCR). PCR was performed using the primer pair MonkeyGluF1 (5′-CCATGACTAATGATATGAAAARCC-3′) and MonkeyProR1 (5′-AGAATSTCAGCTTTGGGTGTTG-3′) (Boubli et al., 2018) for amplification of 1,148 bp fragment of the mitochondrial cytochrome b gene (MIT-*CYTB*) from *Sapajus nigritus*. To *Callithrix* spp., was utilized the primer forward (5'-CAAAACGCCGCATCYCCAATC-3') and reverse (5'-GGCCTGGTCGTATGGAAG-3') to amplify 600 bp fragment of the mitochondrial cytochrome oxidase 2 gene (*COX2*) (Carvalho et al., 2013).

For DNA samples extracted from *S. nigritus* feces, the PCR solutions consisted of a final volume of 25 µL using GoTaq® Colorless Master Mix with a final concentration of 1X, 0.5 µM of each primer and DNA concentrations of approximately 25 and 50 ng $\mu L^{-1}$, completing the volume with ultrapure water. A second PCR was prepared with DNA concentration below 20 ng $\mu L^{-1}$. The amplification reactions occurred at an initial temperature of 94°C for 2 min, followed by 34 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 2 min, ending with a final extension at 72°C for 10 min.

For amplification of DNA samples extracted from *Callithrix* spp. feces, the PCR reactions consisted of a final volume of 25 µL, containing GoTaq® Colorless Master Mix with final concentration of 1X, 2 µM of each primer, and DNA concentration of approximately 25 and 30 ng $\mu L^{-1}$. The amplification followed the temperature cycles: 94°C for 3 min, followed by 35 cycles of 92°C for 1 min, 45°C for 1 min e 72°C for 1 min, with a final extension of 72°C for 10 min. The amplifications were performed in a ProFlex™ PCR System thermocycler (Applied Biosystems ® Thermo Fisher Inc.)

All PCR products were analyzed in 5% polyacrylamide gel, using positive controls, obtained from DNA extracted from blood samples of the respective species. A negative control was also prepared, containing water instead of DNA. PCR products (4 µL) were applied to each well of the polyacrylamide gel along with a loading buffer containing bromophenol blue. For all reactions, the size of amplified fragments was compared with a molecular weight marker, DNA Ladder of 100 bp.

## Results and discussion

In test 1, seven fecal samples from *Sapajus nigritus* and one from *Callithrix* spp., collected in 2015, 2018, and 2019, and stored in 70% ethanol, failed to yield DNA with either of the two protocols. When using the Finger (2015) protocol, both *S. nigritus* and *Callithrix* spp. samples showed a yellowish supernatant, and after the DNA precipitation step, a gel-like precipitate (Figure 1) was observed, making dilution in TE 1X

impossible. When using the Doyle & Doyle (1987) protocol, a yellowish supernatant was also observed, though lighter in color, and a precipitate with less of a gel-like appearance, but DNA was not obtained.

**Figure 1.** Gel-like precipitate into the plastic tubes after DNA extraction from two fecal samples of *Sapajus nigritus* using the Finger's protocol.

In tests 2 and 3, using fresh fecal samples and samples stored for a month at -20°C (three from *Sapajus* and two from *Callithrix*), no DNA was obtained using the Finger (2015) protocol. The samples still showed yellowish supernatant and a gel-like precipitate. However, when using the Doyle & Doyle (1987) protocol, DNA was extracted in both tests, with bands observed at an estimated concentration of 16 ng $\mu L^{-1}$ (1S and 3S samples) and 8 ng $\mu L^{-1}$ (2S) for *S. nigritus* samples, and 10 ng $\mu L^{-1}$ (1C) and 12 ng $\mu L^{-1}$ (2C) for *Callithrix* spp. fecal samples (Figure 2). The five DNA samples quantified in agarose gel were submitted to PCR.

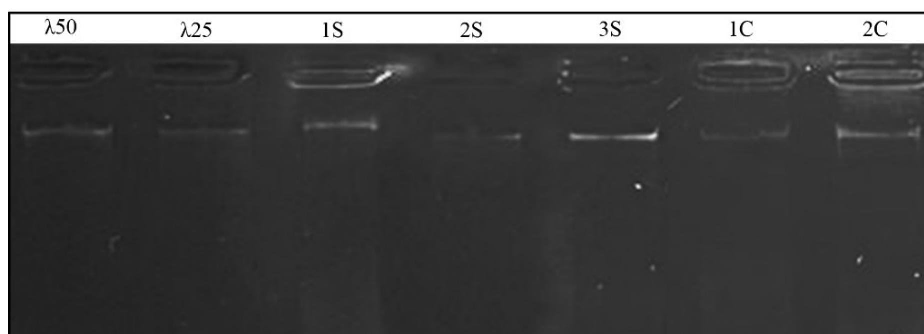


**Figure 2**. Quantification of DNA extracted from fecal samples of *Sapajus nigritus* and *Callithrix* spp. using the Doyle & Doyle (1987) protocol. DNA was stained with GelRed and visualized on a 0.8% agarose gel under ultraviolet light. From left to right: Lambda DNA marker, λ at 50 ng $\mu L^{-1}$; λ phage DNA at 25 ng $\mu L^{-1}$; 1S, 2S, and 3S: fecal samples of *S. nigritus* (S), with approximately 16 ng $\mu L^{-1}$ (1S), 8 ng $\mu L^{-1}$ (2S), and 16 ng $\mu L^{-1}$ (3S); 1C and 2C: fecal samples of *Callithrix* spp., 1C (10 ng $\mu L^{-1}$) and 2C (12 ng $\mu L^{-1}$).

When visualizing PCR products in a 5% polyacrylamide gel, bands were observed for the two positive controls of *S. nigritus* and *Callithrix* spp., with approximate sizes of 1,148 bp and between 500 and 600 bp, respectively. Among the tested samples, only 1C, extracted from *Callithrix* spp. feces, generated a band of the same size as its positive control. Amplification occurred with DNA concentration around 25 ng $\mu L^{-1}$ (Figure 3). No amplification was observed for any of the three *S. nigritus* fecal DNA samples.

For the *S. nigritus* fecal DNA samples that did not result in amplicons in the first PCR (1S, 2S, and 3S), reactions were prepared with increased DNA concentration to approximately 50 ng $\mu L^{-1}$ (1+, 2+, and 3+), and three others with reduced DNA concentration to approximately 15 ng $\mu L^{-1}$ (1-, 2-, and 3-). The reduction in DNA concentration aimed to decrease the concentration of potential PCR inhibitors, as a yellow coloration had been observed in the precipitates during DNA extraction, suggesting contamination by polyphenols. With

this change, amplification of an expected fragment around 1,148 bp was obtained for sample 1S (corresponding to 1-, that is, more diluted and therefore with a lower DNA concentration) (Figure 4). For samples 2S and 3S, no PCR success was achieved with any of the DNA concentrations used (Figure 4).

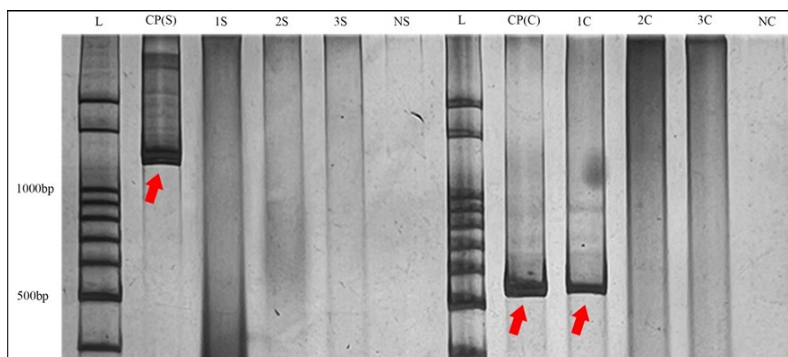


**Figure 3.** PCR products in 5% polyacrylamide gel, obtained from DNA samples extracted from the feces of *Sapajus nigritus* and *Callithrix* spp. using the Doyle & Doyle (1987) protocol. L: 100 bp DNA Ladder; CP(S): positive control of *S. nigritus* with a band of approximately 1,148 base pairs (bp) from the mitochondrial cytochrome b gene; samples 1S, 2S, and 3S: did not amplify; NS: negative control of *S. nigritus*; CP(C): positive control of *Callithrix* spp. with a band between 500 and 600 bp from the mitochondrial cytochrome oxidase 2 gene; sample 1C: band around 600 bp; sample 2C: did not amplify; NC: negative control of *Callithrix* spp.

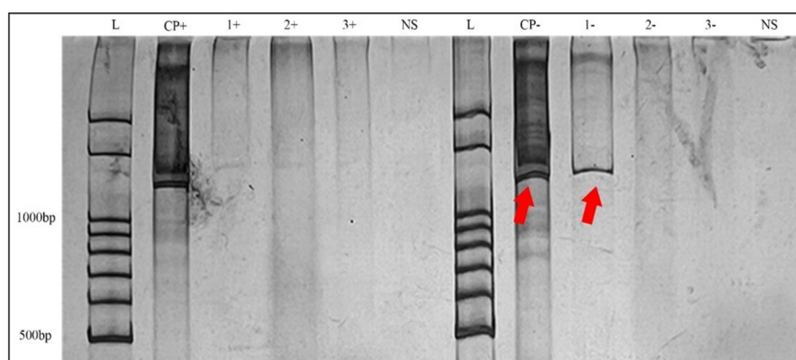


**Figure 4**. 5% polyacrylamide gel with fragments of the mitochondrial cytochrome b gene amplified by PCR from DNA obtained from fecal samples of *Sapajus nigritus*, using higher (+) DNA concentration (above 20 ng $\mu L^{-1}$) and lower (-) DNA concentration (below 20 ng $\mu L^{-1}$). L: 100 bp DNA Ladder; CP: positive control of *S. nigritus*; 1, 2, and 3: fecal samples of *S. nigritus*; NS: negative control of *S. nigritus*.

The excess of poorly digested fruits present in the feces of *S. nigritus* interfered with PCR amplification of DNA extracted from these samples. This was probably due to the persistence of polyphenols that gave the yellowish coloration observed in the supernatant and final precipitate, both known inhibitors of PCR. The fecal washing step prescribed in the Finger (2015) protocol, originally developed for human feces, was insufficient to remove these inhibitors from capuchin monkey and marmoset feces, which may explain the failure of this protocol in all three tests.

In contrast, the Doyle & Doyle (1987) protocol yielded a more translucent supernatant, suggesting reduced polyphenol contamination, and the absence of a gel-like appearance in the precipitate, indicating more efficient removal of polysaccharides (Schenk et al., 2023). This improvement is likely attributable to the use of the CTAB detergent, which facilitated the recovery of purer DNA (Schenk et al., 2023). The persistence of contaminants in the DNA samples of *S. nigritus* may reflect the digestive physiology of this species. As omnivores, capuchin monkeys have a limited capacity to digest fibers, resulting in poor digestion of fruits (National Research Council, 2003) and, consequently, higher levels of polysaccharides and polyphenols in their feces. Marmosets, on the other hand, have a long intestine and a complex cecum, which may enhance polysaccharide digestion (Rylands et al., 1993), thereby reducing the presence of PCR inhibitors in their feces. Thus, although dilution reduced the initial amount of DNA in the PCR reaction, it also diluted contaminants, which in this case was sufficient for successful amplification.

Our results are consistent with Lathuillière et al. (2001), who reported that CTAB was more efficient for obtaining DNA from *Macaca sylvanus* feces. Similarly, Vallet et al. (2008), demonstrated that combining phenol-chloroform-isoamyl alcohol with CTAB improved PCR success in gorillas and Barbary macaques' feces

by reducing polyphenols and polysaccharides contamination, an important consideration in herbivorous species, which harbor a higher load of plant-derived compounds and therefore require more robust protocols. Notably, in some cases, CTAB has proven more effective for obtaining high-quality DNA than commercial extraction kits (Liu et al., 2024). Together, these findings highlight the importance of adapting extraction protocols to the digestive and dietary characteristics of the studied species.

Beyond digestive and dietary factors, variables such as sample collection time and storage conditions also play a crucial role in DNA preservation and PCR success. Our findings corroborate Hale et al. (2015) that while analyzing spider monkey fecal samples, concluded that freezing fecal samples at -20-°C preserved DNA more efficiently than ethanol when extraction tests were performed after 4 weeks. Overall, those results highlight that successful non-invasive genetic investigations require careful attention not only to extraction protocols but also to sample collection and storage conditions.

## Conclusion

The Doyle & Doyle (1987) protocol has proven efficient for obtaining DNA suitable for PCR, enabling the removal of polysaccharides and polyphenols from fecal samples of *S. nigritus* and *Callithrix* spp., while the Finger (2015) protocol was ineffective. Using the Doyle & Doyle method, DNA can be isolated from fresh feces up to 30 days after collection if stored at -20°C, with successful PCR amplification. However, adjustments may be needed for other primate species. Overall, Doyle & Doyle protocol is an effective, low-cost alternative to commercial kits, enabling broader sampling for genetic analysis of non-human primates.

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