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SHORT COMMUNICATION

Amplification of hypervariable simple sequence repeats (microsatellites) from excremental DNA of wild living bonobos (*Pan paniscus*)

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Abstract

We show that nuclear DNA extracted from faeces of free living bonobos (*Pan paniscus*) can be used to amplify hypervariable simple sequence repeats, which can be used for paternity analysis and kinship studies. Of 130 DNA extractions of samples from 33 different animals, about two-thirds yielded PCR products at the first attempt. For several samples only a second extraction resulted in positive amplifications. Consistency tests revealed that in some cases only one of the two alleles was amplified. Presumably this is due to a very limited amount of bonobo DNA in the sample and we suggest therefore that a sample found to be homozygous at a given locus should be typed repeatedly for verification.

Keywords: bonobos, excremental DNA, microsatellites

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Introduction

Determination of kinship using DNA markers has become a crucial technique for many behavioural studies on wild living animals (Burke *et al.* 1991; Martin *et al.* 1992; Queller *et al.* 1993). However, obtaining direct blood or skin samples for the isolation of DNA can lead to severe perturbation of the animals under study. This is a particular problem when working with higher primates, since one would risk losing acceptance by a habituated study group if one took samples forcefully. In the case of chimpanzees, Morin *et al.* (1994a,b) have therefore suggested the use of hair samples, from nests, which may contain enough DNA for use in subsequent PCR amplification of simple sequence or microsatellite loci (Queller *et al.* 1993). However, from field studies on chimpanzees and bonobos it is known that night nests are not only used by the individual which has constructed the nest, but also by other group members for social contact (Fruth & Hohmann 1993) or mating (Nishida 1994). Consequently, nests might be occupied temporarily by more than one individual and the identity of the hair samples collected from a given nest may thus be questionable. We have therefore tested the possibility of using faeces as a source for DNA for the amplification of microsatellite markers. It has been shown previously that it is possible to amplify mitochondrial DNA, as well as nuclear DNA from bear droppings (Höss *et al.* 1992; Kohn *et al.* 1995). We show here that faeces can also be a source of DNA for the amplification of highly polymorphic simple sequence repeat or microsatellite loci and can thus be used for kinship and paternity studies. Since the initial submission of this manuscript, a similar method was described by Constable *et al.* (1995).

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Material and methods

Samples

Faeces samples were obtained from wild bonobos (*Pan paniscus*) of the Lomako forest (Zaire). Samples were col-

lected from identified members of the Eyengo community (Badrian & Badrian 1984) that was previously habituated to the presence of human observers. Samples came from nesting sites tracked in the evening and revisited the next morning. Animals drop their faeces usually shortly after waking up. Observing individuals during this period allows collection of fresh samples and unequivocal assignment to known individuals. About 10 g was taken and directly transferred into about 40 mL of undiluted ethanol. Care was taken to avoid the possibility of contaminating the samples with human DNA by touching the faeces only with fresh leaves. The samples were then kept at ambient temperature for up to 4 years. Multiple samples were taken for most animals. As positive controls, we also used blood samples of two individuals from the Zoo in Leipzig.

DNA extraction

Our DNA extraction procedure is based on the protocols of Boom *et al.* (1990) and Höss & Pääbo (1993). However, instead of silica matrix we used diatomaceous earth particles as a DNA binding medium (Carter & Milton 1993). These particles were easier to handle and the DNA yield was found to be slightly higher. During the whole procedure (including the preparation of the buffers) we took care to avoid contamination with human DNA. The glassware, the plasticware and the chemicals were taken from freshly opened packages and handled only with gloves. To isolate the DNA, a piece of about 50–100 mg of faeces was transferred into 1 mL of lysis buffer using forceps which had been flamed until red-hot and then allowed to cool, macerated with a pipette tip and incubated overnight on a rotating wheel [lysis buffer: 50 mM Tris-HCl (pH 7.0), 25 mM EDTA (pH 8.0), 1.25% Triton X 100, 5-M guanidinethiocyanate (Fluka, Cat. No. 50990; note that buffers containing guanidinethiocyanate should never be mixed with acids, since this would release highly poisonous HCN gas; such buffers may be safely disposed by mixing them with 1/10th volume of a 10-M NaOH solution). Insoluble matter was removed by 10 min centrifugation at 16 500 *g* in a microcentrifuge. The supernatant (about 700 μL) was removed, combined with an equal volume of DNA binding solution and incubated for 2 h on a rotating wheel (DNA binding solution prepared according to Carter & Milton (1993): 1 g of size fractionated diatomaceous earth (Sigma, Cat. No. D-5384) resuspended in 100 mL of 50 mM Tris-HCl (pH 7.0), 25-mM EDTA (pH 8.0), 5-M guanidinethiocyanate]. The diatomaceous earth particles were sedimented by 1 min centrifugation at 16 500 *g* and were washed twice with 50-mM Tris-HCl (pH 7.0), 5-M guanidinethiocyanate and twice with 70% ethanol. The ethanol was removed and the particles were dried in a heating block at 56 °C. DNA was eluted from the particles by resuspending them in 120 μL of TE buffer [TE: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)] and incubating them for 10 min at 56 °C. The particles were then sedimented by 5 min centrifugation at 16 500 *g* and the supernatant containing the DNA was transferred into a new tube, taking care that no particles were carried over. The success of the procedure can be monitored by running an aliquot of the extract on an agarose gel. However, one should be aware that the DNA that is visible under these conditions is mostly of bacterial origin or residual DNA of the food plants.

PCR amplification

We used primers for microsatellite loci that had originally been isolated from human DNA and that were found to yield amplification products in several primates (T. Cooke & M. W. Bruford personal communication). The primers of the following human GT/CA repeat loci (Gyapay *et al.* 1994) yielded a polymorphic microsatellite amplification product: D1 S 207, D6 S 271, D7 S 503, D16 S 402 and D17 S 791 (the sizes of the amplification products in bonobos lie between 135 and 202 bp). PCR reactions were set up in a volume of 20 μL according to the recommendations of the supplier of the *Taq* polymerase with 1.35 mg/mL BSA (Biolabs New England) and 5 μL of DNA extract. All loci were amplified using the same standardized cycling profile on a Perkin–Elmer thermocycler: initial denaturation for 3 min at 93 °C, followed by seven cycles (93 °C for 30 s, 50 °C for 60 s, 72 °C for 90 s) and 30 further cycles (89 °C for 30 s, 54 °C for 60 s, 72 °C for 60 s) (T. Cooke & M. W. Bruford personal communication).

Microsatellite detection

The amplified fragments were resolved on denaturing polyacrylamide gels as described in Tautz (1989). Detection was carried out by blotting the gels onto a nylon membrane (Hybond-N+, Amersham) either by using a direct blotting apparatus as described in Schlötterer (1993) or by employing the following blotting procedure: after electrophoresis the dry nylon membrane was directly placed onto the gel, followed by two sheets of Whatman 3MM paper and placing a glass plate with an about 2 kg weight on top of the stack. After 30 min, the membrane was taken off, washed in 2 x SSC (20 x SSC: 3-M NaCl, 0.3-M sodium citrate) and the fragments were UV cross-linked with a total energy of 0.5 J/cm^2. The membranes were then hybridized using a [^{32}P]-end-labelled 15-mer oligonucleotide consisting of a CA repeat (hybridization and washing conditions: 5 x SSC at 39 °C). This indirect detection method by hybridization has the advantage that only the bands from the amplified microsatellite will be visible and not any amplified artefact bands.

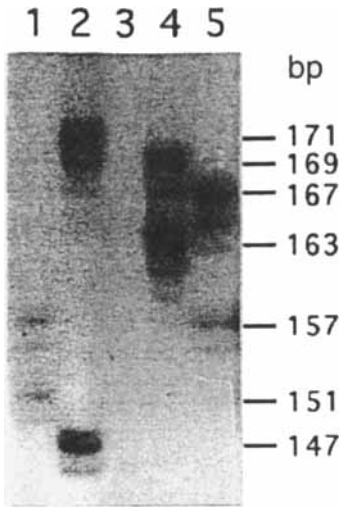

Fig. 1 PCR amplifications of microsatellite locus D1 S207 from DNA extracted from faeces. PCR products were separated on a denaturing sequencing gel and transferred onto a nylon membrane (see Methods). The membrane was then probed with a CA repeat oligonucleotide to detect microsatellite bands only. The sizes of the alleles in nucleotides are indicated to the right. Note that the additional bands represent the typical slippage products generated during the PCR process (Tautz 1989). Lanes 1, 2, 4 and 5: unrelated animals, lane 3: negative control. All individuals show two alleles only and are different, making the possibility of contamination by human DNA highly unlikely.

Results

The primary goal of this study was to evaluate the use of PCR-amplified microsatellites from excremental DNA for paternity analysis and kinship studies. For this purpose it is essential that as many individuals as possible can be reliably typed for multiple alleles. In the following we want to focus therefore on two aspects, first the success rate of PCR amplification from excremental DNA and second on the reproducibility of the results.

Success rate

To ensure that bonobo DNA could indeed be extracted from the faeces, we have first amplified the mitochondrial control region from several samples. Sequencing of the resulting fragments was then used to verify that they were not derived from contamination with human DNA (results not shown). However, the concentration of mitochondrial DNA may be in thousand-fold excess over nuclear DNA in a given sample. The reliable amplification of nuclear loci can therefore be more problematical. To obtain an estimate for the success rate for amplifications of microsatellite loci from DNA isolated from faeces, we have tested several different extracts with several primer sets. Only PCR reactions that resulted in an amplification of hybridizable bands (see Methods) were counted as positives.

On average, we found that about two-thirds of the extracted DNA samples (about 130 extractions from 33 individuals) yielded amplification products for all primers tested in the first attempt. Since almost all samples differed in their allele combinations (Fig. 1), we conclude that these amplification products were indeed derived from bonobo DNA and not from contaminating human DNA. Only a single reaction showed more than two alleles, which was presumably due to contamination by human DNA.

For several samples that did not yield an amplification product at the first attempt, we found that a second extraction of the same sample of faeces resulted in positive amplifications. However, we also found that these more problematical samples did not always yield an amplification product with all primers tested. For two samples we could not obtain any amplification for any of the primers in spite of repeated extraction of the faeces. These observations indicate that the amount of specific DNA extracted may indeed be very small and may depend on the particular sample collected. Multiple collections for each individual are therefore advisable.

Reproducibility

A highly critical parameter for PCR based microsatellite analysis from faeces is the reliability of the results obtained. A consistency test was performed with locus D1 S207 for faeces from 14 individuals which had been independently extracted three times. For 10 individuals we

Table 1 Inconsistent PCR amplifications for four animals. Locus D1 S207 was amplified one or two times from three different DNA extracts of the same faeces sample. The numbers refer to the observed allele sizes in nucleotides

	animal A	animal B	animal C	animal D
1st extraction	151/157	151/157	151 only	147 only 147/171
2nd extraction	151 only 151/157	151/157	147/151	147/171
3rd extraction	151/157	151 only	147/151	147 only

found fully consistent results for each PCR reaction. However, four individuals yielded in some reactions only one of the two alleles (Table 1). This finding indicates again that the amount of bonobo DNA in the extracts may be very limited. Possibly it is so highly diluted that, when removing a small aliquot for the PCR, it may happen that only one allele is sampled. Another possibility is that larger alleles can have a lower likelihood for amplification, since the DNA might be highly degraded. This would at least explain the pattern found for animal D, where the larger allele was missing in two reactions (Table 1). However, for animal C, it was the smaller allele that was missing in one case. Whatever the cause for the occasional nonamplification of the alleles may be, these results suggest that one should be cautious with individuals that were typed homozygous for a given locus. Additional PCR reactions should be employed to verify that only one allele is present at the respective locus.

Conclusion

Our results clearly demonstrate that faeces can be a source for amplifiable DNA markers in bonobos. Since the dropping of faeces can be directly observed, we believe that this is potentially the most reliable source for noninvasively obtaining samples from defined individuals. Moreover, in contrast to hair samples, faeces do not require permission for collection and transport under the CITES agreement, thus facilitating extensive sample collections. The availability of such samples will greatly enhance our capacity to combine studies of the social behaviour among individuals with a knowledge of their genetic relationships.

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The bonobo community in Lomako has been studied by Gottfried Hohmann and Barbara Fruth since 1990, who have also collected the samples. The bench work was done in the laboratory of Diethard Tautz at the Department of Zoology of the University of Munich and is part of several ongoing projects that utilize simple sequence polymorphisms for the analysis of populations. The first studies on showing that bonobo faeces are in principle a source for amplifiable DNA markers were done by Kornelia Rassmann, Irene Rambold and Diethard Tautz. The systematic studies with multiple loci and with samples from the whole community were done by Ulrike Gerloff as part of her Diploma-Thesis under the supervision of Christian Schlötterer.