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Title

Highly Versatile, Non-Invasive Method for Collecting Buccal DNA from Free-Ranging Non-Human Primates

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ABSTRACT

Non-invasive techniques for collection of DNA samples of suitable quality and quantity are important for improving the efficiency of genetic wildlife research. The development of a non-invasive method for collection of DNA samples from wild stump-tailed macaques (*Macaca arctoides*) is described herein. Polyester rope was cut into 10 cm pieces, which were then soaked in a 20% sugar solution to bait

individuals. Rope swabs were immediately collected and transferred to a lysis buffer solution after subjects had picked up, chewed, and discarded them. DNA was later extracted from the buffer. Quantitative real-time PCR and both allelic dropout and genotype failure rates were used to compare the quantity and quality of the buccal DNA samples to those of intestinal slough cell DNA samples collected from freshly dropped feces. The buccal samples yielded significantly more DNA (27.1 ± 33.8 ng/μL) than did the fecal samples (11.4 ± 15.4 ng/μL) and exhibited lower allelic dropout and genotyping failure rates for the 10 autosomal microsatellites investigated. Buccal cell collection was also simple, inexpensive, reliable, and less time-consuming compared to fecal sampling. Thus, this method should facilitate genome-wide studies of non-human primates and other wildlife species.

Keywords

Non-invasive DNA collection, Microsatellite markers, Quantitative real-time PCR, Allelic dropout

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Introduction

Wildlife, including non-human primates, has been subject to genetic analyses in a wide variety of research fields, such as evolutionary biology (e.g., Blancher et al. 2008, Osada et al. 2015, Tosi et al. 2002), population genetics (e.g., Evans et al. 2001, Kanthaswamy et al. 2013, Trask et al. 2013), phylogeography (e.g., Bunlungsup et al. 2016), pedigree analysis (e.g., Barelli et al. 2013), and conservation biology (e.g., Minhós et al. 2013), using a variety of DNA markers. Mitochondrial DNA (mtDNA), for example, is generally used for investigating maternal relationships and phylogeography (Avise 2004), whereas Y-chromosome genes of mammals are used to investigate paternal relationships and male dispersal (Tosi et al. 2000, Tosi et al. 2002). Meanwhile, autosomal markers, such as microsatellite and single-nucleotide polymorphism (SNP) markers, are often used to investigate population genetics and genomic diversity (Bruford and Wayne 1993, Brumfield et al. 2003).

As a result of recent advances in DNA analysis technology and growing concerns over animal welfare (Russell and Burch 1959), genetic studies of wildlife frequently use DNA samples that have been collected by non-invasive means (e.g. Woodruff 1993, Taberlet et al. 1999). For example, DNA samples have been collected from egg shells (herring gull, *Larus argentatus*; Egloff et al. 2009), blood-fed mosquitos

(Ejiri et al. 2011), koala feces (*Phascolarctos cinereus*; Wedrowicz et al. 2013), and bug-bite blood (Sumatran rhinoceros, *Dicerorhinus sumatrensis*; Rovie-Ryan et al. 2013). DNA has similarly been collected non-invasively in genetic studies of wild, non-human primates, for example, from trapped hairs (white-headed langur, *Trachypithecus leucocephalus*; Wang et al. 2016), semen (Japanese macaques, *Macaca fuscata*; Domingo-Roura et al. 2004), urine (Japanese macaques; Hayakawa and Takenaka 1999), and saliva (mountain gorillas, *Gorilla beringei beringei*, and Grauer's gorillas, *Gorilla beringei graueri*; Smiley et al. 2010, Chimpanzee, *Pan troglodytes*, Inoue et al. 2007). Among these DNA resources, fecal samples have been most commonly used (Blair and Melnick 2011, Buchan et al. 2003, Nietlisbach et al. 2012, Städele et al. 2015, Vigilant et al. 2001). However, fecal samples generally yield low quantities of low-quality DNA, and even though the markers used in some studies (e.g., mtDNA markers) can be amplified successfully owing to their high copy numbers (Bunlungsup et al. 2016), enormous efforts are required when examining nuclear markers (Navidi et al. 1992, Taberlet et al. 1996). One major problem with using fecal DNA samples for nuclear genotyping is allelic dropout, a phenomenon in which one of two autosomal alleles is not amplified by PCR, causing heterozygous genotypes to be misinterpreted as homozygous (Pompanon et al. 2005, Tebbutt and Ruan 2008). Allelic

dropout is problematic in paternity and kinship analyses using autosomal microsatellites (Vigilant et al. 2001).

As such, development of non-invasive DNA sampling methods that allow researchers to obtain large quantities of high-quality DNA samples with low levels of contamination is needed. Buccal cell collection methods, such as collecting sugarcane wedges or pith of terrestrial herbaceous vegetation after their chewing by wild bonobos (*Pan paniscus*, Hashimoto et al. 1996, Ishizuka et al. 2018), taking oral swabs from anesthetized mountain and Grauer's gorillas (Smiley et al. 2010), and attaching ropes to saliva-collecting devices near free-ranging Tibetan macaques (*Macaca thibetana*, Simons et al. 2012), have been reported previously. Collecting DNA from wedges of sugar cane or other plants is a non-invasive method that does not require manipulation of animals and is thus applicable to other study sites with appropriate modification according to certain factors, such as the environment of the study and the feeding patterns of the subjects. However, methods that require anesthesia are impossible in most of field studies, and methods that require specialized equipment takes time and cost to produce the device. Especially, in the wild condition, using specific devices is less flexible to collect multiple samples from several monkeys at once due to mobilities. Such methods were inapplicable to the stump-tailed macaques at our study site because

of the difficulty in preparation and storage of the bite materials. Thus, we designed another method for collecting buccal cells reporting here.

Herein, a non-invasive method for collecting buccal DNA samples using rope swabs is described as simple, reliable, inexpensive, and less time-consuming than other commonly used methods. To test the effectiveness of this method, two experiments were conducted. The first was a quantitative comparative test of host DNA in 41 fecal and 41 buccal DNA samples randomly selected using real-time PCR. In addition, gel electrophoresis ("gel tests") were also used to quantitatively test DNA samples cheaply and conveniently, and their results were compared with those of costlier real-time PCR to verify their accuracy. The second experiment was a qualitative comparison based on allelic dropout and genotype failure rates in 30 fecal and 30 buccal DNA samples selected using gel tests.

Materials and methods

Study site

The present study was conducted at the Khao Krapuk Khao Taomor Non-Hunting Area, Phetchaburi Province, Thailand (12°47′59.2″ N, 99°44′31.1″ E), which harbors five free-ranging groups of stump-tailed macaques (*Macaca arctoides*). There are five

groups: Ting group, 115 individuals; Nadam group, 91 individuals; Third group, 71 individuals; Fourth group, 75 individuals; Wngklm group, 43 individuals (Toyoda and Malaivijitnond 2018). This survey area is mainly a mountainous area composed of secondary forests and bamboo forests, and open areas coexist including temple and houses of local people. The moving area of monkeys was divided between north and south by large roads, and food provisioning by locals or visitors was occasionally observed along the road or at temple ground. As information on environmental conditions, mean annual temperature and annual rainfall are 27°C and 1070 mm, respectively, in the data of the nearby national park, named Keang Krachan National Park, about 30km far from this study site (Wijitkosum 2012). This site consists primarily of secondary forest, including stands of bamboo and agricultural areas. Food provisioning by locals or visitors was occasionally observed.

Collection and extraction of DNA samples

Buccal cells were collected using baited ropes (hereafter *rope swabs)*. Polyester ropes (6 mm in diameter; Takagi Corporation, Kagawa, Japan, JAN code: 4943 956 261 513) were cut into approximately 10 cm pieces, autoclaved, and dried (Figure 1). To bait individuals, the rope swabs were soaked in a 20% sugar solution (70 g cane sugar

139 dissolved in 350 mL distilled water) for at least 30 min, and then scattered on the open
140 ground where the monkeys were found. After being chewed (Figure 2) and discarded by
141 a monkey, the rope swab was quickly collected and transferred to a 5 mL carrying tube
142 containing 3 mL lysis buffer (0.5 % (w/v) in SDS, 100 mM EDTA pH 8.0, 100 mM
143 Tris-HCl pH 8.0, and 10 mM NaCl) (Hayaishi and Kawamoto 2006). To compare the
144 quantity and quality of the buccal DNA with that of other commonly used DNA sources,
145 intestinal slough cells from freshly dropped fecal samples were also collected. A sterile
146 cotton bud, which was soaked in 2 mL lysis buffer, was used to swab the surfaces of
147 feces, following the protocol of Bunlungsup et al. (2016). To increase DNA yields, the
148 surfaces of the feces were swabbed at least three times.

149 The buccal and intestinal cells that were transferred to the lysis buffer were
150 kept at room temperature for at least five months until DNA extraction. DNA was
151 extracted following the procedure of Kawamoto et al. (2013). Potential PCR inhibitors
152 were removed by adding 600 mg of hydrolyzed starch (Wako, Osaka, Japan) to 1.5 mL
153 of lysis buffer per sample. The samples were incubated at 36 °C for 10 min, and then
154 centrifuged at 1000 ×*g* for 15 min. Finally, 750 μL of each supernatant was processed
155 using a commercially available DNA clean-up system (Wizard SV Gel and PCR
156 Clean-Up System; Promega, Madison, WI, USA), and the DNA was finally eluted with

50 μL pure water. Study had been conducted from September 25th, 2015 to June 15th, 2017, and 74 fecal samples and 579 buccal samples were collected.

DNA quantification

The amount of host DNA was quantified by quantitative real-time PCR (Morin et al. 2001). Forty-one DNA samples extracted from buccal and 41 from fecal samples were selected randomly from all of the extracted DNA samples. The real-time PCR method was used because both the buccal and intestinal DNA samples were contaminated with other exotic DNA sources, such as bacteria, eukaryotic parasites, and dietary materials (e.g., plants, insects, or small animals), which could not be differentiated using conventional spectrophotometry. The sequences of the real-time PCR primers and c-*myc* probe were 5'-GCCAGAGGAGGAACGAGCT-3' (CMYC_E3_F1U1), 5'-GGGCCTTTTCATTGTTTTCCA-3' (CMYC_E3_R1U1), and 5'-FAM-TGCCCTGCGTGACCAGATCC-TAMRA-3' (CMYC_E3_TMV), respectively (Morin et al. 2001). Real-time PCR was performed using a StepOnePlus real-time PCR System (Thermo Fisher Scientific, Waltham, MA, USA), and each 20 μL reaction contained 2 μL DNA template, 1× TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific), 250 nM probe, and 900 nM of each primer. In addition, the

PCR amplification conditions included an initial denaturation step of 95 °C for 20 s, followed by 45 cycles of 95 °C for 1 s and 60 °C for 20 s. Host DNA quantity (concentration) was determined using a standard curve made by a duplicate set of DNA with known quantity. The standard set was made from DNA extracted from the blood of a northern pig-tailed macaque (*Macaca leonina*) reared in the Primate Research Unit, Chulalongkorn University (Bangkok, Thailand). The DNA was quantified using a spectrophotometer and diluted to 10 ng/μL, 2.5 ng/μL, 625 pg/μL, 156 pg/μL, 39.1 pg/μL, and 9.8 pg/μL with deionized water. The mean DNA yields obtained from the buccal and fecal samples were compared using the Wilcoxon rank sum test in R Ver. 3.4.2 (R Core Team 2016).

Real-time PCR provides an accurate host DNA concentration for each DNA sample, and thus was appropriate for comparing the DNA yields of the buccal and fecal samples. However, real-time PCR analysis is expensive. Therefore, to select suitable samples for microsatellite genotyping, the usability of the 82 DNA samples was roughly screened using conventional PCR and agarose gel electrophoresis following the procedure of Kawamoto et al. (2013) and Ball et al. (2007) (gel electrophoresis). For the gel test, the c-*myc* gene was PCR-amplified in 12.5 μL reactions of 1 μL template DNA, 1× PCR Buffer for KOD FX, 400 μM dNTPs, 0.25 U KOD FX (Toyobo, Osaka, Japan),

and 0.015 pM of both the forward and reverse real-time PCR primers, using the following conditions: initial denaturation step of 94 °C for 2 min, 45 cycles of 98 °C for 10 s, 58 °C for 30 s, and 68 °C for 30 s. The resulting amplicons were electrophoresed on 2% agarose-TAE gels, stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific), and visualized using UV transilluminators to determine the intensity of the target band. To estimate the amount of buccal and intestinal DNA, a series of human placental DNA (Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 500, 300, and 100 pg/μL were used as reference controls. When the luminous intensity of a PCR product was > 300 pg/μL of the control, the sample was considered to have sufficient yield and was used in the next step for microsatellite amplification. We used human placental DNA as a reference as following Kawamoto et al. (2013), that was different from the *Macaca leonina*'s DNA used in the real-time PCR. This was because of the difference of availability of the DNA standard in Japan and Thailand, and the difference of the species was considered not to affect the substantial results (Smith et al. 2002). The accuracy of the real-time PCR analysis and gel test screening were compared using the Wilcoxon rank sum test with continuity correction.

DNA quality analysis

To determine DNA quality, the 30 paired buccal and intestinal DNA samples that passed the gel test were randomly selected for microsatellite genotyping. Ten microsatellite loci (D3S1768, D6S2793, D7S2004, D8S1106, D11S2002, D13S765, D14S306, D17S1290, D18S537, and D19S582; Aarnink et al. 2011, Barelli et al. 2013, Kawamoto et al. 2008, Nair et al. 2000, Smith et al. 2000) were amplified using a modified version of the two-step multiplex method described by Arandjelovic et al. (2009). During the first step of PCR, all microsatellite loci were amplified in a single 20 μL reaction that included 1 μL template DNA. During the second step, the 10 loci were divided into three subsets and were amplified in 12.5 μL multiplex PCR reactions that each included 1 μL of non-diluted amplicon from the first multiplex PCR reaction. The PCR thermocycling conditions were the same as those from the gel test, except that 35 cycles were used for the first PCR, and 45 for the second PCR. Allelic dropout rates and false allele rates were calculated using PEDANT Ver.1 (Johnson and Haydon 2007, available from http://sites.google.com/site/pcdjohnson/home/pedant). In the program, the results of two independent PCR products per sample per locus were used to estimate the allelic dropout and false allele rates. The allelic dropout and false allele rates of the buccal and fecal sample DNA were compared using the Wilcoxon signed-rank test ($p < 0.05$) in R. In addition, the genotype failure rate (a phenomenon in which the peak of an allele is

detected at extremely low levels or is not detected) of each locus was calculated based on the duplicated PCR results, and the genotype failure rates of the buccal and fecal DNA samples were compared using the Wilcoxon signed-rank test ($p < 0.05$) in R.

Results

DNA quantity

Analysis of the 82 DNA samples (41 buccal and 41 intestinal DNA samples) revealed that the buccal samples yielded significantly more host DNA (27.1 ± 33.8 ng/μL) than did the fecal samples (11.4 ± 15.4 ng/μL; $W = 473$, $P < 0.001$). Although 68% (28/41) of intestinal samples yielded concentrations less than 10 ng/μL, only 29% of buccal samples produced such low concentrations (12/41) (Figure 3).

The determination by the gel test was possibly made the presence/absence of the band (Figure 5, as an example of gel-test judgment). Of the 41 fecal and buccal DNA samples tested, 22 (53.7%) and 35 (85.4%) met the criterion for sufficient yield (≥ 300 pg/μl), respectively. The concentration of host DNA that passed and failed the gel tests as measured by real-time PCR was a significant difference ($W = 991$, $p < 0.01$), indicating that either real-time PCR or the gel test can be used for DNA quantification.

DNA quality

For the 30 monkeys whose samples passed the gel test, the allelic dropout rate of the 10 microsatellite loci was significantly lower for the buccal (0.00%, range: 0.00 – 6×10^{-6} %) than for the fecal DNA samples (18.12 ± 16.12%, range: 0.00–55.96%; Wilcoxon signed-rank test, $V = 44$, $p < 0.01$; Table 1). Estimated dropout rates were used to calculate the amount of repetition necessary for accurate results at the 99.99% certainty level (Morin et al. 2001). At least 6 repetitions were needed for fecal sample analysis to produce reliable genotype data, whereas one repetition was sufficient for buccal samples.

Similarly, the genotyping failure rate was significantly lower for buccal DNA samples (2.70% ± 3.88, range: 0.0–13.3 %) than for fecal DNA samples (35.67% ± 15.35, range: 18.3–65.0%; Wilcoxon signed-rank test, $V = 55$, $p < 0.01$), although the rate was variable among the loci examined (Table 1).

Discussion

Advantages from sampling point of view

When fecal sample are used as genetic resources, the success depends largely on the skill of the collector, the state of the feces, and other factors. Using the rope swab

method in our study, the collection of high-quantity and quality DNA samples would be possible without much training, providing a more versatile option that is not dependent heavily on the level of experience of the sample collector. Our rope swab method may also be useful for collecting samples from infants. Indeed, our method was capable of collecting samples from infants aged 2–3 weeks, even though the feces of infants were often soft, diarrhea-like or very small and often difficult or almost impossible to collect. Thus, we strongly believe that our method would be a powerful alternative to overcome the difficulty of collecting fecal samples from infants which are indispensable for genetic analysis such as paternity test. The rope swab method is also less time consuming than fecal collection. Since the quality of DNA samples cannot be checked in-situ study, multiple fecal samples must be collected to ensure collection of an adequate sample from the target animals. On the other hand, most of buccal samples provided usable DNA, and thus, fewer specimens need to be collected from each animal. Additionally, to collect fecal samples, researchers must patiently follow the targeted animals until they defecate, which is time-consuming. Therefore, the rope swab method presented in this study has great potential to save time and mitigate these factors.

Advantages from analysis point of view

Our study showed that the rope swab method is more effective, in terms of both quantity and quality of recovered DNA, compared to extraction from fecal samples. The rope swab method yielded up to 2.4 times more host DNA than did fecal samples and exhibited much lower allelic dropout and genotype failure rates, indicating that our method possibly facilitates genotyping analyses with fewer repetitive PCR trials, which could save time, labor, and money. This is because low DNA quantity increases genotyping errors that affect the reliability of genotyping in microsatellite analysis (Taberlet et al. 1999), and thus repeating experiments for each locus and extract is recommended (Goossens et al. 1998).

Important notice using rope swab method

Although our method would be useful, there are several cautionary notes while collecting samples. Firstly, in the initial phase, monkeys may not chew on the rope swabs. In this case, a habituation period using fruit juice instead of sugar water to increase the attractiveness of the swab rope is recommended. From experience, however, it seems better to switch to sugar water during the sample-collection phase. Genotyping results were not stable when using DNA samples collected with orange juice, probably due to the acid or other chemical compounds present in the fruit juice.

Secondly, the collection of samples shortly after monkeys have consumed food should be avoided, especially at provisioned sites or when targeting captive animals, as fruits are the main food items given and contain acids or other chemical compounds that may inhibit PCR. Complex polysaccharides possibly originating from vegetable material in the diet are also considered potential PCR inhibitors (Monteiro et al. 1997). Thus, time of sampling may affect the quality of the sample rather than the duration for which the monkey chews the rope.

Thirdly, adjustments to the soaking time of the rope swab in the sugar-water solution and the concentration of sugar according to the condition of the subject animals or study site may be needed. Extended soaking times or high sugar concentrations could encourage monkeys to chew the rope swabs for longer periods, which may lead to greater DNA yields. However, the potential downside of a longer chewing period is that the target monkeys may move while chewing, making retrieval of the rope swabs more difficult for the researcher. Although some individuals spent significant time chewing the swabs and occasionally broke them into small fragments, no monkeys accidentally ate the rope swabs during this study period, demonstrating their safety in application.

Fourth, the rope swabs should be well-distributed among the troop, otherwise higher-ranking males will take multiple ropes at once. When samples from subordinate

individuals are needed, spreading the rope swabs over a wide area to attract high-ranking individuals, and then casting some swabs to the target individual may be an effective strategy.

Lastly, because this method requires that the rope swabs be provided to the animals, it may not be suitable for use with non-habituated, wild animals. This method also cannot be used in research sites where access to wildlife or provisioning is prohibited. Since this method involves material once contained in the mouths of animals, researchers must be aware of the possibility of touching saliva to prevent zoonosis (e.g., Kelesidis and Tsiodras 2010). When conducting behavioral observation at the same time, the possibility of influencing the behavior of the target animals must also be considered. Ultimately, the applicability of this method will depend on the specific needs and conditions of the research.

Furthermore, we must note about the standard range of quantitative real-time PCR. In this study, the standard range of quantitative real-time PCR could not cover the sample concentration range due to the fact that the quantity of DNA was extracted at a higher concentration than our assumption. We followed the protocol of Wizard SV Gel and PCR Clean-Up System and used 50 μL of water for the final elution step, though 200 μL is used in Morin et al. (2001). This difference of the final elution volume should

have resulted in the higher concentration of DNA both from buccal and fecal samples in our study.

Future possibility of application

The successful DNA collection and genotyping of *M. arctoides* using our method can be further applied to different conditions, for example, for populations kept in captive conditions at research institutions or individuals kept in cages in laboratories but also for provisioned or well-habituated free-ranging primates such as populations living near temples which are widely seen in most Southeast Asian countries, as long as researchers pay attention to risks and take precautions. This is a very useful method for researchers who have to obtain samples from specific individuals in a limited research period in the wild. Furthermore, with some modifications, this method can be applied for hormone and veterinary analysis (e.g., detecting a specific virus in the saliva; Musso et al. 2015, Huff et al. 2003). The non-invasive buccal cell collection method described by this study may further facilitate animal population genomic studies in both captive and field environments. Further integration of genetic information with behavioral and ecological data is expected to provide more insights into *M. arctoides*, including genetic structure and socioecological characteristics such as reproductive strategy and kinship structure.

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Table 1. Allelic dropout and genotype failure rates of 10 microsatellite loci for fecal and buccal DNA samples of stump-tailed macaques in Khao Krapuk Khao Taomor.

Loci	Allelic dropout rate (%)		Genotype failure rate (%)	
	Fecal	Buccal	Fecal	Buccal
D3S1768	10.54	0.00	38.33	0.00
D6S2793	25.00	0.00	58.33	2.00
D7S2204	8.80	0.00	31.67	13.33
D8S1106	13.04	0.00	45.00	0.00
D11S2002	0.00	0.00	65.00	1.67
D13S765	29.20	0.00	23.33	0.00
D14S306	0.00	0.00	18.33	1.67
D17S1290	28.45	0.00	33.33	5.00
D18S537	55.97	0.00	25.00	3.33
D19S582	10.21	0.00	18.33	0.00

Figure

Fig 1 Rope swabs cut into 10cm length and 3ml of lysis buffer in 5ml tube

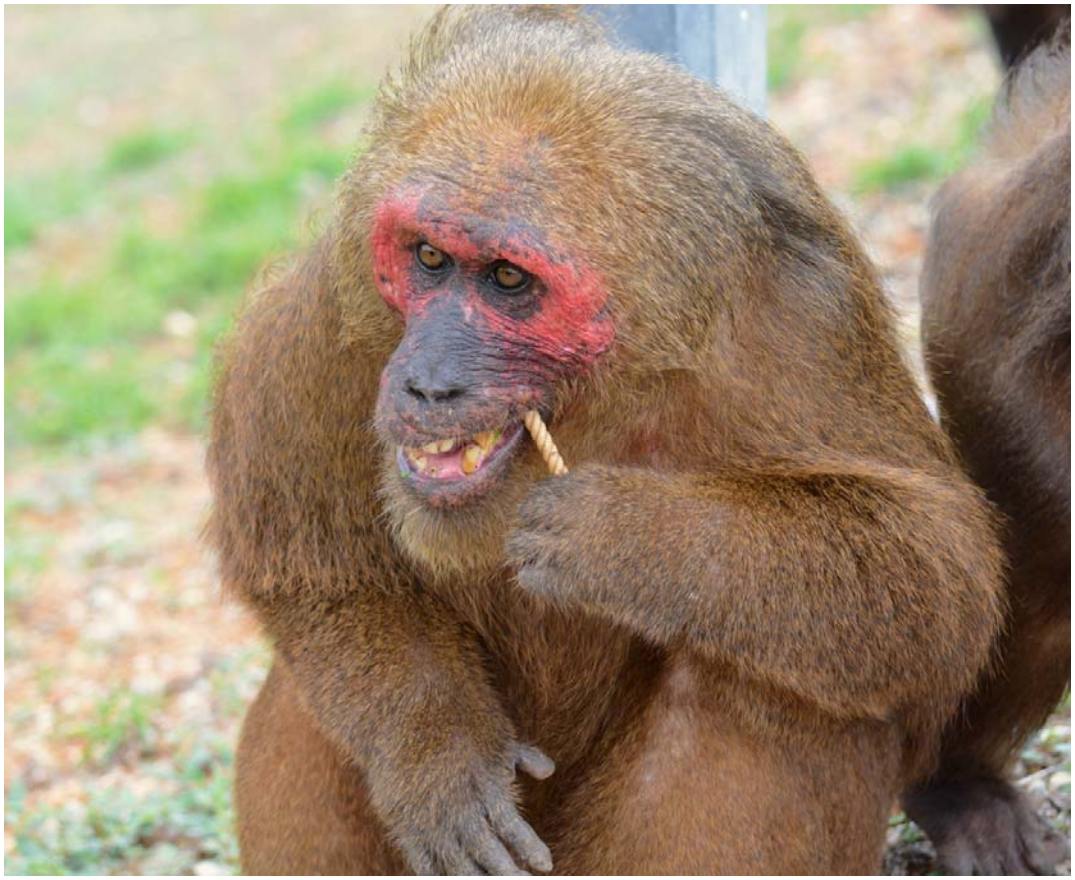

Fig 2 Monkey chewing a rope swab

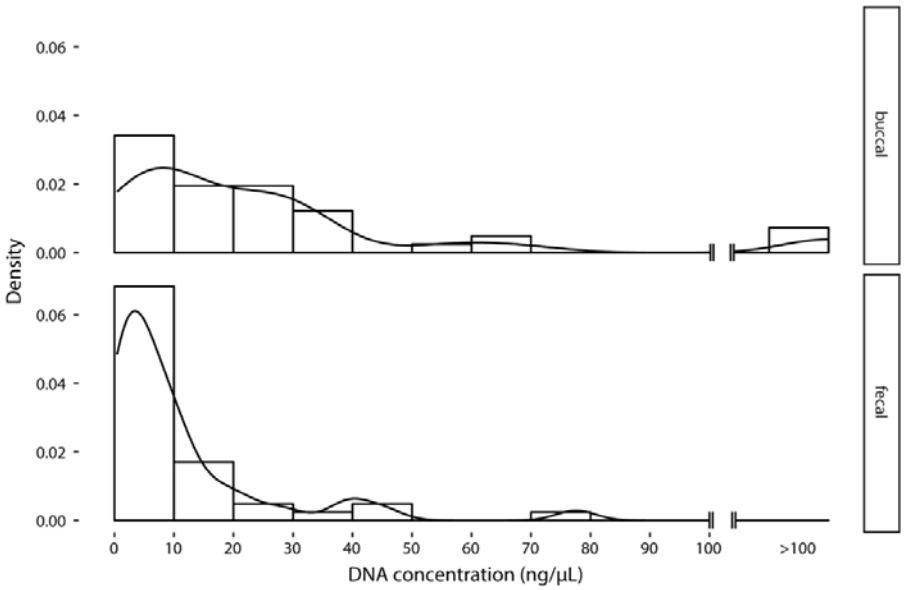

Fig 3 Frequency of buccal and fecal DNA in each DNA concentration zone. Although many fecal samples are dense in the low concentration zone, meaning that the sampling efficiency is not good, buccal samples shows a gentle peak overall, indicating that samples with high concentration can be more easily obtained.