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SHORT COMMUNICATION

Factors affecting the amount of genomic DNA extracted from ape faeces and the identification of an improved sample storage method

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Abstract

Genetic analysis using noninvasively collected samples such as faeces continues to pose a formidable challenge because of unpredictable variation in the extent to which usable DNA is obtained. We investigated the influence of multiple variables on the quantity of DNA extracted from faecal samples from wild mountain gorillas and chimpanzees. There was a small negative correlation between temperature at time of collection and the amount of DNA obtained. Storage of samples either in RNAlater solution or dried using silica gel beads produced similar results, but significantly higher amounts of DNA were obtained using a novel protocol that combines a short period of storage in ethanol with subsequent desiccation using silica.

Keywords: chimpanzee, genetic tagging, gorilla, microsatellite genotyping, noninvasive samples, quantitative PCR

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Introduction

The effective use of noninvasive samples such as faeces for molecular genetic analyses of wild animal populations is currently hampered by the typically poor quality and low quantity of the nuclear DNA obtained (Taberlet *et al*. 1999). Such DNA is more difficult to amplify using the polymerase chain reaction (PCR), and even when amplified tends to produce erroneous results at a non-negligible rate (Goossens *et al*. 2000; Morin *et al*. 2001). Although accurate results can still be obtained through the application of rigorous standards for the replication of results (Taberlet *et al*. 1996; Morin *et al*. 2001), this is at the cost of increased time, effort and expense (Vigilant 2002).

Although some researchers report relatively high average rates of successful PCR amplification of microsatellite loci from faecal DNA (e.g. > 99% from captive Asian elephants, Fernando *et al*. 2003; 98% from dolphins, Parsons 2001; 95% from domestic sheep and reindeer, Flagstad *et al*. 1999), the majority are rather less successful (65% from captive brown bears, Murphy *et al*. 2003, and wild orangutans, Goossens *et al*. 2000; 67 and 80% from wild and captive bears, respectively, Wasser *et al*. 1997; 67% from wild bonobos, Gerloff *et al*. 1995; 70% from wild baboons, Bayes *et al*. 2000, and wild langurs, Launhardt *et al*. 1998; 75% from wild mountain lions and bobcats, Ernest *et al*. 2000; and 89% from wild chimpanzees, Morin *et al*. 2001, and badgers, Frantz *et al*. 2003). These studies not only represent multiple species with varied diets and living conditions, but also employ various sample storage, DNA extraction and PCR amplification methods. Furthermore, use of the average frequency with which PCR products are obtained as a measure of success obscures the considerable variation in the amplification success compared across samples, loci and individuals. The PCR is a powerful tool precisely because it can be done using as little as a single or a few copies of template DNA (Hofreiter *et al*. 2001), and so simply considering success or failure of PCR amplification gives little information on the amount of template DNA obtained.

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The effectiveness of genetic analysis using faecal samples would be improved if some of the factors affecting DNA yield could be identified and used as guidelines in sample collection. Analysis of samples collected from captive animals would allow greatest control over potential factors, such as diet, that might influence DNA yield. However, the very different ecological and social environments of captive and wild animals mean that results from captive studies may not always be valid for extrapolation to the wild, and research on the genetics of wild populations is the most common application of noninvasive sampling. Therefore, we used two approaches to investigate the factors influencing DNA yield from faeces of wild apes. In the first, we analysed the results from more than 300 extractions of DNA from wild mountain gorilla faeces in order to examine the roles of factors such as fruit presence in diet, sample age, length of time of storage, sex and approximate age of donor, ambient temperature, and storage method upon success of DNA extraction. Our expectations were that fruit in the diet, increased sample age, higher ambient temperatures at the research site and increased storage time would have a negative effect upon the success of DNA extractions. We also compared the success of extractions from samples collected in parallel using a standard method of faeces storage, desiccation with silica (Wasser *et al*. 1997), with those collected into a preservative buffer, RNAlater (Ambion). In a second study, we compared results obtained from samples of wild mountain gorillas and wild chimpanzees also collected in parallel and stored either with silica, or subjected to a novel treatment employing short-term storage in ethanol followed by silica desiccation (Roeder *et al*. submitted). Rather than considering the percentage of positive PCR amplifications, an approximate measure of success, for both studies we estimated success by the concentration of amplifiable DNA recovered as measured using quantitative PCR (Morin *et al*. 2001). Estimation of the amount of DNA recovered is useful as it not only enables quantitative comparison of results from different extracts, but can also be related to the probability of successful PCRs and frequency of PCR errors such as allelic drop-out (Morin *et al*. 2001).

Materials and methods

Sample collection

For the first study of factors affecting DNA yield, a total of 319 faecal samples were collected from wild mountain gorillas (*Gorilla beringei beringei*) living in the Bwindi Impenetrable National Park, Uganda. Information associated with each sample included the approximate age of the sample (newly produced or up to 12 h old — nest sample), age class and sex of individual, average daily maximum and minimum temperature, local rainfall amount during the previous 24 h and known presence of fruit in the diet the previous day. The majority of the ~5 g faecal samples were preserved in 50 mL tubes containing 20 g of silica gel beads (Sigma S7625) (Wasser *et al*. 1997) but a subset was collected into tubes containing 10 mL of RNAlater preservative solution (Ambion). Although field conditions did not allow precise measurement of the amount of faeces collected, any resultant error can be assumed to be randomly distributed. For the second study comparing two storage conditions, samples were collected in parallel into silica tubes as described, as well as into tubes containing 30 mL of 97% ethanol. The ethanol samples were mixed by inversion and after 24–36 h the ethanol was carefully poured off and the remaining solid material was transferred to tubes containing silica. This procedure of brief storage in ethanol followed by silica treatment we term the 'two-step' storage procedure (Roeder *et al*. submitted). Although samples were stored in ethanol for varying periods, any resulting variance in results should not hinder the goal of the experiment, which was to see whether an overnight treatment in ethanol under field conditions resulted in significantly improved results compared with storage in silica. A total of 42 samples of Bwindi mountain gorillas and 20 samples of western chimpanzees (*Pan troglodytes verus*) from the Taï National Park, Côte d'Ivoire were collected in duplicate in as consistent a manner as possible using both the silica and two-step storage methods. All samples were kept at ambient temperature in the field and at 4 °C (silica storage) or –20 °C (RNAlater storage) after arrival in the laboratory.

DNA extraction and quantification

Approximately one fifth of the entire sample, that is, either 100 mg of dried sample or 2 mL of sample in RNAlater solution, was extracted using the QIAamp DNA Stool kit (Qiagen) according to the manufacturer's instructions with the following modifications. In the first step, the dried samples were vortexed in 1.6 mL of ASL buffer and left overnight (12–16 h) in an agitating heat block at 25 °C. For the samples in RNAlater solution, 2 mL of the mixture was first centrifuged for 15 min at 3000 ***g*** and the supernatant removed, followed by a second centrifugation for 15 min at 500 ***g*** and removal of supernatant. The pellet was then resuspended in 1.6 mL ASL, vortexed and incubated for 5 min at room temperature. The intermediate steps followed the manufacturer's protocol. The final step in the procedure, in which buffer AE elutes the DNA, included an incubation step of 20 min followed by centrifugation for 2 min. Up to 12 samples were extracted at one time, and duplicate samples collected using varied storage methods were extracted together to minimize any potential differences arising out of variation in handling among extraction sets. The amount of amplifiable DNA present in 2 µL (1/100 of the total) of DNA extract was estimated

using quantitative PCR as previously described (Morin *et al*. 2001). In brief, this 5′ nuclease assay is similar to conventional PCR, but includes a probe oligonucleotide that emits a fluorescent signal as the reaction proceeds. Comparison of the amount of fluorescence emitted during reactions using unknown amounts of template with amounts generated in reactions containing standard templates of known DNA amounts allows estimation of the concentrations of the templates of interest. Although the target segment of the quantitative PCR assay is rather short (81 bp), it has been shown that this assay provides information on the probability of amplification of product lengths more typical of microsatellite markers (Morin *et al*. 2001; Smith *et al*. 2002). PCR amplification of a sex-identifying segment of the amelogenin locus was conducted as previously described (Bradley *et al*. 2001) on the 297 (of 319) extracts measuring > 1 pg/μL from the first set of mountain gorilla faeces.

Data analysis

A general linear model was used to investigate the influence of several categorical factors (age class of individual, sex of individual, preservation method, fresh or nest sample, known fruit in diet) and continuous independent variables (rainfall, maximum temperature, length of storage time) on the dependent variable (DNA amount) (Grafen & Hails 2002). All ecological data (rainfall, temperature, fruit) were available for about half (156 of 319) of the samples. To meet the assumptions of normality and equality of variances, the DNA amounts were transformed to natural logarithms [ln(DNA amount + 1)]. The best model was selected based on comparison of the adjusted R^2 of various models consisting of different subsets of the independent variables. The adjusted R^2 takes the number of independent variables into account and corresponds to the proportion of variance registered by the respective models. All analyses were carried out using SPSS 11 for Windows.

Results

Factors affecting DNA yield

The data from the DNA extracts obtained from a total of 319 mountain gorilla samples were examined. The concentration of these extracts was extremely variable (mean = 39.4 pg/μL, SD = 107.2) with a minimum and maximum value of 0 and 1315.5 pg/μL, respectively. The general linear model revealed that of the five categorical factors and the three continuous independent variables under consideration, only temperature was selected in the best model. In other models, other independent variables in addition to temperature were occasionally significant, but only as the result of interaction and the effect size compared with temperature, based on comparison of partial ε^2, was much lower. The maximum daily temperature at the research site ranged from 14 to 28 °C. DNA concentration decreased significantly with increasing temperature at time of collection but only a limited amount of the variation is explained by the model ($F(1, 155) = 28.267$, $P < 0.001$, $R^2 = 0.156$) (Fig. 1).

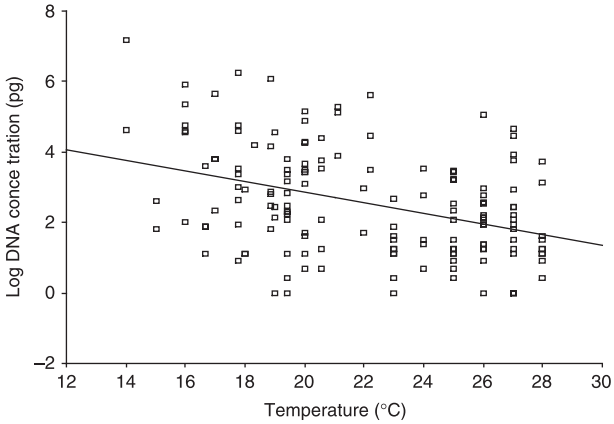

Fig. 1 Negative correlation between the amount of DNA obtained and the maximum temperature at the time of collection of faecal samples. The small squares represent the 156 extracts for which the maximum temperature on the day of sample collection was known. The relationship between temperature and amount of DNA is described by the equation: ln(DNA + 1) amount = −5.88 − 0.151 × temperature.

Although the previous analysis did not find a significant effect of storage method (silica vs. RNAlater) upon DNA amount, direct comparison of the 37 samples collected in parallel might be more likely to detect a difference. The concentrations of the extracts from silica samples averaged 55.3 pg/μL (SD = 109.9), while the extracts from RNAlater samples averaged 62.9 pg/μL (SD = 217.2). A paired *t*-test revealed that the yield from samples stored in silica as compared to that of samples stored in RNAlater did not differ ($t(36) = 0.192$, $P = 0.849$).

Overall, 21 (7%) of 319 mountain gorilla faecal samples yielded no measurable amplifiable DNA. Almost half (45%, 144 of 319) of the extracts contained very low amounts of template DNA (1–10 pg/μL), and 48% (154 of 319) contained > 10 pg/μL. In order to assess the relationship between DNA amount and PCR success rate, we used a subset of 279 extracts to examine the success rate of amplification of a sex-identifying segment of the amelogenin gene located on the X and Y chromosomes (Bradley *et al*. 2001). We found that PCRs containing 2 μL of the very low template concentration extracts had a significantly lower amplification success rate than those made with 2 μL of extracts with concentrations > 10 pg/μL (66.9% (87/130) vs. 96.6% (144/149); Fisher's exact test, $P < 0.001$). In other words, the 48% of the extracts containing > 10 pg/μL could be routinely amplified at a nuclear locus.

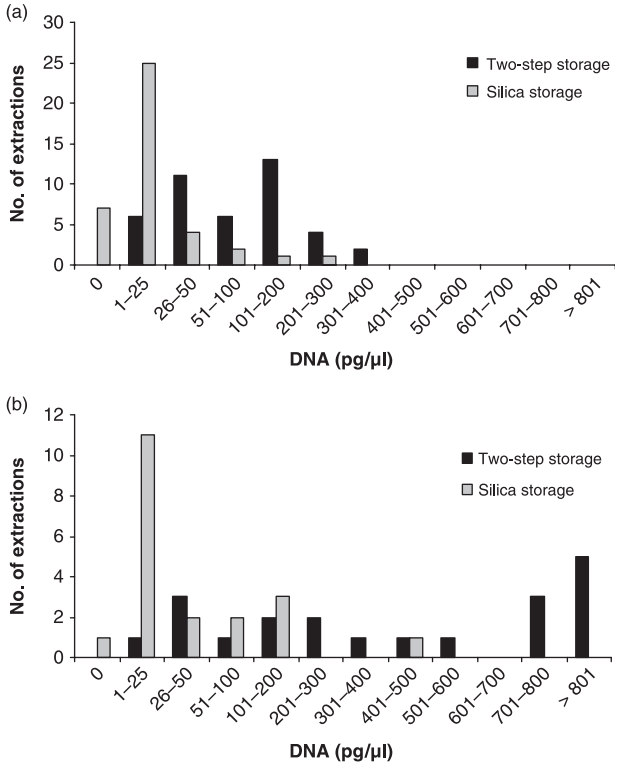

Fig. 2 Estimated concentrations of DNA obtained from (a) 42 gorilla samples, and (b) 20 chimpanzee samples collected in parallel and stored either on silica or using the two-step storage protocol, as indicated.

Silica storage vs. two-step storage

Forty-two pairs of matched samples from mountain gorillas and 20 pairs of samples from chimpanzees were used in this analysis. The average DNA concentrations of the extracts from two-step storage samples were several times higher than that of the silica-stored samples (gorilla samples, 101.1 vs. 21.1 pg/µL; chimpanzee samples, 550.1 vs. 60.8 pg/µL) (Fig. 2). Paired *t*-tests showed that in both cases the differences between samples were significant (gorilla samples, $t(41) = 6.833$, $P < 0.0001$; chimpanzee samples, $t(19) = 4.512$, $P < 0.001$). If we consider that only extracts measuring > 10 pg/µL are likely to amplify consistently (see above), our projected PCR success rate from the combined set of two-step storage samples is 95.2% (59 of 62 extracts), whereas for the silica samples it is 51.6% (32 of 62 extracts). Furthermore, if we assume that up to 4 µL of template can be included in a PCR, some 66% (41 of 62) of the reactions using extracts of the two-step storage samples would contain > 200 pg, a threshold previously shown to be associated with a reduced rate of allelic drop-out (Morin *et al*. 2001). The corresponding figure for the extracts of silica preserved samples is only 16% (10 of 62).

Discussion

It can be imagined that two main factors affect the amount of 'donor' DNA that can be extracted from faeces. The first is the amount of DNA initially present in the sample and the second is the amount still present after collection and storage. Until now, research focusing on the former topic was limited to one recent study that employed controlled feeding of captive brown bears and found a significant effect of diet upon success of amplification of nuclear DNA from faeces, with poor success using samples from individuals eating salmon as compared with a vegetarian diet (Murphy *et al*. 2003). Relative amounts of dietary fibre were not the obvious cause of this difference, because when the bears were limited to a diet of deer meat, this did not produce poor results. Our measure of diet for mountain gorillas in this study was necessarily crude, and consisted of whether fruit had been observed to be eaten the previous day. We found no effect of diet, nor of other factors intrinsic to the individual such as sex or age class, upon the amount of DNA extracted from the samples. This study did not directly examine whether species differences exist in the amount of DNA that can be retrieved from faeces, but it is worth noting that five times as much DNA was obtained from the chimpanzee samples compared with the gorilla samples collected using the two-step storage protocol. Further investigation controlling for differences in field collection practices, diet, and climate would be needed to determine the cause(s) of any species differences.

DNA is an extraordinarily fragile molecule (Lindahl 1993), and so it is to be expected that collection and storage conditions will influence survival of DNA in samples. Degradation of DNA by endonucleases is most likely to be avoided under conditions of low temperature, rapid desiccation and high salt concentration (Hofreiter *et al*. 2001). We found that the temperature at the time of sample collection partially influenced the amount of DNA obtained from the gorilla faecal extracts, with reduced amounts retrieved from samples collected on warmer days. Other researchers have also reported a higher success rate of PCR using DNA from faecal samples collected in cool rather than warm seasons (Lucchini *et al*. 2002). Although immediate freezing of faecal samples is likely to be favourable for DNA preservation, the maintenance and shipping of frozen samples would present difficulties at many field research sites. The use of a proprietary storage buffer containing unknown ingredients, RNAlater, produced similar results to that obtained using silica storage.

It is possible that degradation of DNA may be merely slowed, but not stopped, during storage. We analysed samples up to 33 months in age, and found no effect of storage time upon the amount of DNA recovered. In contrast, a recent study compared various storage methods for faeces and found that silica storage produced poor results,

with the success rate for 1-month-old samples lower than that for 1-week-old samples (Murphy *et al*. 2002). However, as the authors noted, they encountered difficulties drying the samples, and the drying process, which in our experience requires only 12–24 h, in their case required up to 10 days and was accompanied by mould. The results obtained by these researchers indicated that once the samples dried, the success rate stabilized at around 30% for the 1-, 3- and 6-month time points. Both our results and the results of Murphy *et al*. (2002) are consistent with a scenario in which DNA is prone to degradation when the sample is incompletely desiccated, but is stable after thorough sample desiccation.

The two-step storage protocol, in which the samples were soaked in ethanol prior to silica desiccation, resulted in a > 5-fold increase in DNA yield compared with simple drying on silica. We suggest that the improvement obtained by presoaking the sample is a result of the ethanol itself acting to quickly desiccate the sample. The subsequent continued desiccation of the ethanol-soaked sample on silica then maintains the already 'dry' state of the sample. This raises the question whether continued storage on ethanol might be preferable to the two-step procedure. We believe that this is unlikely for several reasons. First, we analysed a small set of eight samples stored in ethanol in parallel with the two-step and silica storage procedures. We found that the samples stored solely in ethanol produced modest amounts of DNA (mean = 34.5 pg/μL, SD = 15.0) similar to amounts produced from the silica samples (mean = 48.3 pg/μL, SD = 37.0), and so results from both storage methods were inferior to those from the two-step storage samples (mean = 152.6 pg/μL, SD = 91.0). Second, PCR success rates using DNA from faecal samples from wild animals stored in ethanol are typically well under 90% (Gerloff *et al*. 1995; Launhardt *et al*. 1998; Goossens *et al*. 2000; Frantz *et al*. 2003) and so are below what can be expected for the success rate for the two-step samples extracted in this study. Although the two-step storage method demands extra sample manipulation in the field, the substantially higher success rate in the laboratory (95.2 vs. 51.6% of samples yielded concentrations of DNA above the 10 pg/μL threshold for reliable amplification for the two-step vs. silica storage methods, respectively) means that fewer samples in total would need to be collected to obtain usable DNA from all subjects, and laboratory expenses would also be reduced. A final reason for preferring the two-step method over continued storage in ethanol is that of practicality, because transport of the relatively lighter, nonleaky, nonflammable silica samples is usually easier and less subject to restriction by airlines and shipping companies. In conclusion, we suggest that researchers studying other taxa should consider testing the two-step protocol for preservation of faecal samples for DNA analysis. We also note that although this study focused on quantification of DNA amounts, a topic that deserves further study is whether the two-step storage method also improves the quality of the DNA obtained and allows amplification of longer target segments than are usually feasible using DNA from noninvasive samples.

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The authors have a common interest in the application of genetic analysis to research on the social behaviour, population history, and conservation of wild animals, especially primates. This project was part of AM Nsubuga's PhD research using genetic analysis to elucidate the reproductive system and social structure of wild mountain gorillas in the Bwindi Impenetrable National Park. MM Robbins conducts field research on the behaviour and ecology of wild gorillas. AD Roeder studies conservation and population genetics in a number of species. PA Morin directed the Laboratory for Conservation Genetics at the MPI-EVA, developing and applying genetics technologies for conservation. His current laboratory at the SWFSC conducts genetics research for conservation of marine mammals. C Boesch has studied the behaviour of wild chimpanzees in the Taï National Park, Côte d'Ivoire, for more than 20 years. L Vigilant is involved in a variety of projects using genetic analysis to address questions on reproductive strategies, kinship, dispersal and population histories of wild primates.
