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Full Length Research Paper

Variation of faecal genomic DNA amount between West African Colobine and Cercopithecine monkeys

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DNA extracted from non-invasive samples, in particular feaces, is usually of poor quality and the trace amount of target DNA often leads to PCR errors. Such errors are especially problematic for genetic census studies and can create a false individual or species designation. To evaluate the non-invasive faecal samples of West African primate species as a valuable source of DNA, faecal samples of nine primate species were collected in Taï National Park and in three forest groves in Côte d'Ivoire. DNA was extracted from faecal sample using Qiamp Stool kits. The amounts of amplifiable genomic DNA obtained in extracts were measured using a real-time quantitative PCR assay. PCR products with the expected range of fragment length were treated as successful amplification. Based on 12S assay, faecal samples collected from Cercopithecine (*Cercopithecus campbelli, Cercopithecus petaurista, Cercopithecus diana*) provide less amplifiable DNA (mean: 60.88 pg/µL; range: 0.5 - 445 pg/µL) when compared to Colobine (*Procolobus verus*, *Piliocolobus badius*, *Colobus polykomos*, *Colobus vellerosus*) with mean of 26 306.69 pg/µL and range of 18.5 - 210 365.5 pg/µL) (P < 0.0001). The initial amount of DNA found in colobine monkey fecal sample is far greater than that found in Cercopithecine faecal samples and suggests that the diet composition of these monkeys contribute to the difference in the amount of their faecal genomic DNA.

Key words: Non-invasive sampling, quantitative PCR, DNA, colobine, cercopithecine.

INTRODUCTION

Non-invasive sampling of faeces has become an important tool for molecular genetic studies in free-ranging animals. Conservation biologists in particular have shown interest in these techniques, which for instance are now routinely used for the monitoring of mammal populations throughout the world (Woods et al., 1999; Palomares et al., 2002; Paetkau, 2003; Eggert et al., 2003; Mainguy and Bernatchez, 2007; Wehausen et al., 2004; Baldwin et al., 2010; Steiner et al., 2013; Caniglia et al., 2014). Although several studies demonstrate the use of these methods in African great apes (Taberlet et al., 1997; Morin et al., 2001; Guschanski et al., 2008, 2009), non-invasive large-scale studies in West African monkeys using faeces collected in the field have been undertaken by few studies (Gonedelé et al., 2008; Petit et al., 2010; Rodrigues, 2012; Minhos, 2012; Haus et al., 2013).

Demonstration of the utility of faeces in genetic studies (Höss et al., 1992; Kohn et al., 1995, 1999; Taberlet et al., 1997; O'Reilly et al., 2008; Gillett et al., 2008; Ramon-Laca et al., 2014), along with studies comparing storage and extraction methods of faeces for DNA analysis have made it clear that faecal samples provide a potentially reliable source of genomic DNA for population studies (Wasser et al., 1997; Frantzen et al., 1998; Flagstad et al., 1999; Piggott, 2004; Broquet et al., 2007; Arandjelovic et al., 2009; Reddy et al., 2012). Reports of

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primate studies utilizing faeces have become more prevalent in recent years (Gerloff et al., 1999; Launhardt et al., 1998; Constable et al., 2001; Smith et al., 2000; Chu et al., 2006; Lathuilliere et al., 2001; McGrew et al., 2004; Chaves et al., 2006; Langergraber et al., 2010) although truly comprehensive studies of wild animal populations using non-invasive samples are still few (Taberlet et al., 1999; Morin et al., 2001; Broquet and Petit, 2004; Broquet et al., 2007; De Barba et al., 2010; Cullingham et al., 2010).

DNA extracted from non-invasive samples, in particular faeces, is usually of poor quality (that is, degraded DNA, the presence of PCR inhibitors and DNA contamination). The trace amount (often in picogram range) of target DNA often leads to PCR errors such as allelic dropout and false alleles that result in incorrect genotype designation (Taberlet et al., 1996, 1999; Smith et al., 2000; Bonin et al., 2004; Hoffman and Amos, 2005). Such errors are especially problematic for genetic census studies even if the error rate is low since a single error can create a false individual or species designation (Taberlet et al., 1996, 1999; Creel et al., 2003; Ebert et al., 2010).

Taberlet et al. (1999) suggested taking care when dealing with non-invasive sample, since non-invasive population genetics is linked to numerous potential problems, especially at the stage of data production in the laboratory. They advice to perform a pilot study in order to estimate genotyping error rates. These rates can, in turn, be used to predict the number of times each sample must be amplified before accepting its genotype at a particular locus (Miller et al., 2002; Valière, 2002; Hausknecht et al., 2010; Ruiz-González et al., 2013). Such pilot studies, however, may not address a number of important questions related to the potential success of surveys based on non-invasive sampling. Parameters such as fragment length, the repeat motif of microsatellite loci, the source of DNA or even the diet of the focal species may influence amplification and genotyping success (Bradley et al., 2001; Murphy et al., 2003; Broquet et al., 2007).

Measurement of the DNA content of non-invasive sample extracts can, in principle, allow classification of extracts by DNA content, and thus expedite the genotyping process while increasing the reliability of the results. For example, extracts of high DNA content could be identified and be subject to less replication, extracts containing low amounts of DNA would be subject to rigorous repetition, and extracts containing no detectable DNA would not be used (Morin et al., 2001). Not assessing the potential of non-invasive sampling prior to extensive analysis can lead to the collection of useless samples, and delay the achievement of the study by many years. Therefore, constraints involved in collecting samples in the field and laboratory costs of using non-invasive sampling must be weighed. Moreover, the validity of non-invasive genetic sampling may vary among species. When adopting the approach, species-specific pilot study is required beforehand (Taberlet et al., 1999).

With regards to West African monkeys, in the context of intensive human pressure, where field studies and direct observation of the remaining primate populations are difficult, non-invasive genetic approach can greatly help in conservation studies. Here we evaluate non-invasive faecal samples of several West African primate species as a valuable source of DNA for population genetic studies. Faecal samples were collected as DNA sources for PCR amplification. Then success rate of PCR amplification from fecal DNA extracts were examined.

MATERIALS AND METHODS

Sampling

Faecal samples of nine primate species were collected between January to February 2001 in Taï National Park and in three forest groves (Guetitapia, Soko and Dinaoudi) (Figure 1). Faecal samples were collected from individual species (*Cercopithecus diana*, *Cercopithecus campbelli*, *Cercopithecus petaurista*, *Procolobus verus*, *Piliocolobus badius*, *Colobus polykomos*, *Colobus vellerosus*) shortly after defecation. Samples of approximately 0.5 g were placed in individual vials (50 mL size) containing silicagel, and stored at room temperature for several weeks. Samples were transferred to fresh tubes of silica upon receipt in the laboratory and kept at 4°C. Faecal samples were stored over five months before extraction.

DNA extractions

DNA was extracted from faecal sample using Qiamp Stool kits (QIAGEN, Germany), following the manufacturer's instructions. In order to optimize the amount of DNA in faecal samples and reduce the time of extraction, 100 mg faecal samples were rehydrated for 2 h at 4°C followed by rehydration overnight instead of 3 days of rehydration as indicated by the manufacturers.

Final volume of DNA extract was 200 µl for each sample. Contamination was monitored by including two extraction blanks in every extraction round.

Quantitative PCR

The amounts of amplifiable genomic DNA obtained in extracts were measured using a real-time quantitative PCR assay as previously described (Morin et al., 2001). Briefly, the forward primer (CMYC_E3_F1U1)/reverse primer (CMYC_E3_R1U1) and probe (CMYC_E3_TMV) designed by these authors were used. The 5' nuclease assay was performed in 20 mL PCR containing 'TaqMan Universal PCR Mastermix' (PE Biosystems), 300 nm each primer, 200 nm probe, 8 mg BSA (Boehringer-Mannheim) and 2 mL DNA extract. PCR amplification

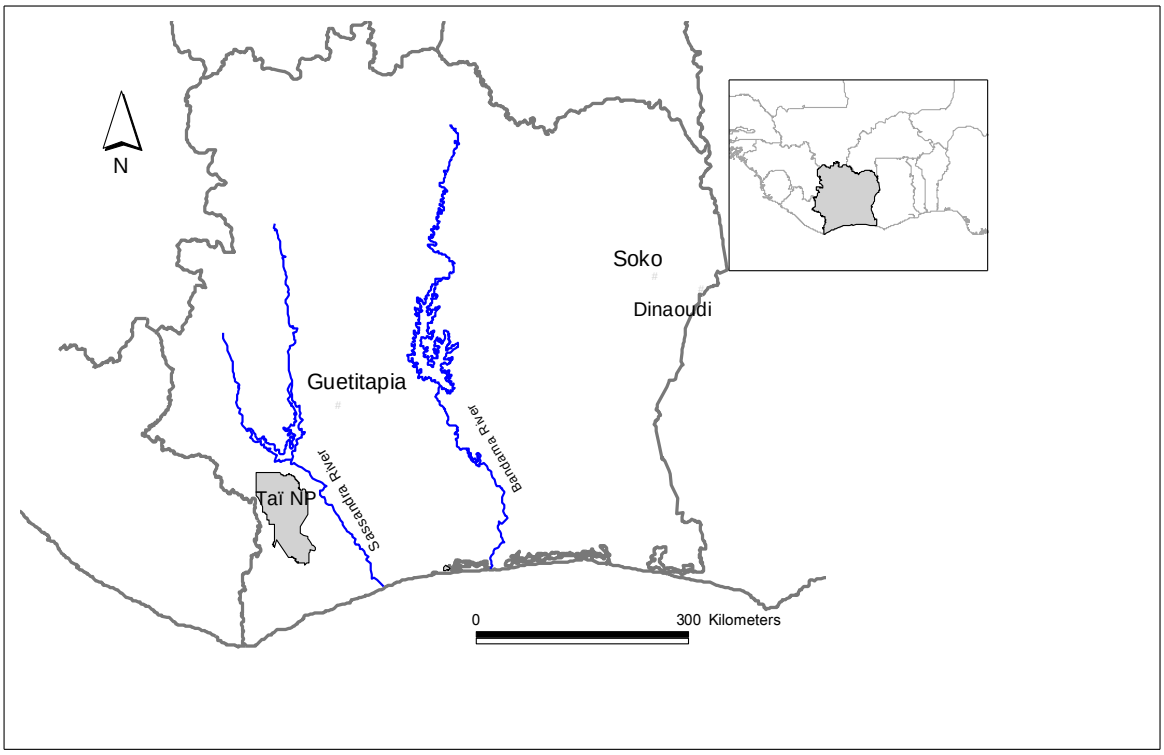

Figure 1. Faeces sampling sites.

was performed in an ABI Prism™ 7700 Sequence Detector (PE Biosystems), with initial incubations for 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of 95°C for 15 s and 59°C for 30 s. Analysis was performed using the ABI Prism™ 7700 Sequence Detector software. PCR products were visualized on 2% agarose gels stained with ethidium bromide (EtBr). PCR products with the expected range of fragment length were treated as successful amplification. Success rates of PCR amplification at each locus and for the overall reactions were calculated by dividing the number of successful reactions to the total number of PCR.

The quantity of DNA extract of 200 µl for each sample was estimated as individuals per species and averaged for each species and each subfamily. We tested differences in faecal DNA quantity among Colobine and Cercopthecine monkeys species and between Colobine and Cercopthecine monkeys subfamilies via Kruskal-Wallis tests.

PCR success rate

PCR success rate based on the size of the amplicons was tested with primer L15996/H14698 amplifying 600 bp and the Amelogenin primer amplifying 100 bp. PCR amplification was carried out in a total volume of 15 µl consisting of 2 µl DNA extract, 2 mM MgCl2, 6 µg bovine serum albumin (BSA), 250 µM each dNTP, 200 nM each primer (AmelA/AmelB, Sullivan et al., 1993; L15996/H14698), 0.375 U AmplitaqGold and 1 × PCR buffer (Perkin-Elmer). Reactions were carried out in a Peltier thermal cycler, PTC 200 (MJ Research).

For both the primers pair, amplification conditions were: initial denaturation at 95°C for 3 min; 45 cycles of 30 s at 95°C, 30 s at 60°C for Amelogenin primer and at 50°C for primer L15996/H14698, 30 s at 72°C, final extension of 30 min at 72°C. Blank controls were used alongside all PCR reactions.

RESULTS

PCR success rate of the non-invasive fecal samples from wild monkeys using Amelogenin primer (100 bp) varies among species. Within cercopithecines (*Cercopithecus diana*, *Cercopithecus campbelli*, *Cercopithecus petaurista*), PCR success rate extends from 60 to 75% with *C. diana* having the highest success rate (Figure 1). Within the four colobine species analysed (*Procolobus verus*, *Piliocolobus badius*, *Colobus polykomos*, *Colobus vellerosus*), except for *C. vellerosus* that has a success rate not surpassing 50%, PCR success rate of the other species fecal samples exceeds 80%, with fecal DNA extracts of *P. verus* and *C. vellerosus* having a success rate of 100% (Table 1 and Figure 3).

Based on 12S assay, faecal samples collected from cercopithecine (*Cercopithecus campbelli, Cercopithecus*

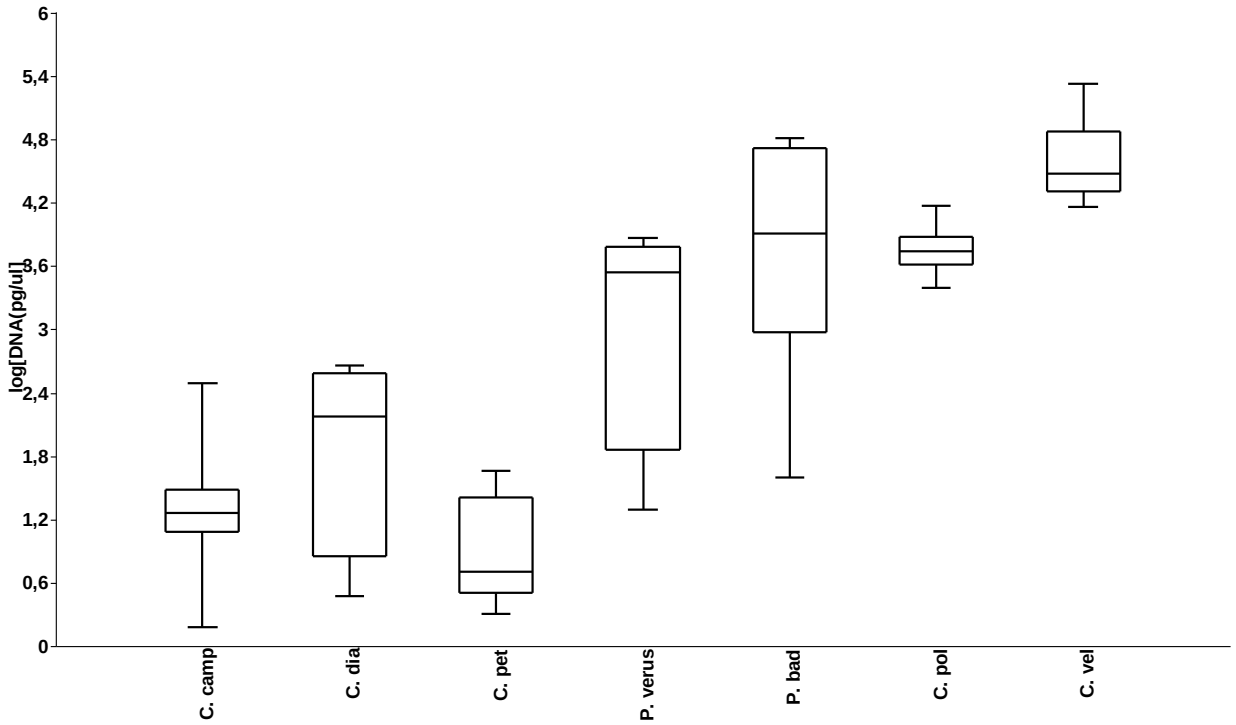

Figure 2. DNA quantity extracted from 7 species of West African primate using 12S methods.
C. camp: *Cercopithecus campbelli*; C. dia: *Cercopithecus diana*; C. pet: *Cercopithecus petaurista*; P. verus: *Procolobus verus*; P. bad: *Piliocolobus badius*; C. pol: *Colobus polykomos*; C. vel: *Colobus vellerosus*.

Table 1. Success rate of PCR amplification from fecal DNA extracts of West African primates with amelognin primer (100 bp) and universal primer L15996/H14698 (600 bp).

Species	Mean DNA quantity (pg/µL)		PCR success rate		# sample
	Amplicon c-myc	Amplicon 12S	Amelogenin primer(100 bp)	Universal primer L15996/H14698(600 bp)	
Cercopithecus campbelli	44.44 (±160.43)	42.78 (±77.17)	68.75%	0.25%	16
Cercopithecus diana	1.63 (±2.36)	192.75 (±192.72)	75%	0%	4
Cercopithecus petaurista	1.7 (±2.49)	13.30 (±18.01)	60%	0%	5
Procolobus verus	0 (±0)	3141.25 (±3587.39)	100%	0%	5
Piliocolobus badius	97.83 (±224.97)	24350.11 (±26425.93)	88.89%	0%	9
Colobus polykomos	10.88 (±26.0143)	6267.75 (±3691.52)	50%	0%	8
Colobus vellerosus	235.7 (±343.76)	55655.10 (±59713.06)	100%	0%	10

petaurista, Cercopithecus diana) provide less amplifiable DNA (mean: 60.88 pg/µL; range: 0.5 - 445 pg/µL) when compared to colobine (*Procolobus verus*, *Piliocolobus badius*, *Colobus polykomos*, *Colobus vellerosus*) (mean: 26 306.69 pg/µL; range: 18.5 - 210 365.5 pg/µL) ($P < 0.0001$) Figure 2. A similar magnitude of difference is obtained when using c-myc assay (Cercopithecine: mean: 29.04 pg/µL; range: 0 - 645.5 pg/µL– Colobine: mean: 103.89 pg/µL; range: 0 - 690 pg/µL) ($P < 0.05$).
DNA concentrations did not vary significantly between species within Cercopithecine monkeys (Kruskall–Wallis test, $P>0.05$). Within Colobine monkeys, DNA

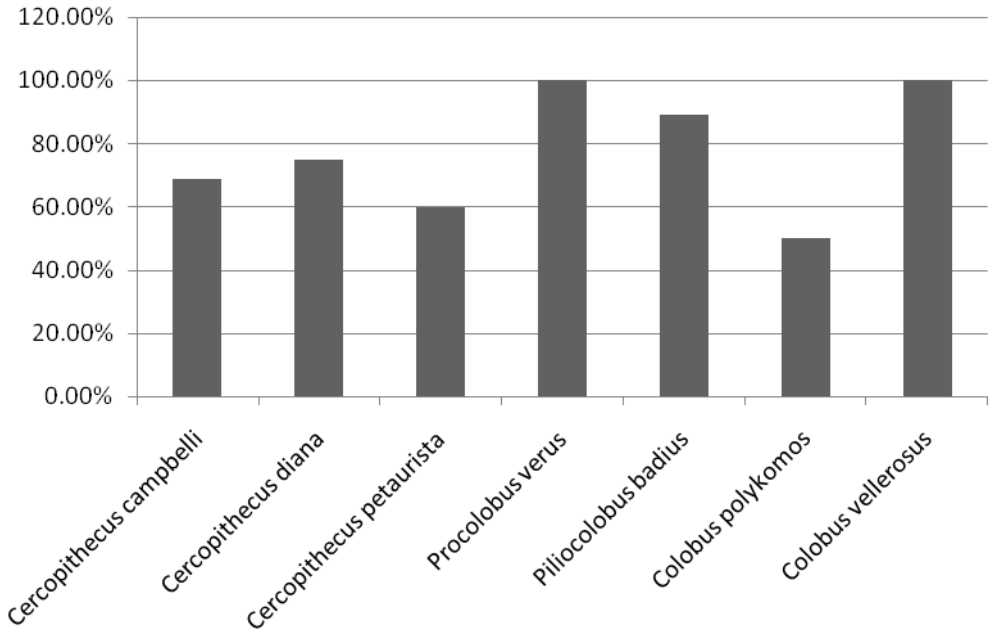

Figure 3. Success rate of PCR amplification from fecal DNA extracts of West African primates with Amelognin primer (100 bp).

concentrations vary significantly between *Colobus vellerosus* and *Procolobus verus* or *Colobus polykomos* (Kruskall–Wallis test, P<0.01).

DNA amplification success was correlated with the length of target DNA sequences (Table 1). Shorter repeat motifs of 100 bp led to higher amplification success than longer motifs of 600 bp. Amplification of the 600 bp fragment was successful in 7.02% of 57 fecal DNAs, whereas amplification of the 100 bp fragment was successful in 77.19% of the total sample (Table 1).

When using the c-myc 5' nuclease assay to quantify the amount of DNA, the average DNA concentration found in *Colobus vellerosus* faecal extracts was by far the highest, with 235.7 pg/µL (range: 8-1175), followed by *Piliocolobus badius* with 97.83 pg/µL (range: 2-690). The lowest DNA concentrations are found within *Procolobus verus*, *Cercopithecus diana* and *Cercopithecus petaurista* with a mean DNA concentration not reaching 2 pg/µL (respective range: 0-5; 0-6). With the exception of *Colobus polykomos* and *Procolobus verus*, the other two colobine monkeys have a mean DNA concentration exceeding 50 pg/µL.

Quantification process using the mitochondrial 12S assay to quantify the amount of DNA, gives an average DNA concentration of > 3000 pg/µL within colobine monkeys whereas this rate does not reach 200 pg/µL within cercopithecines. Within colobine monkeys, the amount of DNA found in *Piliocolobus badius* faecal extracts was by far the highest, with 24,350.11 pg/µL (range: 38.5 - 63,580), followed by *Colobus vellerosus* with 55,655.10 pg/µL (range: 14,202 - 210,365.5). The lowest DNA concentrations are found within *Procolobus verus*, with a mean DNA concentration not reaching 3,141.25 pg/µL (range: 18.5 - 7,272.5). The quantity of fecal DNA correlates positively with PCR success rate within primates examined ($r^2 = 0.777$, $p<0.05$).

When using DNA quantification methods based on the c-myc 5' nuclease assay, 42% of Cercopithecine and 50% of Colobine faecal extracts measured as zero contain amplifiable DNA. PCR success rate slightly increase to 50% and 60% respectively in Cercopithecine and Colobine for faecal extracts having 0.1 to 3 pg/µL. Within both subfamilies, all the faecal extracts that have >3 pg/µL contain amplifiable DNA (Figure 4). Both assays indicate a far greater DNA amount in Colobine faecal sample as compared to those of Cercopithecine (Figure 5).

DISCUSSION

We show a strong relation of PCR success rate to initial amounts of DNA used. Approximately one-third of the 57 faecal extracts had undetectable amounts of DNA. A strong dependence of PCR success on the initial template amount was demonstrated, but with a number of positive reactions (21%) produced from reactions containing non-detectable (0 pg) amounts of template DNA. Accurate quantification of DNA extracts can help to decide protocols and hasten work when dealing with non invasive fecal samples within Colobine and Cercopithecine.

Our study indicates that 3 pg of template DNA represented a critical threshold value below which PCR failure could reach high proportions within Colobine and

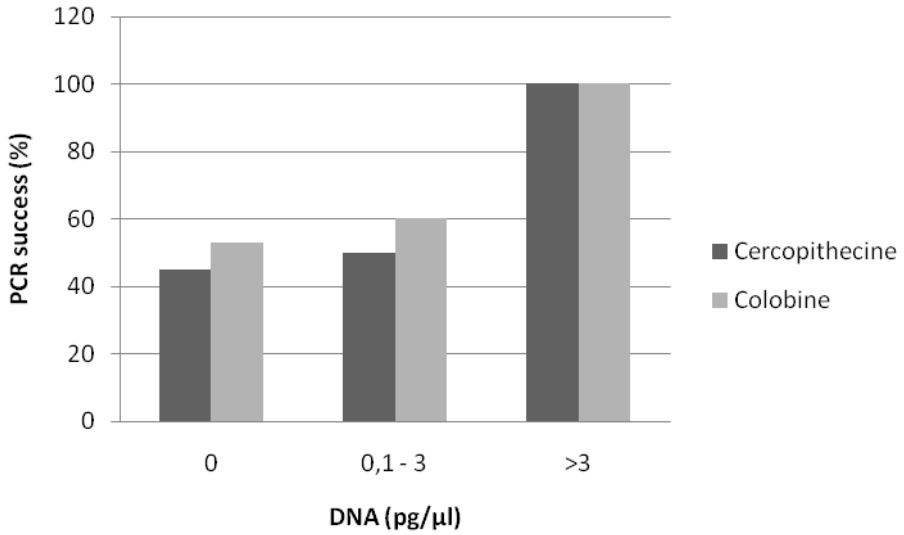

Figure 4. PCR success for various categories of DNA template amount within cercopithecine and colobine monkeys.

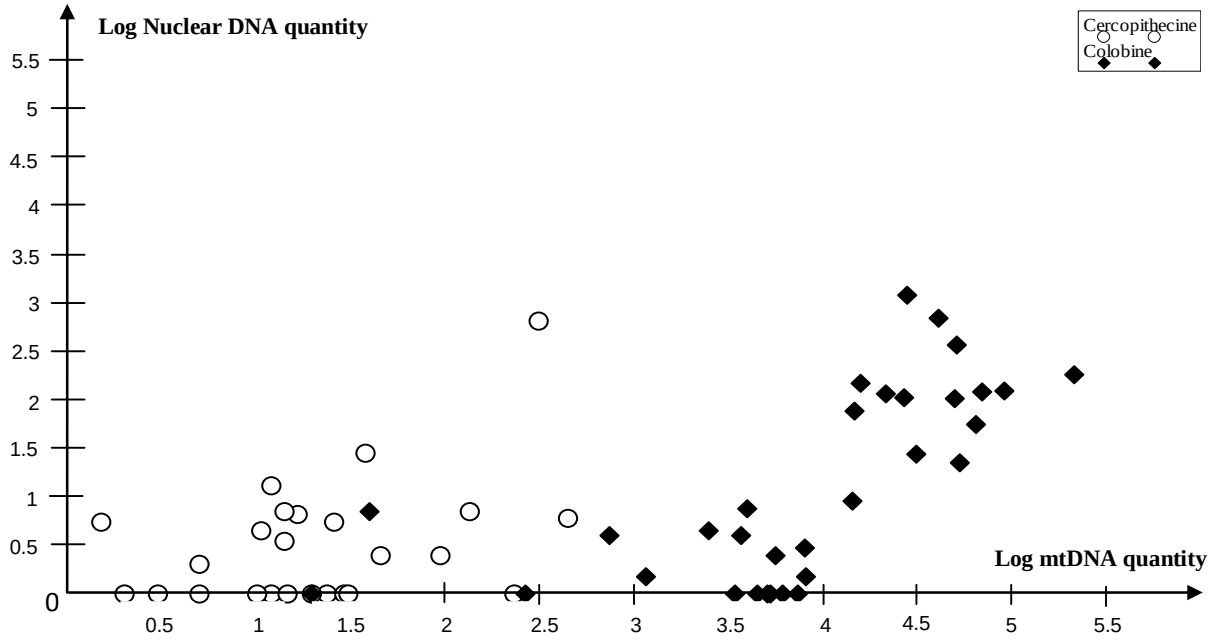

Figure 5. Log(X+1) of DNA quantity pg/µL for the mtDNA 12S and the c-myc assay.

Cercopithecine monkeys. Comparatively, earlier studies by Taberlet et al. (1996), Morin et al. (2001) within chimpanzee and gorilla, respectively found that 56 and 25 pg of template DNA has a critical threshold value below which PCR and allelic dropout could reach high proportions. Our data within West African Colobine and Cercopithecine monkeys indicate a lower critical threshold value compared to these indicated within chimpanzee and gorilla. These differences could be explained by the presence of more PCR inhibitors in the faecal samples of great apes compared to Colobine and Cercopithecine monkeys.

The value found in this study lends support to the idea that the categories of DNA amounts presented here could be applicable to PCR amplification/sequencing and microsatellite genotyping studies within West African monkeys.

In contrast to nuclear DNA, mitochondrial DNA

(mtDNA) is present in many copies per cell and has therefore been the target of many investigations using noninvasive samples. A 5' exonuclease assay targeting a portion of the mitochondrial 12S ribosomal RNA (rRNA) gene was able to detect still lower concentrations of total genomic DNA than the c-myc assay described here, and in fact several samples had positive readings despite containing no detectable DNA using the c-myc assay. The excess of mtDNA relative to nuclear DNA is most likely to be the result of different initial amounts of these DNAs in the samples, and perhaps also the consequence of differential stability of the two types of DNA during storage (Morin et al., 2001). Quantification of DNA for mtDNA studies is essential to avoid errors arising from nucleotide misincorporation in the first cycles of amplification from few starting molecules (Handt et al., 1996). Thus, a strategy of quantification such as the one outlined here, followed by appropriate repetition, should also be useful for mtDNA studies using noninvasive samples within West African Colobine and Cercopithecine monkeys.

Amplification success drastically decreased with increasing amplicon length. Smaller mtDNA sequences may therefore be preferred in noninvasive genetic studies of Colobine and Cercopithecine monkeys as indicated by studies conducted on other wild fauna (Smith et al., 2002).

In addition to the low amount of DNA in fecal samples, success of PCR amplification are often influenced by other factors such as diet of species (Bradley et al., 2000; Murphy et al., 2003), preservation methods (Frantzen et al., 1998; Nsubuga et al., 2004) and freshness of fecal samples (Nsubuga et al., 2004). Bradley et al. (2000) found that the success rate of fecal DNA PCR was lower in gorilla than in chimpanzee, and suspected that different dietary components between the two species might be associated with different PCR inhibitors. Murphy et al. (2003) directly quantified the influence of 6 diets (grass, alfalfa, carrots, white-tailed deer, blueberries and salmon) on fecal DNA amplification in captive brown bears and concluded that some diets (for example, salmon) lead to a significant decrease in PCR success rate of nuclear gene.

The differential quantity of the faecal DNA within the two subfamilies highlighted the difference in their diet composition. Though they also eat fruits, Colobines are leaf-eating specialists, more than any Cercopithecines that are mostly omnivorous, with diets ranging from fruits, leaves, seeds, buds, and mushrooms to insects and spiders to smaller vertebrates (Strier, 2007).

Although the diet of Colobus monkeys is mainly composed of leaves, buds and fruits can also be included(Johnston, 1920). About 35-75% of their diet consists of young leaves which are easier to digest and are less toxic (Usongo and Amubode, 2001). At times they may not have the choice of young leaves and so have to feed on more difficult to digest mature leaves.

However they possess a multichambered stomach with special microbes that break down cellulose over an extended time allowing fermentation to occur (Tovar et al., 2005).

Study conducted by Nijboer (2006) on the effect of dietary composition on faeces consistency indicates that high intakes of crude fibre are correlated with high amounts of crude fibre in faeces leading to well-shaped faeces. The author suggested that crude fibre might have a superior water-binding capacity. Alternatively or additionally, the increase in the amount of fibre in faeces may be responsible for the improvement of the faeces quality (Nijboer, 2006). Indeed, field observation indicates that fecal samples of colobine monkeys (particularly, Black and white colobus monkeys and red colobus) are properly formed and generally solid (Gonedelé Bi, University FHB, personnal communication). Hence, the higher faeces quality of colobine monkeys may greatly contribute to quantity of the faecal DNA.

Conclusion

This study highlights the variation in success rate of PCR amplification between Colobine and Cercopithecine monkey and provides valuable indices for non-invasive genetic studies of West African monkeys. The initial amount of DNA found in Colobine monkey fecal sample is far greater than that found in Cercopithecine faecal samples.

The relatively higher success rate of mtDNA PCR indicates that the non-invasive genetic sampling method can be used in mtDNA-based studies with West African monkeys and is suitable for practical concern. The use of updated technical improvements in fecal sample preservation and PCR procedure could greatly increase the success rate in the application of non-invasive sampling method with West African monkeys.

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