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PRESERVATION AND EXTRACTION OF DNA FROM FECES IN HOWLER MONKEYS (*ALOUATTA CARAYA*)

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Introduction

Techniques of molecular genetics are increasingly used to study various aspects of the social systems of human and wild non-human primates (Altmann *et al.*, 1996; Gagneux *et al.*, 1999; Nievergelt *et al.*, 2000; Paabo, 2003; Di Fiore, 2003). In the past, studies of primate molecular genetics were limited by the availability of blood or tissue samples for DNA extraction. Today, samples such as hair and feces, obtainable through non-invasive methods, are preferred for genetic analysis. This strategy avoids the capture of the animals, minimizing any undesirable impact on their behaviour as well as preventing injuries and infectious diseases either to the animal or the sample collector (Constable *et al.*, 2001; Sibal and Samson, 2001). As a result, it is becoming safer and easier to obtain information on kinship, sex ratio, effective population size and gene flow in undisturbed populations of arboreal and threatened species.

Although a number of recent studies have used non-invasive sampling to examine aspects of the social structure of several Old World primates (Gagneux *et al.*, 1999; Gerloff *et al.*, 1999; Goossens *et al.*, 2000; Constable *et al.*, 1995, 2001; Vigilant, 2002), only a handful of studies have used feces from New World monkeys as a source of DNA for molecular studies (SurrIDGE *et al.*, 2002; Escobar-Paramo, 2000; Böhle and Zischler, 2002). The main goal of this study was to test alternative methods for preserving and subsequently extracting DNA from fecal samples of a New World primate, in order to identify a low-cost solution that might be broadly applied in molecular ecological research on platyrrhines.

Materials and Methods

Sampling

We collected fecal samples from two groups of black-and-gold howler monkeys in two habitats: flooded forest on Brasileira Island, near the confluence of the Río Paraná and Río Paraguai in the Chaco region of northern Argentina (27°20'S, 58°40'W), and the semi-deciduous forest of the basin of the Río Riachuelo, a tributary of the Río Paraná, further to the southwest (27°30'S, 58°41'W) near the southern margin of the geographic range of *A. caraya*. Samples were collected from a total of five different individuals immediately after defecation. Individuals 1 and 2 were from the flooded forests, and individuals 3, 4 and 5 were from the riparian forests. In all cases one sample (10 g) was taken from each individual and subdivided into four sub-samples of approximately 2 g each, which were then preserved according to the following protocols:

1. In paper envelopes kept in shadow at approximately 20°C (68°F);

2. in paper envelopes dried under the sun;
3. in paper envelopes dried at 60°C in an oven (30°-120°C); and
4. in sterile tubes containing 34 g of technical grade solid salt (NaCl).

Samples were stored in these conditions for one month and then used for extraction by three different methods.

DNA Extraction

We tested three methods for DNA extraction on each of the four preservation protocols. Duplicate extractions were made in all cases.

1. CTAB (Corach *et al.*, 1995)

For each extraction, ~300 mg of feces were added to 2.5 ml of Cetyl Trimethyl Ammonium Bromide (CTAB, Carlo Erba RPE) and 10 µl Proteinase K (20 mg/ml). Samples were then incubated overnight at 56°C with constant agitation. Organic solvent extractions were carried out with 2.5 ml of Chloroform: Iso-Amil Alcohol (24:1); the mixture was thoroughly shaken for 15 minutes and then centrifuged for 25 minutes at 2000 g. The aqueous phase was carefully removed and this procedure was repeated once more. The aqueous phase was transferred to a fresh tube and 2/3 of the volume of the aqueous phase of 2-Propanol (Carlo Erba RPE) was added. Tubes were frozen overnight and then centrifuged cold for 15 minutes at 2000 g. The supernatant was then carefully transferred to a fresh tube; 1 ml of 70% ethanol was added to wash the pellet, centrifuged again, and the ethanol carefully removed. The pellet was then dried at 37°C for 2 hours and resuspended in 200 µl of deionized water.

2. Guanidinium Thiocyanate/Silica (Boom *et al.*, 1990)

For each extraction, ~300 mg feces were added to 3 ml lysis buffer (10 M GuSCN, 0.1 M Tris-HCl, pH 6.4, 0.02 M EDTA, pH 8.0, 1.3% Triton X-100). After 15 minutes of constant agitation and 15 minutes of centrifugation at 12000 g, 1.8 ml of the aqueous phase were transferred to a fresh tube and 40 µl of silica suspension described in Boom *et al.* (1990) were added. The mixture was immediately vortexed for 5 seconds, incubated at room temperature for 10 minutes, vortexed again (5 seconds) and centrifuged (30 seconds, 12000 g). The supernatant was then disposed of by suction, and the silica pellet was washed twice with 1 ml washing buffer (10 M GuSCN, 0.1 M Tris-HCl, pH 6.4), twice with 1 ml 70% ethanol and once with 1 ml acetone. The pellet was then dried at 56°C for 30 minutes and nucleic acids were eluted at 56°C for 10 minutes with 200 µl TE (10 mM Tris-HCl, 10 mM EDTA, pH 8.3). The tube was centrifuged (5 minutes, 12000 g) and 50 ml of the supernatant were carefully removed (to avoid pipetting silica particles) and transferred to a fresh tube.

3. TEC/Guanidinium Thiocyanate/Silica

For each extraction, ~300 mg feces were added to 3 ml of TEC (10 mM Tris-HCl, pH 8, 10 mM EDTA, 100 mM NaCl), 10 µl of Proteinase K (20 mg/ml) and 3 µl of

DiThioThreitol 1M. After incubation for four hours at 56°C with constant agitation, the mixture was centrifuged for 20 minutes at 7000 g. One ml of the supernatant was added to 2 ml lysis buffer (10 M GuSCN, 0.1 M Tris-HCl, pH 6.4, 0.02 M EDTA, pH 8.0, 1.3% Triton X-100). The tube was vortexed for 30 seconds and then centrifuged (20 minutes, 7000 g). The supernatant was recovered and transferred to a fresh tube, where 40 µl silica suspension were added. The following steps are the same as those described for silica extraction in Protocol 2.

PCR Amplification

PCR reactions were carried out using 2 µl of each extracted sample. In all cases, extraction and amplification reactions included negative controls. In addition, blood samples from zoo specimens were used as positive controls.

Two PCRs were carried out for each extract obtained. As we used three extraction methods for each of four subsamples from each of five howler monkeys, we ran a total of 120 PCR reactions. The presence of nuclear DNA from howler monkeys was tested by PCR amplifications of one microsatellite isolated from *Alouatta palliata* (AP74) using primers (5'-GCACCTCATCTCTTTCTCTG-3') and (5'-CATCTTTGTTTCCTCATAGC-3') (Ellsworth and Hoelzer, 1998). These primers were used in 25 µl PCR reactions containing the following: 2 µl of template, 0.2 mM each dNTP, 0.5 µM of each primer, 1.5 U Taq DNA Polymerase (Invitrogen), 20 mM Tris-HCl, 50 mM KCl, and 1.5 mM MgCl₂. The PCR cycling profile consisted of 40 cycles of denaturing for 1 minute at 95°C, annealing for 1 minute at 52°C, and extension for 1 minute 30 seconds at 72°C. PCR products were separated by electrophoresis on 2% agarose gels stained with ethidium bromide.

Results

Amplification products were obtained from samples preserved with two of the four methods tested—those desiccated in NaCl and those dried in paper envelopes exposed to the sun. However, the success rate was greater with the former rather than the latter procedure (4/5 and 2/5, respectively). The only extraction procedure that yielded DNA suitable for amplification was that using Guanidinium Thiocyanate/Silica (Protocol 2), with a success rate of 80% for NaCl preserved feces (Fig. 1) and 20% for feces dried in the sun (Table 1). No amplifications were obtained from the samples preserved by drying at 60°C or at room temperature, no matter which extraction method was used (Table 1).

Gel electrophoresis of the straight DNA extraction products revealed that the TEC/Silica extraction method (Protocol 3) and CTAB (Protocol 1) ostensibly yielded a high quantity of DNA, but serial dilutions of this template in new AP74 PCR reactions failed to yield any product (Table 1), suggesting a low concentration of howler monkey DNA in these extractions. This is probably due to the fact that the CTAB extraction and TEC pre-treatment lead to improved lysis of vegetable cells present in the feces and thus

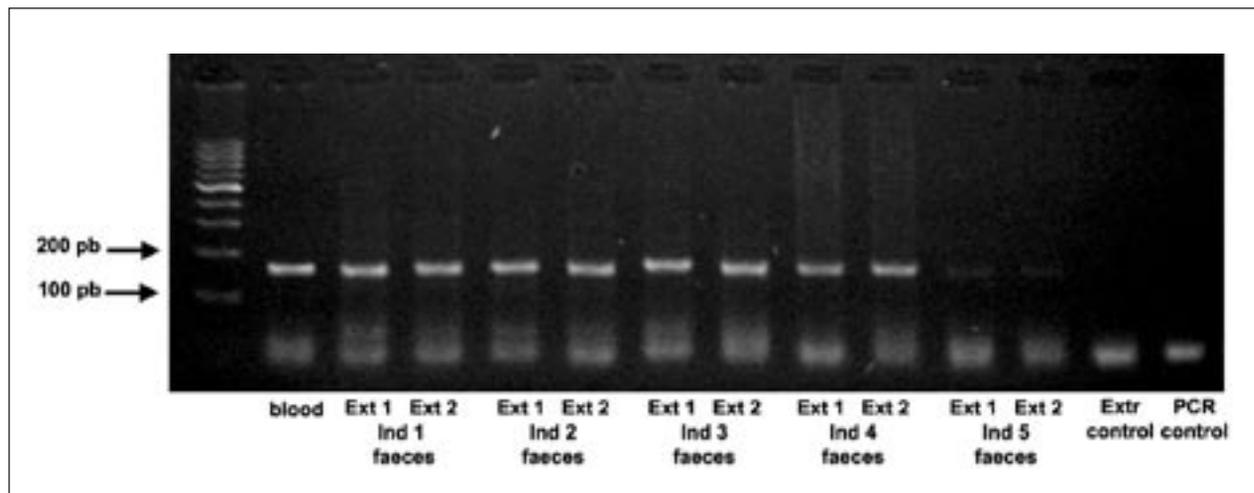


Figure 1. Amplification products obtained for combination of the conservation method in NaCl and the extraction with Guanidinium Thiocyanate/Silica. Duplicate extractions and amplifications are shown.

Table 1. Results of PCR amplifications of microsatellite AP74 from five black-and-gold howler monkeys. One sample from each individual was subdivided into four sub-samples with different preservation protocols and extracted by three different methods.

Preservation	Extraction Method	Ind. 1		Ind. 2		Ind. 3		Ind. 4		Ind. 5	
		Ext. 1	Ext. 2								
RT	1	No									
	2	No									
	3	No									
Sun	1	No									
	2	No	No	Yes	Yes	No	No	Yes	Yes	No	No
	3	No									
60°C	1	No									
	2	No									
	3	No									
NaCl	1	No									
	2	Yes	No	No							
	3	No									

Ind. 1, 2: Inhabiting the flooded forest on the island of Brasilera (27°20'S, 58°40'W).

Ind. 3, 4, 5: Inhabiting the semi-deciduous forest of the Río Riachuelo basin (27°30'S, 58°41'W).

Preservation: preservation methods: RT: paper envelopes kept in shadow and room temperature; Sun: paper envelopes dried under the sun; 60°C: paper envelopes dried at 60°C; NaCl: sterile tubes containing 34 g of technical grade solid salt (NaCl).

Extraction: DNA extraction methodologies tested: 1: CTAB; 2: Guanidinium Thiocyanate/Silica; 3: TEC – Guanidinium Thiocyanate/Silica.

Ext. 1/ Ext. 2: Duplicate extractions for the same individual.

Ind. #: Presence or absence of amplification products for each individual.

co-extraction of plant DNA from species in the howlers' diet, in addition to DNA from the animals' own cells sloughed off in the intestine. The direct silica extraction, on the other hand, is a fast-extraction procedure, and thus only animal cells are expected to lyse. Since the black-and-gold howler monkeys are folivore/frugivores (Rumiz *et al.*, 1986), the only animal cells that might be present in feces are those of their gastrointestinal tract.

Discussion

How to acquire samples is a pivotal issue in genetic studies of arboreal primates. Current methods in molecular biology

allow for the use of non-invasive sampling of hair or feces. In *Alouatta caraya*, as in many other arboreal species, hair sampling is extremely difficult, although possible (Ascunce *et al.*, 2003). In contrast, fecal samples are easy to obtain and to identify. Non-invasive sampling methods are limited, however, due to the low quantity and quality of the DNA obtained, which may lead to incorrect results (Taberlet *et al.*, 1996, 1999; Vigilant, 2002; Gagneux *et al.*, 1997, 2001). The quantity and quality of DNA obtained will improve when samples are collected immediately after defecation (Frantzen *et al.*, 1998; Wasser *et al.*, 1997). Therefore, the ability to obtain nuclear DNA from feces depends primarily on the method of sample storage (Vigilant, 2002). Condi-

tions in the field are also important, since the samples must be collected and preserved until they can be moved to the laboratory (Frantzen *et al.*, 1998). Non-human primates usually live in habitats where the climate is extremely humid, and generally there are no drying ovens or freezers near the field sites to preserve the samples. Thus, the difficulty and the expense of having cryopreservation or drying systems in the field will determine the need for appropriate and inexpensive systems for prolonged room-temperature preservation.

Our results indicate that feces may provide samples amenable to molecular research in *Alouatta caraya*. Duplicate extractions and amplifications yielded reproducible results. Of the four tested methods of fecal sample preservation, the most appropriate seems to be in solid salt (NaCl), since it presents no difficulties in the field, and yielded the best results in the amplifications. As for the extraction methods, the Guanidinium Thiocyanate/Silica method was the only one that provided useful results (from samples preserved in NaCl and those dried in the sun). As Boom *et al.* (1990) described, most viruses and mammalian cells are expected to lyse in the first step of the silica extraction. The quick lysis of this protocol avoids the extraction of vegetal DNA, also present in the samples, which might saturate the DNA-binding capacity of the silica particles. In addition, the DNA purified by this method is essentially free of potential inhibitors of the Taq Polymerase that might prevent PCR amplifications. In contrast, CTAB extraction and pre-treatment with TEC prior to Guanidinium Thiocyanate/Silica extraction increases the yield of vegetable DNA that may dilute the animal DNA obtained. The absence of amplifiable DNA in the samples stored at 20°C could be explained by bacterial proliferation. On the other hand, the samples dried in the oven at 60° suffer a rapid and intense dehydration, reducing the ability of the Guanidinium Thiocyanate solution to moisten and homogenize the tissue during its short exposure (15 minutes).

Our purpose in this note has been to describe simple and inexpensive methods to sample feces from New World primates and extract DNA suitable for molecular analysis. Although there are commercial kits, buffers, and other methods which allow the extraction and preservation of DNA from feces (Nsubuga *et al.*, 2004), they are very expensive, even prohibitively so, for researchers in developing countries.

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associada, que pode ser o reflexo de uma estreita co-evolução. Ao mesmo tempo, análises detalhadas das relações com esses parasitas são importantes para a avaliação da conservação e da qualidade do habitat utilizado pelos primatas (Stuart e Strier, 1995). Para Martins (2002) e Santa Cruz *et al.* (2000), a infecção parasitária pode ser agravada pela degradação e fragmentação de habitats. Com a fragmentação das florestas os animais tendem a ocupar áreas menores e, conseqüentemente, permanecem um maior tempo nas mesmas árvores (Kowalewski e Zunino, 1999), aumentando a exposição e as possibilidades de infecção e re-infecção de parasitas (Freeland, 1976, 1980; Gilbert, 1994a, 1994b). Stuart e Strier (1995) ressaltaram que o entendimento do processo de co-evolução, entre os parasitas e seus hospedeiros primatas, pode nos proporcionar *insights* a respeito de eventos filogenéticos e de especiação destes animais.

Assim, a quantificação da prevalência de diferentes parasitas em uma população de primatas pode auxiliar os primatologistas a identificar fatores ecológicos e comportamentais limitantes que estariam atingindo, de diversas maneiras, populações inteiras, grupos, genealogias ou indivíduos. Fatores como umidade da área de uso, período do ciclo reprodutivo das fêmeas, densidade populacional e tamanho de grupo do hospedeiro, ou diferenças comportamentais entre os indivíduos do grupo, podem influenciar o tipo de infecção do primata hospedeiro (Stuart e Strier, 1995). Outro fator importante está relacionado à maneira como os animais utilizam seu habitat. Stuart *et al.* (1990) verificaram que os grupos de *Alouatta palliata palliata* que usavam repetidamente as mesmas rotas durante o forrageamento apresentaram uma maior contaminação do que os outros grupos.

Baseado nas inúmeras oportunidades de contaminação dos agentes infecciosos, tanto em animais de cativeiro como os de vida livre, torna-se indispensável o estudo parasitológico nos animais envolvidos em projetos de re-introdução que vêm sendo desenvolvidos com o objetivo de re-povoamento de áreas naturais. Estes estudos deveriam avaliar a área escolhida para a reintrodução bem como acompanhar o processo de habituação dos animais com a área escolhida. Desta forma, poderíamos minimizar os possíveis comprometimentos não somente dos animais envolvidos na re-introdução, mas, também, das espécies já existentes na área (Santini, 1986; Magnusson, 1995; Martins, 2002).

INFECÇÃO POR ENDOPARASITAS EM UM GRUPO DE BUGIOS-PRETOS (*ALOUATTA CARAYA*) EM UM FRAGMENTO FLORESTAL NO ESTADO DO MATO GROSSO DO SUL, BRASIL

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Introdução

A importância dos estudos parasitológicos realizados com primatas é reforçada pelo consenso de que esses animais podem possuir uma fauna de parasitas, característica e

No entanto, alguns estudos constataram que os primatas possuem comportamento de defesa no uso do seu habitat natural numa tentativa de diminuir os riscos de contaminação (Stuart e Strier, 1995). Um comportamento de defesa dos *Alouatta*, relacionado principalmente em evitar infecções parasitárias, é de defecarem em conjunto e em locais pré-estabelecidos. Esses primatas, após um período de descanso pela manhã e à tarde, deslocam-se para galhos intermediários, o que possibilita defecarem diretamente no solo. Este comportamento, possivelmente, minimizaria a re-infecção de parasitas, evitando a contaminação de fontes alimentares e o contato com os patógenos em suas fezes (Montilha *et al.*, 2002; Gilbert, 1997). Além disso, em um estudo com