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Short Communication

Noninvasive genetic sampling of endangered muriqui (Primates, Atelidae): Efficiency of fecal DNA extraction

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Abstract

The muriqui (*Brachyteles*) is one of the most endangered primates in the world, however little is known about the viability of the remaining populations. We evaluated the technique of extracting DNA from wild muriqui feces for PCR applications. In order to determine the effect of the DNA in subsequent amplifications, we analyzed five different extracts. The importance of the recommended BSA and the HotStarTaq DNA polymerase was tested. The minimal conditions to successfully amplify highly degraded fecal DNA were determined, showing that the recommended reagents are not required. We envision that this method may be useful in further conservation management studies.

Keywords: *Brachyteles*, conservation genetics, endangered species, fecal DNA, noninvasive sampling.

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The genus *Brachyteles* (muriqui) represents the largest neotropical nonhuman primate and comprises two endemic species occurring in the Brazilian Atlantic Rainforest. The species *B. hypoxanthus* (Kuhl, 1820) or northern muriqui can be found in the States of Bahia, Minas Gerais and Espírito Santo, and *B. arachnoides* (É. Geoffroy, 1806) or southern muriqui is distributed along over the States of Rio de Janeiro, São Paulo and Paraná (Aguirre, 1971; Lemos de Sá *et al.*, 1990, Lemos de Sá *et al.*, 1993; Martuscelli *et al.*, 1994). Their small population size and the deforestation of the Atlantic Forest have led to the classification of muriqui as an "endangered" primate since 1982, and as "critically endangered" since 2000 (Rylands *et al*., 2003). *B. hypoxanthus* is also listed as one of the 25 most endangered primates of the world since the year 2000 (Mittermeier *et al*., 2005). Historically, the muriqui species roamed throughout the Atlantic Brazilian Rainforest, but now it is estimated that there are no more than 1200 individuals living in a few dozen remaining forest fragments (Strier and Fonseca, 1996/1997). Thus, questions about the consequences of habitat fragmentation in the genetic structure of populations, gene flow and probability of extinction are frequently addressed with regard to muriqui (Leigh and Jungers, 1994; Strier, 1995; Strier, 2000). DNA assessment is critical for investigating these questions, and genotyping for molecular markers like microsatellites is essential to conduct more realistic population viability analyses (PVAs), improving the currently available data (Strier, 1995).

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Until recently, molecular genetic analyses of primates have been limited by the availability of blood or tissue samples for DNA extraction (Surridge *et al.*, 2002). Despite the fact that noninvasive DNA sampling usually yields low quantities of DNA (Taberlet *et al.*, 1996; Taberlet *et al.*, 1997; Constable *et al.*, 2001), the advent of the polymerase chain reaction (PCR) technique has been successfully used to assess the genetic composition of social groups and populations, and to evaluate both species and genealogical relationships based on such small samples (Höss *et al.*, 1992; Morin *et al.*, 1994; Constable *et al.*, 1995; Gerloff *et al.*, 1995; Taberlet *et al.*, 1996; Reed *et al.*, 1997; Constable *et al.*, 2001).

Boom *et al.* (1990) presented the first study that was successful in isolating DNA from shed epithelial cells mixed with feces. Since then, studies in conservation genetics using DNA from fecal samples have been carried out in threatened species, including bears (Taberlet *et al.*, 1997) and wolves (Creel *et al.*, 2003). In spite of this scenario, in the last few years, fecal samples from muriquis have been used exclusively to monitor ovarian cycle hormones in females, and testosterone and cortisol levels in males (Strier and Ziegler 1997; Ziegler *et al.*, 1997; Strier *et al.*, 1999). In the present study, our primary goal was to test the reliability of results obtained from muriqui fecal DNA by downstream PCR. The conclusions reached may be a starting point for future population genetic studies in this species.

Feces were collected from 28 individually identified muriquis that have been the subjects of long-term observational field research at the "Estação Biológica de Caratinga" (EBC/RPPN-FMA) in Minas Gerais, Brazil. Approximately 5 g of feces per individual were transferred into a sterile 50 ml polypropylene conical tube containing silica gel beads. About 20 g of humidity-sensitive silica beads and a fine layer of cotton were placed underneath and above the feces, to completely fill the tubes, in order to isolate and quickly dehydrate the samples. Until DNA extraction, the dehydrated samples were conserved at 4 °C, and the silica beads were changed whenever humidity was detected.

DNA was extracted from 200 mg of dried feces, using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's protocol. All procedures were carried out using a face mask. Few extractions were manipulated simultaneously, in order to avoid cross-contamination and contamination by exogenous DNA. Muriquis have a vegetarian diet (Strier, 1991; Olmos *et al.*, 1997), which eliminates the need to remove prey parts, such as bones and hair, as in carnivore fecal extractions (Paxinos *et al.*, 1997; Wasser *et al.*, 1997; Farrell *et al.*, 2000). After extraction, DNA was qualitatively evaluated in 0.8% agarose gel and quantified in a spectrophotometer (260 nm of wavelength and 1:25 μL of dilution). DNA concentration was calculated as described by Sambrook *et al.* (1989), and the yields varied from 18 to 140 ng/μL. Three of the 28 samples presented DNA concentrations below the detection threshold of the spectrophotometer.

We tested the quality and quantity of the DNA template, and the influence of bovine serum albumin (BSA, New England Biolabs), which has been considered essential in downstream PCR applications using fecal DNA as template, resulting in 90 different amplification mixtures. Five muriqui DNAs with different levels of degradation (quality) in six different quantities (5 ng, 10 ng, 20 ng, 50 ng, 100 ng, and 200 ng), and three final concentrations of BSA (0.0, 0.1, and 0.2 μg/μL), were tested in the PCR mixture.

A total volume of 25 μL PCR mixture was used in a PTC-100 Thermocycler (MJ Research), including 10% of 10X PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 0.2 mM of each dNTP, 2.5 U of "Taq Brazilian Origin" DNA polymerase (Invitrogen), 2.0 mM of $MgCl_2$, and 0.2 μM of each primer: L6955 (5'-AACCATTTCATA ACTTTGTCAA-3') and H7766 (5'-CTCTTAATCTTTA ACTTAAAAG-3'). These primers were originally designed to amplify the subunit II of the human cytochrome *c* oxidase (*COII*) mtDNA gene (Ashley and Vaughn, 1995), and successfully tested in the closely related genus *Ateles* (Collins and Dubach, 2000). The PCR conditions included a denaturing phase at 92 °C for 5 min, followed by 35 cycles of 92 °C for 1 min, 48 °C for 45 s, and 72 °C for 1 min, and a final extension step at 72 °C for 5 min. The PCR mix was prepared in a special chamber, to avoid contamination. High-molecular-weight human DNA (200 ng) was used as positive control and distilled water instead of DNA as negative control. All of the 90 reactions were carried out in duplicate, to validate the results. The PCR products of four out of the five muriqui DNAs were sequenced as control (accession numbers DQ118288, DQ118289, DQ118290, DQ118291), to exclude false species-specific amplification from contamination with exogenous DNA (human, plant, protozoa, bacteria and others). The human positive control was also sequenced and deposited in the GenBank (Accession number DQ118287). DNA sequencing was performed in an automated MegaBACE 1000 sequencer, using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences). First, sequences were compared through BLAST on the GenBank database, subsequently they were automatically aligned, and a neighbor-joining tree was drawn using the MEGA 3.0 package (Kumar *et al.*, 2004).

Here, we were able to demonstrate the usefulness of a suboptimal source of DNA such as the feces of the endangered wild muriquis for further PCR applications. We analyzed three variables that could affect the efficiency of PCR using fecal DNA samples: (i) Five DNA qualities; (ii) six DNA quantities (5 ng, 10 ng, 20 ng, 50 ng, 100 ng, and 200 ng); and (iii) three final concentrations of BSA (0.0, 0.1 and 0.2 μg/μL); totaling 90 PCR tests. Only 17 (19%) of the 90 reactions failed to amplify a *COII* fragment, indicating a high success rate for mtDNA, with a doubly longer amplicon (~800 bp), as compared to previous analyses of ursids (Wasser *et al.*, 1997).

BLAST query resulted in a 96% similarity of our four muriqui sequences with *Brachyteles arachnoides hypoxanthus* (AF216253), 88% with *Ateles paniscus* (AF216247), and 87% with *Lagothrix lagothrica* (AF216251). The human control sequence crossed with 99% of human mtDNA. None of the four muriqui sequences showed any similarity with any organisms other than nonhuman neotropical primates. Sequences were also aligned with the complete mtDNA genome of *Cebus albifrons* (AJ309866), a neotropical primate. The muriqui sequences matched at the correct *COII* position, which is

7016-7703 bp (Arnason *et al*., 2000). The absence of contamination is graphically shown in Figure 1a.

With regard to DNA quality, the most degraded DNA (6* in Figure 1b) presented the lowest amplification efficiency (44%), but the negative results were obtained almost exclusively in reactions containing 5-20 ng of DNA template. The most intact templates (5* and 7* in Figure 1b) presented 95% efficiency in amplifying the *COII* segment. Comparatively, the best results were achieved using non-degraded DNA templates, as observed in extracts 4* (89%), 5* (95%) and 7* (95%).

A minimum of nine out of 15 reactions (60%) resulted in positive amplifications when 5-20 ng of DNA template were used. The optimal amount of DNA was found to be above 50 ng. Nevertheless, positive amplification was achieved with 5 ng, using good quality fecal DNA (*e.g.*, extracts 4* and 5*).

In order to test the importance of using BSA, we evaluated the amplification efficiency of all the reactions with at least 50 ng of DNA (optimal amount) and moderately to highly intact DNA (qualities of extracts 4*, 7* and 5*). All of these samples (27/27) showed positive amplifications, suggesting that the concentration of BSA did not affect amplification, under any of the evaluated conditions. Additionally, the results remained practically unchanged after modification of the BSA concentration, when reactions which contained suboptimal amounts of DNA template, such as 5-20 ng, were also counted.

Otherwise, previous studies of other mammals had reported the increasing of the PCR product after addition of BSA (Pääbo, 1990; Kohn and Wayne, 1997; Al-Soud and Rådström, 2000; Palomares *et al*., 2002). Potentially, BSA can counteract to PCR inhibitors or avoid the adsorption of PCR reagents to the tube wall, making them available to the amplification reaction (P. Taberlet and Qiagen Scientific Support, personal communication). The Qiagen protocol also strongly recommends the addition of BSA to the PCR mixture, in a final concentration of 0.1 μg/μL.

However, other authors have also reported the dispensability of BSA, or did not report its use (Höss *et al*., 1992; Takasaki and Takenaka, 1991; Sugiyama *et al*., 1993; Wasser *et al*., 1997). Our results imply that despite some studies pointed out that fecal DNA can be contaminated with PCR inhibitors, BSA was not essential in PCR (Farrell *et al*., 2000; Creel *et al*., 2003). We believe that in-

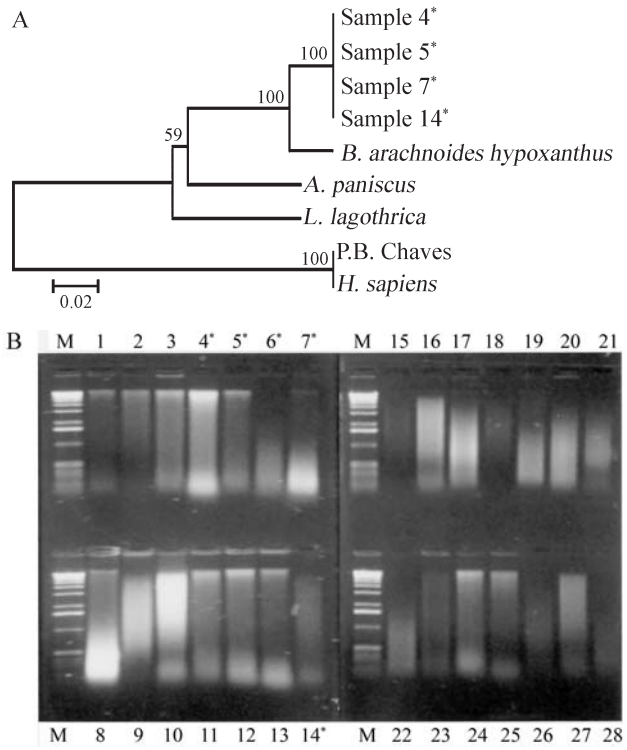

Figure 1 - A. Neighbor-joining tree showing DNA sequences of *Brachyteles hypoxanyhus* (accession numbers DQ118288, DQ118289, DQ118290 and DQ118291 corresponding to Samples 4*, 5*, 7* and 14*, respectively, this paper) clustered together, and closely related to *Brachyteles arachnoides hypoxanthus* (AF216253), *Ateles paniscus* (AF216247) and *Lagothrix lagothrica* (AF216251). Human control (accession number DQ118287, this paper) is grouped with *Homo sapiens* (AP008260). Bootstrap values (1000 replicates) are shown above branches B. DNA profiles (6 μL/lane), numbers correspond to 28 DNA extracts. "M" identifies the 1 kb ladder. Asterisks refer to samples 4, 5, 6, 7 and 14 used in PCR amplification.

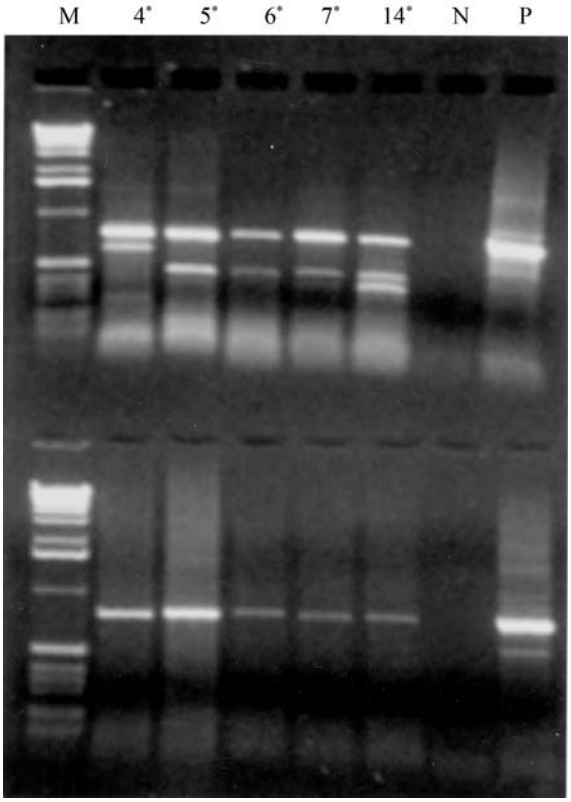

Figure 2 - PCR products of the DNA extracts numbers 4*, 5*, 6*, 7*, and 14*, respectively. N and P stand for negative and positive controls, respectively. No BSA was added in the reactions, primer concentrations of 0.4 μM and 0.2 μM were used for upper bands and lower bands, respectively. "M" identifies the 1 kb ladder.

hibitors were eliminated during the extraction procedure, and thus the activity of BSA was not significant.

The Qiagen protocol also recommends the use of the Qiagen HotStarTaq DNA polymerase. However we obtained excellent results using a much cheaper polymerase (Brazilian Taq DNA polymerase, Invitrogen Inc). We achieved satisfactory results in minimizing nonspecific PCR bands by reducing the primer concentration from 0.4 μM to 0.2 μM (Figure 2). Additionally, digestion reactions with endonucleases have shown the suitability of these PCR products for further analysis using Restriction Fragment Length Polymorphisms (Fagundes *et al.*, unpublished data).

Our results provide additional information to optimize the PCR reactions using noninvasive fecal DNA samples as template, minimizing both cost and time of standardization in further genetic studies. In conclusion, appropriate fecal DNA extraction methods make molecular studies feasible for endangered species, such as muriquis. This protocol may also be applicable to a large variety of primate and non-primate mammals in upcoming genetic approaches.

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Internet Resources

GenBank, http://www.ncbi.nih.gov/Genbank/

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