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Applicability of Non-Invasive Sampling in Population Genetic Study of Taiwanese Macaques (*Macaca cyclopis*)

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ABSTRACT: This paper presents a pilot study conducted to test the applicability of non-invasive sampling approach in population genetic studies of Taiwanese macaques (*Macaca cyclopis*). Monkey feces were collected in the field and used as non-invasive DNA sources. PCR success rates of both microsatellite and mitochondrial DNA markers were examined. When compared with other studies by non-invasive genetic sampling of different mammal species, success rate of microsatellite PCR amplification is low (42.4%, N = 181) while that of mtDNA PCR amplification is acceptable (66.5%, N = 334). The low PCR success rate and poor PCR repeatability of microsatellite alleles due to allelic dropout and false alleles make it difficult to obtain a reliable microsatellite data set. However, the difficulties may be overcome by new techniques.

KEY WORDS: ***Macaca cyclopis*, Non-invasive sampling, Microsatellite, mtDNA d-loop.**

INTRODUCTION

In conventional population genetics sampling, animals have to be captured or sacrificed. Most trapping and handling procedures are stressful to subject animals while destructive sampling is not preferred or allowed in some circumstances. Alternative approaches are therefore encouraged in wildlife studies (Woodruff, 1993). During the last decade, it has been demonstrated possible to amplify DNA from non-invasive samples such as feces, urine, wadges, ejaculates, menstrual blood or shed hairs (e.g. Hashimoto et al., 1996; Goossens, et al., 1998; Chu et al., 1999; Hayakawa and Takenaka, 1999; Constable et al., 2001; Domingo-Roura et al., 2004; Hung et al., 2004). The approach is desired to exploit the full potential of DNA analysis and to provide the same information as DNA extracted from blood or tissue samples. Application of the approach is valid in PCR amplification of mitochondrial DNA (mtDNA) for species identification (Hansen and Jacobsen, 1999; Valiere and Taberlet, 2000; Murakami, 2002) and intraspecific phylogeographic studies (e.g. Morin et al., 1993; Taberlet and Bouvet, 1994). However, it is restricted and problematic when amplifying nuclear genes for gender (Taberlet et al., 1993; Murphy et al., 2003; Hedmark et al., 2004), paternity and individual identification (Bradley et al., 2001).

DNA extracted from non-invasive samples, in particular feces, is usually of poor quality (i.e. degraded DNA, the presence of PCR inhibitors and DNA contamination) and trace quantity (often in picogram range). The trace amount 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; Taberlet et al., 1999; Smith et al., 2000). Such genotyping errors are especially problematic for genetic census studies even if the error rate is low since a single error in a multi-locus genotype creates a false individual designation (Taberlet et al., 1996; Taberlet et al., 1999; Creel et al., 2003).

Some authors recommended a 'multiple-tubes PCR procedure' (Navidi et al., 1992; Taberlet et al., 1996) to improve the reliability of microsatellite genotyping. However, the procedure increases total cost of laboratory work 5 to 10 times higher than when using blood or tissue samples (Taberlet et al., 1999). Therefore, constraints involved in capturing animals 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).

This study is a pilot experiment to examine the applicability of non-invasive sampling in population

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genetic studies of Taiwanese macaques (*Macaca cyclopis*). Fecal samples were collected as DNA sources for PCR amplification. Success rate of PCR amplification, repeatability of genotyping and validity of producing reliable genotype profiles from fecal DNA extracts were examined. The updated technical improvements in non-invasive genetic sampling are also discussed.

MATERIALS AND METHODS

In this study, success rates of PCR amplification of microsatellite and mitochondrial genes from fecal DNA were examined. For each fecal sample, the surface was smeared with a sterilized swab several times and was then immersed in 1 mL 1 X STE buffer (10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl pH = 8.0) pluses 1 mL pure ethanol in a 2 mL sample vial (Chu et. al., 2005). The fecal samples used for the microsatellite PCR amplification (total N = 181) were collected from Yushan (N = 116), Ershui (N = 32) and Lichia (N = 33). For the mtDNA PCR amplification, 334 fecal samples collected from 31 localities in Taiwan were used. Sampling localities are indicated in Chu (2004). Success rates of PCR amplification of microsatellites from blood and hair DNA were evaluated as reference. Blood samples (total N = 63) were collected from monkeys at the Eastern Wildlife Rescue Center in I-Lan (N = 43) and Taipei Zoo (N = 20). Hair samples (total N = 139) were collected from wild animals in Nanhua (N = 13) and Shoushan (N = 126).

Total genomic DNA was extracted from blood, hair and fecal samples by the phenol-chloroform procedure in Kocher et. al. (1989) with slight modification. DNA extracts derived from feces were further eluted by a silica pellet method (Geneclean III, Bio101). Microsatellite PCR mixture contained 10 pmole of the primer pair, 50-400 μM dNTP, 2-4 mM $MgCl_2$, 1 X PCR reaction buffer (20 mM Tris-HCl, pH 8.4, 20 mM KCl), 0.25 μL bovine serum albumin (BSA), 0.1 unit *Taq* polymerase and 1-5 μL DNA extract in a total volume of 25 μL. DNA thermal cycler (Biometra) was programmed to perform an initial denaturation at 95ºC for 5 min, 35 cycles of 20 s at 95ºC, 20 s at annealing temperature (Table 1), and 20 s at 72ºC. A 5 min final extension at 72ºC was also included. Negative and positive PCR controls were included in each set of amplification to check for possible contamination and for allele sizing.

Blood and hair DNA extracts were amplified at 9 microsatellite loci (Table 1). Two loci (D1S548 and D5S1470) were excluded in fecal DNA PCR because higher DNA quantity, quality, and a more stringent PCR condition were required during amplification. Another locus, D5S820, produced only one band (185 bp) in most fecal DNA extracts that was unexpected from previous knowledge (Chu et al., 1999). Therefore, we suspected non-specific amplification at locus D5S820 and dropped the locus for analysis. Thus, only 6 loci (D1S533, D1S550, D3S1768, D6S501, D12S67, D17S804) were amplified from fecal DNA extracts.

The applicability of fecal DNA extracts in population genetic studies of Taiwanese macaques was assessed by: (1) the success rates of the first PCR attempt at the 6 microsatellites; (2) the repeatability of microsatellite banding pattern for each sample; and (3) the reliability of genotyping.

Firstly, 181 fecal DNA extracts were amplified at the 6 microsatellite loci. 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. Secondly, 45 fecal DNA extracts from Yushan successfully amplified at 4 or more loci in the previous procedure were selected to determine the repeatability of banding pattern. Three separate PCR trials per locus were conducted for each sample. PCR products were visualized on 8% polyacrylamide gels with silver staining (Tegelstrom, 1986). Banding pattern of a locus was "repeatable" when it was the same in at least two of the three PCRs. The repeatability ratio was the proportion of samples with consistent banding patterns at each locus or over all loci. Finally, genotype was determined from 37 DNA extracts (22 from Yushan and 15 from Lichia) with successful PCR amplification at 4 or more loci. Considering the extensive labor and laboratory costs, only 4 loci with stable PCR performance were amplified (i.e. D1S533, D3S1768, D6S501 and D17S804). Three independent trials of multiple-tubes PCR (Navidi et al., 1992; Taberlet et al., 1996) with two replicates were conducted per locus. For each DNA extract, there were at most 6 positive PCRs (P+s) per locus. Genotype was determined by the following criteria. It was assigned as homozygous when an allele was observed at least 3 times with no other alleles observed more than once, and as heterozygous when two different alleles were observed at least twice. The proportions of allelic dropout and false alleles were also calculated based on the total results of P+s per locus.

Table 1. Characteristics of the microsatellites used to assess the applicability of the non-invasive genetic sampling approach in *Macaca cyclopis*.

Locus	Repeat motif	T_a[1] (°C)	Primer sequence 5'→3'
D1S533	tetra	40	CATCCCCCCCAAAAAATATA
			TTGCTAATCAAATAACAATGGG
D1S548	tetra	40	GAACTCATTGGCAAAAGGAA
			GCCTCTTTGTTGCAGTGATT
D1S550	tetra	40	CCTGTTGCCACCTACAAAAG
			TAAGTTAGTTCAAATTCATCAGTGC
D3S1768	tetra	43	GGTTGCTGCCAAAGATTAGA
			CACTGTGATTTGCTGTTGGA
D5S1470	tetra	57	CATGCACAGTGTGTTTACTGG
			TAGGATTTTACTATATTCCCCAGG
D5S820	tetra	57	ATTGCATGGCAACTCTTCTC
			GTTCTTCAGGGAAACAGAACC
D6S501	tetra	40	GCTGGAAACTGATAAGGGCT
			GCCACCCTGGCTAAGTTACT
D12S67	tetra	51	GCAACAGTTTATGCTAAAGC
			GCCTATGCAGTTCAAATCTA
D17S804	di	57	GCCTGTGCTGCTGATAACC
			CACTGTGATGAGATGTCATTCC

[1] T_a: annealing temperature of PCR

The mtDNA control region (CR) was amplified by the following primer set: DL1 (forward): 5'-CCAGAAATGAACACCCTTCCTAGGGC-3' (Chu et al., 2005), and Saru5 (reverse): 5'-GCCA GGACCAAGCCTATTT-3' (Hayasaka et al., 1991). Length of the PCR product is about 1.4 Kb. PCR mixture contained 10 pmole of both primers, 200 μM dNTP, 1.5 mM $MgCl_2$, 1 X PCR reaction buffer (20 mM Tris-HCl, pH = 8.4, 20 mM KCl), 1 unit *Taq* polymerase and 1-3 μL of DNA extract in a total volume of 50 μL. A DNA thermal cycler (Biometra) was programmed to perform an initial denaturation at 95°C for 10 min, 32 cycles of 1 min at 95°C, 1 min at 58°C, and 2 min at 72°C. A 10-min final extension at 72°C was also included. PCR products were visualized on 1% agarose gels stained with EtBr.

RESULTS AND DISCUSSION

PCR success rate of the non-invasive fecal samples from wild monkeys was lower than that of conventional samples, especially for microsatellites (Table 2). For the blood and hair DNA extracts, 99.1% and 93.7% microsatellite PCRs produced positive and moderate to high-quality products in the first PCR attempt, respectively. However, the overall success rate of microsatellite PCR amplification was only 42.4% for fecal DNA extracts. In total, 115 alleles were found from 9 microsatellite loci in *M. cyclopis*. Allelic size ranges were mostly similar between different DNA sources (blood, hair and fecal samples) at each locus (Table 3). It suggests that microsatellite genotyping from non-invasive genetic sampling can be reliable if under cautious check. As for mtDNA, amplification of the 1400bp fragment was successful in 66.5% of 334 fecal DNAs (Table 2). All the successfully amplified fragments can be sequenced to a range of ca. 350-800bp for further phylogeographic analysis in our recent studies (Chu et al., unpublished data). It indicates that quality of mtDNA PCR product amplified from fecal DNA is comparable to that amplified from blood or tissue DNA.

In this study, success rate of microsatellite PCR amplification for fecal DNA (42.4%) was lower than those reported in other species (36.5-82%, Table 4). However, the success rate of mtDNA PCR (66.5%) is comparable to the reported range (56-100%, Table 4), even though the fragment amplified here was relatively longer (1400bp) than others. The higher success rate of mtDNA PCR amplification excludes PCR inhibitors as the explanation for microsatellite PCR failure. In addition, concentration of PCR inhibitors should have been reduced during the purification process because all the fecal DNA extracts have been purified before PCR.

A possible reason for the low success rate is the small amount of fecal material collected for DNA extraction (approximately 100 mg per sample). Our collection procedure can save time and reduce packing load for field practice. It turned out to be adequate for mtDNA PCR-based studies. However, the samples collected this way provided an extremely low amount of degraded DNA for microsatellite PCR amplification, which may greatly increased the problem of allelic dropout (51.3% in this study) and introduced erroneous information (Taberlet et al., 1996).

Table 2. Summary of overall PCR success rates of microsatellites and mitochondrial DNA d-loop amplified from DNA extracted from different sample sources of *Macaca cyclopis*.

Marker	Sample type	N	Number of loci used	Overall PCR success rate
Microsatellites	Blood	63	9	99.1%
	Hair	139	9	93.7%
	Feces	181	6	42.4%
Mitochondrial DNA d-loop	Feces	334		66.5%

Table 3. Allelic size range of each microsatellite locus obtained from different DNA sources of *Macaca cyclopis*.

Locus[1]		Allelic size range		
		Blood (ER+Zoo)[2]	Hair (Nanhua+Shoushan)	Feces (Yushan+Lichia)
D1S533	(18)	188-236	196-240	172-244
D1S548	(4)	192-208	192-204	N.A.
D1S550	(5)	148-164	152-164	N.A.
D3S1768	(13)	187-223	167-223	187-223
D5S1470	(15)	161-221	161-229	N.A.
D5S820	(14)	185-221	181-233	N.A.
D6S501	(10)	161-193	161-193	157-193
D12S67	(24)	112-293	116-305	N.A.
D17S804	(12)	156-170	154-178	156-164

[1] Number of alleles found at each microsatellite locus is indicated in parentheses
[2] ER, Eastern Wildlife Rescue Center; Zoo, Taipei Zoo
N.A., data not available

Table 4. Success rate of microsatellite and mtDNA PCR amplification from fecal DNA extracts of mammal species in the literature and in *Macaca cyclopis* (bold-typed).

Species[1]	Microsatellite success rate	Number of loci	MtDNA success rate	MtDNA length (bp)
Brown bear[2,3]	36.5-84%	1	56-87%	~146
	80% (captive)	1	65-90% (captive)	398-700
	67% (wild)	1	60-80% (wild)	398-700
Eurasian otter[4]	65%	7	91%	232
Taiwanese macaque	**42.4%**	**6**	**66.5%**	**~1400**
Red wolves and coyotes[5]			75%	200
Baboon[6]			92-100%	100-700
Bobcat and cougar[7]	>75%	12		
Barbary macaque[8]	70%	3		
Bonobo[9]	67%	5		
Chimpanzee[10]	82%	14		
Gorilla[10]	56%	14		
Orangutan[11]	65%	1		
Savannah baboon[12]	70%	8		

[1] Scientific names for the mammal species in the table are Brown bear, *Ursus arctos*; Eurasian otter, *Lutra lutra*; Taiwanese macaque, *M. cyclopis*; Red wolf, *Canis rufus*; Coyote, *C. latrans*; Baboon, *Papio cynocephalus ursinus*; Bob cat, *Lynx rufus*; Cougar, *Puma concolor*; Barbary macaque, *M. sylvanus*; Bonobo, *Pan paniscus*; Chimpanzee, *P. troglodytres verus*; Gorilla, *Gorilla gorilla gorilla* and *G. gorilla beringei*; Orangutan, *Pango pygmaeus abelii*; Savannah baboon, *P. papio*
[2] Murphy et al., 2002; [3] Wasser et al., 1997; [4] Hung, 2004; [5] Adams et al., 2003; [6] Frantzen et al., 1998; [7] Ernest et al., 2000; [8] Lathuilliere et al., 2001; [9] Gerloff et al., 1995; [10] Bradley et al., 2000; [11] Goossens et al., 2000; [12] Bayes et al., 2000

Therefore, performance of fecal DNA extracts was not very satisfactory in our study. Results of microsatellite PCR and genotyping performance are shown in Table 5. PCR success rates of the 6 microsatellites varied in a range of 34.3%-49.7%. The repeatability was the highest at D12S67 (64.4%) and the lowest at D1S550 (4.4%). Allelic dropout was detected 101 times among the 197 P+s (51.3%). The presence of dropout also appeared to vary among the 4 loci, with the highest dropout rate at D3S1768 (63.6%) and the lowest at D1S533 (13.3%). False alleles were observed 25 times among the 197 P+s (12.7%). The appearance of false alleles was also the highest at D3S1768 (21.0%) and the lowest at D1S533 (no false allele). Among the 37 fecal DNA extracts selected from the Yushan and Lichia populations, the consensus genotypes were determined in 11-27 samples at the 4 loci.

Table 5. PCR success rates, PCR repeatability, proportions of allelic dropout and false allele, and numbers of available consensus genotypes of fecal DNAs at 6 microsatellite loci in *Macaca cyclopis*.

Locus	PCR success rate N = 181	PCR repeatability N = 45	Allelic dropout[1]	False allele[1]	Number of consensus genotypes
D1S533	34.3%	26.7%	13.3% (4/30)	None	16/37
D1S550	46.4%	4.4%	N.A.	N.A.	N.A.
D3S1768	44.2%	53.3%	63.8% (67/105)	21.0% (22/105)	25/37
D6S501	34.8%	22.2%	41.7% (10/24)	8.3% (2/24)	11/37
D12S67	45.3%	64.4%	N.A.	N.A.	N.A.
D17S804	49.7%	26.7%	52.6% (20/38)	2.6% (1/38)	27/37
Overall	42.4%	33.0%	51.3% (101/197)	12.7% (25/197)	

[1] Ratios of number of allelic dropout and false alleles to total number of positive PCR in each locus are listed in parentheses
N.A., data not available

In addition to the low amount of DNA in fecal samples, success of PCR amplification and accuracy of genotyping 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; Murphy et al., 2002; Nsubuga et al., 2004) and freshness of fecal samples (Murphy et al., 2002; 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 (e.g. salmon) lead to a significant decrease in PCR success rate of nuclear gene. Their studies highlight a new dimension of variation in success rate of PCR amplification between species and even between individuals. The variation hinders the preparation of a reliable genetic data set from a feces-based non-invasive sampling approach.

Until now, no conclusive evidence has been reported about whether the desiccation or the buffer/ethanol based preservation methods are better for fecal sample storage. Recently, Nsubuga et al. (2004) suggested a 'two-step storage protocol' in which the fecal samples were first soaked in ethanol and then desiccated by silica. The method requires extra sample manipulation in the field. However, the substantially higher success rate of PCR amplification (increasing from 51.6% to 95.2%) reduces the total number of fecal samples required for genetic studies, and thus reduces laboratory expenses (Nsubuga et al., 2004). Other studies indicated that the success rate of PCR amplification tended to be higher for fresh fecal samples collected under cold environment (Flagstad et al., 2004) or preserved for a shorter length of time (Nsubuga et al., 2004). These studies provide valuable guidelines to improve success rate of PCR amplification for future non-invasive genetic studies of Taiwanese macaques.

In this pilot study, the trace amount of amplifiable DNA in each fecal sample not only contributes to the low success rate of microsatellite PCR amplification (42.4%), but also makes PCR repeatability unstable (33.0%) (Table 5). In other words, if non-invasive genetic sampling method is applied to Taiwanese macaques, only 14% of the fecal samples can provide materials for genotyping. Moreover, the result of genotyping may still be inaccurate unless the exhausted 'multiple-tubes PCR procedure' (Navidi et al., 1992; Taberlet et al., 1996) is conducted. Taberlet et al. (1996) suggested a genotype to be heterozygous or homozygous when at least 2 repeats of each allele were observed in 3 independent P+s or an allele was observed in 7 independent P+s in their multiple-tubes PCR procedure, respectively. In this study, we followed their criterion of heterozygote, but adopted a more relaxed criterion of homozygote (observed 3 instead of 7 independent repeats). The relaxed criterion may increase the possibility of identifying a false homozygote up to 13.5% (0.513^3).

To improve the reliability of genotyping, the ultimate solution is to detect the amount of amplifiable DNA in fecal DNA extracts by the quantitative PCR analysis (Morin et al., 2001). The analysis can distinguish between the 'target DNA' and the co-extracted bacterial/plant DNA that is likely to occur in large quantity in fecal DNA extracts. Hence, samples with enough amplifiable DNA (> 100 picogram) for reliable genotyping (Morin et al., 2001) can be selected for further analysis. Up to now, few studies employed the approach because real-time PCR machine is not yet a standard equipment in most molecular ecology laboratories. Piggott et al. (2004) suggested a 'multiplex pre-amplification method' that performed an initial large-volume (50 μL) PCR containing primers for the entire panel of loci to be genotyped. The PCR product can later be used as template in

separate PCRs at each locus. This method can improve the performance of microsatellite PCR, reduce error rate and allow more loci to be analyzed than a regular single-locus PCR approach (Piggott et al., 2004).

In conclusion, in this pilot study on the applicability of non-invasive genetic sampling method in Taiwanese macaques, a low success rate of microsatellite (42.4%) and a moderate success rate of mtDNA (66.5%) PCR amplification was observed. The relatively higher success rate of mtDNA PCR indicates that the non-invasive genetic sampling method can be used in mtDNA-based studies when capturing macaques is not suitable for practical concern. The relatively low success rate of microsatellite PCR undermines a reliable genetic data set. However, many updated technical improvements in fecal sample preservation and PCR procedure may greatly increase the success rate in the application of non-invasive sampling method.

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非侵略性採樣法在臺灣獼猴族群遺傳研究的應用性

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摘　　要

本文為檢驗非侵略性採樣法在臺灣獼猴族群遺傳研究應用性的前導實驗。本研究採集獼猴排遺樣本作為非侵略性之 DNA 樣本來源，並利用微隨體及粒線體 DNA 兩種分子標幟檢驗其 PCR 成功率。在與其他哺乳動物非侵略性遺傳研究的比較後發現，臺灣獼猴的微隨體 PCR 成功率較低（42.4%, N = 181），而粒線體 DNA 的 PCR 成功率則尚可接受（66.5%，N = 334）。微隨體對偶基因的低 PCR 成功率及低可重複性（即高對偶基因遺失率及高假性對偶基因出現率），使得可靠的微隨體資料庫難以建立。然而，日新月異的實驗技術也許能克服以上的實驗困境。

關鍵詞：臺灣獼猴、非侵略性採樣法、微隨體、粒線體 DNA d-loop。

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