Taiwania 62(4): 363-370, 2017
DOI: 10.6165/tai.2017.62.363

Evaluation on the effects of ageing factor, sampling and preservation methods on Asiatic black bear (*Ursus thibetanus*) noninvasive DNA amplification

Chih-Chin SHIH[1,2], Sung-Lin WU[2], Mei-Hsiu HWANG[3] and Ling-Ling LEE[1,*]

1. Institute of Ecology and Evolutionary Biology, National Taiwan University, Taipei, 106, Taiwan ROC.
2. Taipei Zoo, Taipei, 116, Taiwan ROC;
3. Institute of Wildlife Conservation, College of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, 912, Taiwan ROC.
[*] *Corresponding author's phone: +886-2-3366-2471; fax: +886-2-2363-4606; e-mail: leell@ntu.edu.tw*

(Manuscript received 12 June 2017; accepted 31 October 2017; online published 9 November 2017)

ABSTRACT: Noninvasive genetic sampling allows studying wildlife without having to catch, handle or even observe individuals. In this study, factors which may affect the quality of noninvasive samples of Asiatic black bear (*Ursus thibetanus*) in the subtropical areas were identified. We collected hair and faecal samples from captive Asiatic black bears and quantitatively evaluated the effects of hair age (from fresh to 60 days), faeces age (from fresh to 14 days), faeces sampling locations (i.e. sample collected from either the surface, inside or a mixture of both the surface and inside of faeces), and faeces preservation methods (frozen or kept at room temperature in 95% ethanol) on amplification success rates of mitochondrial DNA fragments of different sizes (450bp, 900bp, and 1600bp). The results showed that the amplification success rates decreased with sample age and amplicon size in both hair and faecal DNA. In subtropical environment, there was no significant difference between amplification success of DNA extracted from fresh and 7-day-old samples of either the hair or faeces. The amplification success rates were not influenced by sampling location of faeces. For faeces preserved in 95% ethanol, the amplification success appeared unaffected by frozen at -20 °C or kept at room temperature in shorter mtDNA fragments, but was significantly influenced when amplicon size was 1600bp. The results of this study will reinforce the optimization of noninvasive sampling approaches in Asiatic black bear research, especially in the subtropics.

KEY WORDS: DNA preservation, Faecal DNA, Noninvasive genetic sampling, Hair DNA, *Ursus thibetanus.*

INTRODUCTION

Noninvasive genetic sampling has been proven a powerful tool for investigating populations of wildlife, particularly those elusive, rare, and free-ranging species roaming in large areas (Roon *et al.* 2003; Broquet *et al.* 2007). Through a set of genetic procedures, noninvasive genetic sampling allows the study of the biology of wildlife without having to catch, handle, or even observe individuals (Piggott and Taylor 2003; Broquet *et al.* 2007). Researchers could integrate various noninvasive techniques in monitoring trends of wildlife populations, especially in large carnivores, for the purposes of management and conservation (Waits and Paetkau 2005; Schwartz *et al.* 2007; De Barba *et al.* 2010). Conservation biologists, for instance, have routinely used noninvasive genetic methods to monitor the long-term population trends of the brown bears in North America (Woods *et al.* 1999; Broquet *et al.* 2007).

Noninvasive DNA could be retrieved from various types of wildlife samples include hair, faece, urine, shed feather, buccal cells from food, snake skin, eggshells *et al.* (Sloane *et al.* 2000; Valiere and Taberlet 2000; Vigilant *et al.* 2001; Broquet *et al.* 2007; Beja-Pereira *et al.* 2009). Faeces and shed hair are more easily collected, and thus are often used as the noninvasive genetics materials (Broquet *et al.* 2007; Renan *et al.* 2012). Despite the many advantages, a major limitation of noninvasive faeces or hair samples is the low quantities of host DNA which is often highly degraded (Waits and Paetkau 2005) and often leads to low PCR amplification rates.

The quantity and quality of faecal and hair DNA can be affected by sample age (Murphy *et al.* 2007; Santini *et al.* 2007; Vynne *et al.* 2012), environmental conditions (e.g. humidity, temperature, exposure to the sun or rain) (Murphy *et al.* 2007; Michalski *et al.* 2011), or technical factors, including sampling location, i.e. whether sample were collected from the surface or inside of faeces (Piggott and Taylor 2003; Stenglein *et al.* 2010) and storage method (Santini *et al.* 2007; Panasci *et al.* 2011). DNA extraction protocol and amplicon size, the fragment length of amplified DNA makers can also affect the quantity and quality of faecal and hair DNA extracted, thus the success rate of amplification (Piggott *et al.* 2004; Buchan *et al.* 2005; Hoffman and Amos 2005; Broquet *et al.* 2007). Previous studies which had evaluated factors affecting the DNA quality and amplification success rates of faecal and hair samples suggest that success rates will be the highest when samples are fresh and dry or preserved in low temperature (Farrell *et al.* 2000;

Lucchini *et al.* 2002; Piggott 2004; DeMay *et al.* 2013).

However, DNA degradation rates can differ among taxa and even within species under different climatic or operational conditions (DeMay *et al.* 2013). The lack of a quantitative comparison of studies in various animal-environment systems makes it difficult to decide which protocol is the most suitable for a given system (Beja-Pereira *et al.* 2009; Renan *et al.* 2012). Those general trends are not necessarily transferable across species or study sites (DeMay *et al.* 2013) and may be of limited applicability to new studies. Therefore, pilot studies are still recommended for each system to determine DNA degradation rates and the appropriate noninvasive protocol (Taberlet *et al.* 1999; Renan *et al.* 2012; DeMay *et al.* 2013).

Noninvasive genetic sampling is often applied in Ursid research. Most studies evaluating the quality and DNA amplification success of noninvasive faeces or hair samples were conducted on brown bears (*Ursus arctos*) in temperate regions (Murphy *et al.* 2002; Murphy *et al.* 2007; Stenglein *et al.* 2010), but few were on bears in regions with different climatic conditions. DNA samples collected under high temperature and humidity in the tropics and the subtropics may be particularly susceptible to degradation (Wasser *et al.* 1997; Bayes *et al.* 2000; Vynne *et al.* 2012). Only a few studies comparing storage treatments or extraction methods have been conducted in tropical forests, and most of them were limited to primates, ungulates and canids (Nsubuga *et al.* 2004; Vallet *et al.* 2008; Soto-Calderon *et al.* 2009; Vynne *et al.* 2012). Comparative studies using DNA of faeces and hair in Ursid have not been performed and the effectiveness of methods for preserving samples has not been evaluated in the tropics or subtropics.

Our study focuses on the Formosan black bear (*Ursus thibetanus formosanus*), an endemic subspecies of Asiatic black bear inhabiting Taiwan, a subtropical island (Wozencraft 2005). Similar to all other Asiatic black bear subspecies, habitat degradation and fragmentation, as well as poaching, have caused a decrease in the population and distribution of the Formosan black bear (Hwang and Wang 2006; Hwang and Garshelis 2007; Hwang *et al.* 2010). To formulate proper conservation strategies, it is important to understand the genetic diversity and genetic structure of this subspecies (Shih *et al.* 2009). For efficient application of noninvasive genetic analysis, it is necessary to identify the variables which may affect the DNA quality and further DNA amplification success in this system.

The main objective of this study is to quantitatively evaluate the effect of multiple variables on amplification success rate of mitochondrial DNA (mtDNA) extracted from Asiatic black bear faeces and hair. When using faeces as noninvasive DNA sources, subsamples are often taken from species producing larger faeces instead of collecting the entire faeces in the field (Stenglein *et al.* 2010). Since few studies have experimentally tested samples taken from the different parts of faeces, we examined the impact of sampling locations, e.g. from the surface or inside of faeces. Soaking faeces in ethanol and silica desiccation are widely employed for faecal DNA preservation (Wasser *et al.* 1997; Frantzen *et al.* 1998; Santini *et al.* 2007). In a subtropical region like Taiwan, ethanol preservation should be more preferable than silica desiccation because high temperature and humidity may hinder the effect of desiccation of silica (Murphy *et al.* 2002). Although transportation of frozen samples from the field to the laboratory would be difficult in field research (Nsubuga *et al.* 2004), the effect of immediate freezing of ethanol-soaked samples in DNA preservation was also evaluated in this study.

In this study, we collected hair and faecal samples from captive Asiatic black bears in subtropical Taiwan to assess the effects of sample age (over a 60-day period for hair and a 14-day period for faeces) and faecal preservation methods (frozen or kept at room temperature in 95% ethanol) on amplification success rates of different mtDNA amplicon size. The results of this pilot study will allow us to make recommendations for optimal noninvasive sampling protocols and to provide sampling and storage guidelines for field researchers conducting noninvasive genetic sampling of Asiatic black bears in the subtropics.

MATERIALS AND METHODS

Experiment design, sample collection and preservation

The specimens for different treatments and evaluation were collected from 5 captive Asiatic black bears (2 males, 3 females) at Taipei Zoo and all treatments were done in the zoo as well. These bears were on a mainly vegetarian diet. Faecal samples were collected and treated with an average temperature of 26.4 °C and average relative humidity of 71% (climate data from the Central Weather Bureau, Taiwan). Hair samples were collected and tested in Taipei Zoo with an average temperature and relative humidity of 28.37 °C and 74%, respectively. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Taipei Zoo.

Fresh faeces were collected in less than 12 hours after being deposited by the 5 bears, then immediately transported to a semi-outdoor flat ground where they would not be directly exposed to rain and sunlight (to simulate the condition under canopy in the wild). For age and sampling location treatments, 1-ml of faecal samples were taken with wooden sticks from inside, surface and inside-surface mixture of faeces at 0 (which means fresh), 1, 3, 7, 14 days post collection from the

bear facilities. After each sampling, the remaining faeces were left undisturbed and subsequent samples were collected from undisturbed portions of the faeces. All faecal samples were soaked in 4-ml of 95% ethanol (Wasser *et al.* 1997; Murphy *et al.* 2002; Panasci *et al.* 2011), then frozen at -20 °C or kept at ambient room temperature in the laboratory for 2 weeks to serve as samples to test the effects of 2 different storage conditions. The sample size of each age, sampling location and storage method treatment was 10, with 2 from each of the 5 bears.

In the treatment of hair age, hair specimens with follicles were collected from captive bears while the animals were in narcosis for health check-ups. We designed 5 hair age treatments: fresh hair and hair of 7, 14, 30, and 60-day old, which were hair laying outdoors under partial tree shade for different amount of time after being collected from the bears to imitate hair collected from the hair-trap. Each treatment included 15 samples (10 hair follicles for each sample) which were also collected from different bears equally.

DNA extraction and PCR amplification

All faecal and hair samples of respective treatments were then preserved at -80 °C (Murphy *et al.* 2000) and DNA was extracted from these samples within 2 weeks to reduce the effect of long storage time. Faecal samples were extracted with methods detailed in Hung *et al.* (2004), which was modified from a hexadecyltrimethylammonium bromide (CTAB)-based extraction (Parsons *et al.* 1999). Hair DNA extractions were carried out by the traditional phenol-chloroform procedure (Kocher *et al.* 1989).

The amplification success may depend on the length of target amplified fragment. Thus all extracts were amplified of mitochondrial control region and its flanking regions using 3 primer pairs for different length of amplified segments: (1)1600bp, CB-Z, 5′-ATGAATTGGAGGACAACCAGT-3′ (Matsuhashi *et al.* 1999) and D4, 5′-AGGCATTTTCAGTGCCTTGCTTTG-3′ (Matsuhashi *et al.* 1999); (2) 900bp, CB-Z and Ut-Dr, 5′- TGCGTACATATGCGTACATAT-3′ (designed in this study); (3) 450bp, UT-1, 5′-TGATCACCAGGCCTCGAGAAA-3′ (Ishibashi and Saitoh 2004) and Ut-Dr. PCR amplifications were carried out using an ASTEC Thermal Cycler PC-808 in a total volume of 20 μL reaction mixture containing: 2 μL of faecal DNA extract and 0.5 μL of hair DNA extract respectively, 1× PCR buffer(including 1.5mM MgCl2), 0.5μM of each primer, 200μM dNTP and 0.5 U of Taq DNA polymerase (Supertherm Taq, JMR). The PCR thermal profile included an initial denaturation of 10 min at 95 °C, 35 cycles of 1 min at 95 °C, 1 min at 60 °C and 2 min at 72 °C, and a postcycling final extension at 72 °C for 10 min. A reagent with negative control to test contamination and a positive control to confirm proper PCR conditions were included in each group of PCR reactions.

The PCR products were electrophoresed on 1% agarose gels and visualized using ethidium bromide staining under UV light to score each PCR sample amplification/non-amplification for target DNA fragment. All samples that failed to produce a positive amplification were attempted to amplify for a second time to avoid random non-amplification (Murphy *et al.* 2007).

Data analyses

The amplification success rates for each treatment and each mtDNA fragments were calculated as percentage of the positive amplification number divided by the total number of PCR attempts.

Faecal DNA amplification results were firstly evaluated using the Friedman test to assess the effect of sampling location (sampling from inside, surface and inside-surface mixture of faeces) on amplification success in 6 preservation method and amplicon size combinations (2 preservation methods and 3 mtDNA fragments of different length). Next we used the Wilcoxon test to examine the differences between two faecal preservation methods (frozen at -20 °C and kept at room temperature). The Mann-Whitney U test was used to test the differences between amplification success rates of faecal and hair samples of 0-day and 7-day-old. The Friedman test, Wilcoxon test and Mann-Whitney U test were all computed using StatView 5.0 software (SAS Institute Inc.) and the results were considered statistically significant if the *P*-value was smaller than 0.05. Later the Page's trend test was performed on both faecal and hair DNA amplification results to test whether there were trends across sample ages and amplicon sizes.

RESULTS

Influence of faecal sampling locations

Fresh bear faeces were soft, moist, smelly, and contained indigestible fibers and seeds. One-day-old faeces kept their original shape and remained moist with a slightly dry surface. Three-day-old faeces kept their shape but were dry in the surface and soft inside. Seven-day-old faeces were hard, dry, and moldy. At the 14th day, the faeces became flaky and the remains contained mostly fibers. Therefore, we could collect samples from 3 sampling locations (surface, inside, and surface-inside mixture) successfully for all faecal samples except those that were 14 days old, from which we only collected a sample of surface-inside mixture.

The results showed that sampling locations had no significant effect on amplification success rate regardless of preservation method and amplicon size (Friedman test, $P = 0.145$ - 0.926) (Table 1). Therefore,

Table 1 Comparisons of the effects of sampling locations on the PCR amplification success rates of faecal DNA collected from samples of different ages, stored by different methods and extracts for mtDNA control region fragments of different sizes. S: samples from surface of faeces. I: samples from inside of faeces, and M: samples from inside-surface mixture of faeces; F: frozen at -20 °C and R: kept at room temperature. *P*-values were the results of the Friedman test.

Age (day)	Storage Method	Amplification success rate (%)											
		450bp				900bp				1600bp			
		S	I	M	*P*-value	S	I	M	*P*-value	S	I	M	*P*-value
0	F	100(10/10)	100(10/10)	100(10/10)	0.607	100(10/10)	100(10/10)	80(8/10)	0.926	70(7/10)	40(4/10)	50(5/10)	0.145
1		100(10/10)	100(10/10)	100(10/10)		100(10/10)	90(9/10)	100(10/10)		50(5/10)	30(3/10)	70(7/10)	
3		90(9/10)	100(10/10)	80(8/10)		80(8/10)	100(10/10)	80(8/10)		10(1/10)	10(1/10)	20(2/10)	
7		60(6/10)	70(7/10)	80(8/10)		50(5/10)	70(7/10)	90(9/10)		20(2/10)	0(0/10)	0(0/10)	
14		-	-	40(4/10)		-	-	40(4/10)		-	-	0(0/10)	
0	R	100(10/10)	100(10/10)	100(10/10)	0.607	80(8/10)	100(10/10)	100(10/10)	0.717	30(3/10)	0(0/10)	20(2/10)	0.150
1		100(10/10)	100(10/10)	100(10/10)		90(9/10)	90(9/10)	90(9/10)		30(3/10)	30(3/10)	20(2/10)	
3		90(9/10)	80(8/10)	90(9/10)		90(9/10)	80(8/10)	90(9/10)		10(1/10)	0(0/10)	0(0/10)	
7		40(4/10)	60(6/10)	60(6/10)		20(2/10)	10(1/10)	10(1/10)		0(0/10)	0(0/10)	0(0/10)	
14		-	-	60(6/10)		-	-	40(4/10)		-	-	0(0/10)	

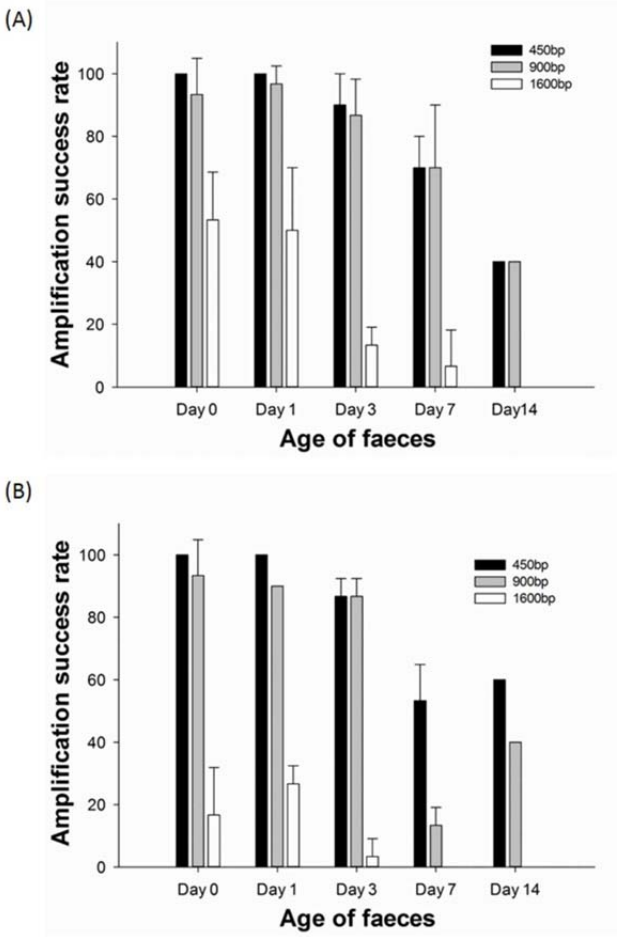

Fig. 1 The amplification success rate of faecal samples of different age kept (A) frozen and (B) under room temperature with different amplicon sizes. Data are the average values of samples collected from 3 different sampling locations, i.e. from the surface, inside, and surface-inside mixture of faeces, except for the 14-day-old faeces, from which only a surface-inside mixture sample was taken.

the data of 3 sampling locations were pooled to calculate the average values before examining the results of faecal preservation method and age of treatments.

Influence of preservation methods, age of faecal samples and amplicon size

The amplification success appeared unaffected by preservation methods (frozen at -20 °C or kept at room temperature in 95% ethanol) in shorter 450bp and 900bp mtDNA fragments from samples within a week (P = 0.330 for 450bp and P = 0.090 for 900bp, Wilcoxon test), but was significantly influenced when amplicon size was 1600bp (P = 0.011). In samples aged from fresh to 7 days, the amplification success rates of 1600bp fragment were higher in frozen samples (53.33% to 6.67%) than the room temperature samples (26.67% to 0%) (Fig. 1). Amplification success rates of 1600bp fragment dropped to zero for DNA extracted from 14-day-old faecal samples regardless of the storage method used (Fig. 1). Although PCR amplification success rates of 450bp and 900bp amplicons of the 7-day old frozen samples were higher than those of 14-day old samples as expected, an unexpected result was found in the PCR amplification success rates of 450bp and 900bp amplicons in 14-day old samples at room temperature, which were higher than that of the 7-day old samples (Table 1 and Fig. 1(B)).

When examining the trends of amplification success rates using average values of subsamples from 3 sampling locations by Page's trend test, both data of frozen and room temperature samples showed a significantly declining trend with increasing age of faeces (frozen samples: $L=163.5 > 160$ ($k=5$, $b=3$, $\alpha=0.001$), $P<0.001$; room temperature samples, $L=162 > 160$ ($k=5$, $b=3$, $\alpha=0.001$), $P<0.001$) and size of amplicon (frozen samples: $L=177>172$ ($k=3$, $b=13$, $\alpha=0.001$), $P < 0.001$; room temperature samples, $L=179.5>172$ ($k=3$, $b=13$, $\alpha=0.001$), $P<0.001$). PCR performances on DNA extracted from fresh versus 14-day-old faecal samples declined from 100% to 40% for 450bp fragments, from 93.33% to 40% for 900bp fragments, and from 53.33% to 0% for 1600bp fragments in frozen samples (Fig. 1(A)); and from

100% to 60% for 450bp, from 93.33% to 40% for 900bp, and from 16.67% to 0% for 1600bp fragments in room temperature samples (Fig. 1(B)).

Influence of hair age and amplicon size

There was no significant difference between mtDNA amplification success of DNA extracted from fresh and 7-day-old samples of either the hair or faeces. In the results of hair treatments, Page trend test also showed a significantly decreasing trend of amplification success rates with both hair age and amplicon size (hair age: $L=157.5>155$ ($k=5$, $b=3$, $\alpha=0.01$), $P<0.01$; amplicon size: $L=68.5>68$ ($k=3$, $b=5$, $\alpha=0.01$), $P<0.01$). The amplification success rate of 450bp fragment was 53.33% even when the hair samples had been in an outdoor environment for 60 days (Fig. 2). But for 900bp fragment, the success rates decreased to 80% for 7-day-old samples, 53.33% for 14-day-old samples and 0% after 30 days (Fig. 2). Furthermore, the 1600bp fragment could only be amplified from fresh hair samples with 86.67% success rate (Fig. 2).

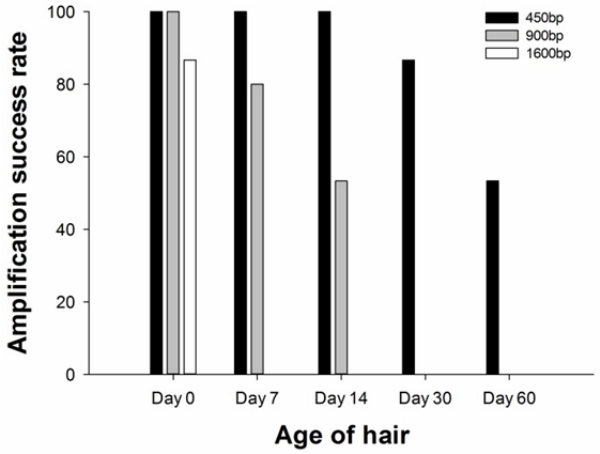

Fig. 2 The amplification success rate of DNA from different hair age with different amplicon sizes.

DISCUSSION

When collecting faecal samples in the field, most molecular scatology studies suggested sampling the outer portions of the faeces because a greater number of intestinal epithelial cells could be present (Albaugh *et al.* 1992; Flagstad Ø *et al.* 1999; Stenglein *et al.* 2010; Wasser *et al.* 2011). Stenglein *et al.* (2010) indicated that the sampling location had a significant effect on nuclear DNA quality of brown bear and wolf scats, and the outer part of the faecal samples had higher DNA quality. Our results show that sampling locations of faeces have no significant effect on mtDNA amplification success rate. Such discrepancy in the results may be due to 2 potential reasons. First, the mtDNA and nuclear DNA may have differential decay rates and patterns (Berger *et al.* 2001; Foran 2006; Soto-Calderon *et al.* 2009; DeMay *et al.* 2013). Second, DNA decay rates and patterns may be different under different climatic condition (Panasci *et al.* 2011). Most of the studies regarding the effects of sampling locations were conducted in the temperate region (Stenglein *et al.* 2010; Wasser *et al.* 2011). Faeces exposed to the subtropical environment of high temperature and humidity in our study might have an effect particularly on the outer portions of faeces, and likely counterbalance the advantage of having more and better quality intestinal epithelial cells on the surface.

Our results showed that preservation methods did not affect the amplification success rates of 450bp and 900bp mtDNA fragments from samples collected within a week. However, the success rates of 1600 bp fragment from the frozen samples was significantly higher than those samples stored at room temperature. Similar to our findings, Santini *et al.* (2007) suggested that wolf scats stored in 95% ethanol at -20 °C had the best nuclear DNA quality comparing to those stored in 95% ethanol at room temperature, dried at -20 °C, and in GUS at room temperature. Santini *et al.* (2007) further indicated the disparities between samples kept frozen and at room temperature increased over time, e.g. 98% positive PCRs at -20 °C and 55% successful PCRs at room temperature after 6 months. However, the non-linear decrease in the amplification success rates of DNA extracted from samples stored at room temperature (Fig. 1(B)) was inconsistent with the results of the frozen samples, even though the declining trend of the amplification success rates of DNA with increasing age was statistically significant, which may suggest that the performances of preservation at room temperature may be less predictable than those of frozen samples when the storage time exceeds one week. Such results suggest that although immediate freezing of faecal samples is often difficult in the field, researchers should consider it especially when amplification of longer mtDNA fragment is critical for their research. In any case, freezing ethanol-soaked samples is highly recommended after the samples are brought back to the laboratory.

Most of the studies regarding the impact of sample age on faecal mtDNA amplification indicated that the amplification success generally decreased over time (Farrell *et al.* 2000; Murphy *et al.* 2007; Soto-Calderon *et al.* 2009; DeMay *et al.* 2013). Our results are consistent with these studies and those studies that showed a decreasing trend in the amplification success rates with increasing amplicon size (Broquet *et al.* 2007; DeMay *et al.* 2013). Furthermore, we found that for the samples as old as 14 days the amplification success rates of mtDNA remained to be at least 40% for the 450bp and 900bp fragments; in contrast, 1600bp fragment could not be amplified from faecal samples older than 7 days (Fig. 1). Information on rates of

faecal DNA degradation regarding sample age and amplicon size in this study allowed researchers to choose better strategies for collecting noninvasive samples and choose suitable markers depending on the conditions of faeces in the field to balance the costs and output of laboratory work. When faecal samples are of older age, smaller mtDNA fragments are expected to have higher amplification success rates and may therefore be favored in genetic studies. If larger mtDNA sequences with increased resolution are needed for phylogenetic research (Waits *et al.* 1999), the noninvasive genetic materials need to be extracted within a certain time frame.

The results of amplification success rates showing no significant difference between hair and faeces at 0 and 7 days in our study are consistent with the comparative review of Broquet *et al.* (2007). Broquet *et al.* (2007) mentioned that greater inhibitor concentrations may counterbalance the advantage of larger target DNA amount in faecal samples. Regarding the effects of hair age and amplicon size on amplification success rates of DNA from hair samples, Roon *et al.* (2003) demonstrated that DNA of hair degraded with time when the samples were preserved using silica desiccant and -20 °C freezing. Broquet *et al.* (2007) reviewed the relationship between mtDNA amplification success and fragment length of hair samples in 2 published papers (Vigilant 1999; Roon *et al.* 2003) and indicated the shorter fragments lead to higher amplification success. However, few studies had measured the rates of hair DNA degradation regarding sample age in outdoor environment without preservation and amplicon size like our study, which indicated that amplification success rates significantly decreased with both hair age and amplicon size. In addition, the amplification success rates we found were lower in comparison to the rates in Roon *et al.* (2003), which might suggest the impact of high temperature and humidity on the quality and degradation rates of DNA of hair samples collected in the subtropics. Researchers conducting noninvasive analyses in the subtropics therefore can consider the DNA amplification success rates from hair samples of different ages revealed in this study and design suitable intervals for hair collection to get appropriate DNA materials.

The mtDNA fragments are useful in addressing questions about species identification, population structure, and phylogenetic research (Waits *et al.* 1999; Murphy *et al.* 2002; Roon *et al.* 2003), whereas the microsatellites of nDNA have utility in individual identification, kinship analysis, gene flow, and demographic studies (Murphy *et al.* 2002; Roon *et al.* 2003; DeMay *et al.* 2013). Mitochondrial DNA and nuclear DNA may have differential decay rates (Foran 2006; Soto-Calderon *et al.* 2009) and some studies have suggested using mtDNA as a screening for further nDNA analyses (Hung *et al.* 2004; Vynne *et al.* 2012). Our study examined the amplification success rates of mtDNA from faecal and hair samples but the decay rates of nDNA in the subtropics remain unanswered. Consequently, it would be necessary to examine the amplification success rates of nDNA from various non-invasive materials in the future.

In addition, some studies have indicated that diet may influence target DNA quantity and genetic analysis of faeces (Murphy *et al.* 2003; Nsubuga *et al.* 2004; Panasci *et al.* 2011; Vynne *et al.* 2012; DeMay *et al.* 2013). Vynne *et al.* (2012) further suggested that the effect of diet should be considered especially in studies of species with highly varied diets. Asiatic black bears are omnivorous animals and the diet of the Formosan black bear in the subtropical Taiwan does change seasonally (Hwang *et al.* 2002). Although the faecal samples of this study were deposited from zoo bears with a mainly vegetarian diet, the components of the diet were not the same as the natural diet of bears in the wild. Therefore, evaluation of faecal DNA degradation under different natural diet of the bears is recommended in future studies.

Our study is the first one to quantitatively evaluate mtDNA degradation of noninvasive hair and faecal samples of Ursid animal in the subtropics. The discrepancy of results between our study and the comparative research in temperate region suggests the importance of pilot study for a new study system. In conclusion, our results demonstrated that faeces and hair could be applied as noninvasive samples for the Asiatic black bears under subtropical climate. We suggest that the amplification success rates are not influenced by sampling location of faeces in subtropical environment. The immediate freezing of ethanol-soaked faecal samples in the field are not so critical in affecting DNA quality of short fragments from samples collected within a week but the effect of immediate freezing is significant for longer mtDNA fragments. We also found that although it may be challenging to amplify longer mtDNA fragments from older faecal and hair samples, shorter fragments could be successfully amplified. Researchers collecting noninvasive samples in similar taxa and field conditions should consider the DNA degradation rates revealed in this study. Careful selection of primers for suitable PCR product sizes depending on sample conditions could optimize success rates of genetic analysis and save both time and financial cost in noninvasive genetic research.

ACKNOWLEDGEMENTS

We would like to acknowledge Taipei Zoo for providing bear samples and Ms. Hui-Lun Chen and Ms. Hsuan-Yi Hou

in Taipei Zoo for their help with the lab work. This study was funded by the Forestry Bureau, Council of Agriculture of Taiwan to LL Lee (95AS-11.1.3-FB-e1) and financially supported by Taipei Zoo to CC Shih. We also thank the permits approving of the Forestry Bureau of Taiwan for using protected wild animals.

LITERATURE CITED

Albaugh, G. P., V. Iyengar, A. Lohani, M. Malayeri, S. Bala and P. P. Nair. 1992. Isolation of exfoliated colonic epithelial cells, a novel, non-invasive approach to the study of cellular markers. Int. J. Cancer. **52(3)**:347-350.

Bayes, M. K., K. L. Smith, S. C. Alberts, J. Altmann and M. W. Bruford. 2000. Testing the reliability of microsatellite typing from faecal DNA in the savannah baboon. Conserv. Genet. **1**:173-176.

Beja-Pereira, A., R., Oliveira, P. C. Alves, M. K. Schwartz and G. Luikart. 2009. Advancing ecological understandings through technological transformations in noninvasive genetics. Mol. Ecol. Resour. **9(5)**:1279-1301.

Berger, A., M. Bruschek, C. Grethen, W. Sperl and B. Kofler. 2001. Poor storage and handling of tissue mimics mitochondrial DNA depletion. Diagn. Mol. Pathol. **10(1)**:55-59.

Broquet, T., N. Ménard and E. Petit. 2007. Noninvasive population genetics: a review of sample source, diet, fragment length and microsatellite motif effects on amplification success and genotyping error rates. Conserv. Genet. **8(1)**:249-260.

Buchan, J. C., E. A. Archie, R. C. Van Horn, C. J. Moss and S. C. Alberts. 2005. Locus effects and sources of error in noninvasive genotyping. Mol. Ecol. Notes. **5(3)**:680-683.

De Barba, M., L. P. Waits, E. O. Garton, P. Genovesi, E. Randi, A. Mustoni and C. Groff. 2010. The power of genetic monitoring for studying demography, ecology and genetics of a reintroduced brown bear population. Mol. Ecol. **19(18)**:3938-3951.

DeMay, S. M., P. A. Becker, C. A. Eidson, J. L. Rachlow, T. R. Johnson and L. P. Waits. 2013. Evaluating DNA degradation rates in faecal pellets of the endangered pygmy rabbit. Mol. Ecol. Resour. **13(4)**:654-662.

Farrell, L. E., J. Roman and M. E. Sunquist. 2000. Dietary separation of sympatric carnivores identified by molecular analysis of scats. Mol. Ecol. **9**:1583-1590.

Flagstad, Ø., K. Røed, J. E. Stacy and S. K. Jakobsen. 1999. Reliable noninvasive genotyping based on excremental PCR of nuclear DNA purified with a magnetic bead protocol. Mol. Ecol. **8(5)**:879-883.

Foran, D. R. 2006. Relative degradation of nuclear and mitochondrial DNA: an experimental approach. J. Forensic Sci. **51(4)**:766-770.

Frantzen, M. A. J., J. B. Silk, J. W. H. Ferguson, R. K. Wayne and M. H. Kohn. 1998. Empirical evaluation of preservation methods for faecal DNA. Mol. Ecol. **7(10)**:1423-1428.

Hoffman, J. I. and W. Amos. 2005. Microsatellite genotyping errors: detection approaches, common sources and consequences for paternal exclusion. Mol. Ecol. **14(2)**:599-612.

Hung, C.-M., S.-H. Li. and L.-L. Lee. 2004. Faecal DNA typing to determine the abundance and spatial organisation of otters (*Lutra lutra*) along two stream systems in Kinmen. Anim. Conserv. **7(3)**:301-311.

Hwang, M.-H., D. L. Garshelis and Y. Wang. 2002. Diet of Asiatic black bears in Taiwan, with methodological and graphical comparison. Ursus **13**:111-125.

Hwang, M.-H., D. L. Garshelis, Y.-H. Wu and Y. Wang. 2010. Home ranges of Asiatic black bears in the Central Mountains of Taiwan: Gauging whether a reserve is big enough. Ursus **21(1)**:81-96.

Hwang, M.-H. and D. L. Garshelis. 2007. Activity patterns of Asiatic black bears (*Ursus thibetanus*) in the Central Mountains of Taiwan. J. Zool. **271(2)**:203-209.

Hwang, M.-H. and Y. Wang. 2006. The status and management of Asiatic black bears in Taiwan. In:Yamazaki, K. (ed) Understanding Asian bears to secure their future. Japan Bear Network, Japan, pp. 107-110.

Ishibashi, Y. and T. Saitoh. 2004. Phylogenetic relationships among fragmented Asian black bear (*Ursus Thibetanus*) populations in western Japan. Conserv. Genet. **5(3)**:311-323.

Kocher. T. D., W. K. Thomas, A. Meyer, S. V. Edwards, S. Pääbo, F. X. Villablanca and A. C. Wilson. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. **86(16)**:6196-6200.

Lucchini, V., E. Fabbri, F. Marucco, S. Ricci, L. Boitani and E. Randi. 2002. Noninvasive molecular tracking of colonizing wolf (*Canis lupus*) packs in the western Italian Alps. Mol. Ecol. **11(5)**:857-868.

Matsuhashi, T., R. Masuda, T. Mano and M. C. Yoshida. 1999. Microevolution of the mitochondrial DNA control region in the Japanese brown bear (*Ursus arctos*) population. Mol. Biol. Evol. **16(5)**:676-684.

Michalski, F., F. P. Valdez, D. Norris, C. Zieminski, C. K. Kashivakura, C. S. Trinca, H. B. Smith, C. Vynne, S. K. Wasser, J. P. Metzger and E. Eizirik. 2011. Successful carnivore identification with faecal DNA across a fragmented Amazonian landscape. Mol. Ecol. Resour. **11(5)**:862-871.

Murphy, M. A., L. P. Waits, K. C. Kendall, S. K. Wasser, J. A. Higbee and R. Bogden. 2002. An evaluation of long-term preservation methods for brown bear (*Ursus arctos*) faecal DNA samples. Conserv. Genet. **3(4)**:435-440.

Murphy, M. A., K. C. Kendall, A. Robinson and L. P. Waits. 2007. The impact of time and field conditions on brown bear (*Ursus arctos*) faecal DNA amplification. Conserv. Genet. **8(5)**:1219-1224.

Murphy, M. A., L. P. Waits and K. C. Kendall. 2000. Quantitative evaluation of fecal drying methods for brown bear DNA analysis. Wildlife Soc. B **28**:951-957.

Murphy, M. A., L. P. Waits and K. C. Kendall. 2003. The influence of diet on faecal DNA amplification and sex identification in brown bears (*Ursus arctos*). Mol. Ecol. **12(8)**:2261-2265.

Nsubuga, A. M., M. M. Robbins, A. D. Roeder, P. A. Morin, C. Boesch and L. Vigilant. 2004. Factors affecting the amount of genomic DNA extracted from ape faeces and the identification of an improved sample storage method. Mol. Ecol. **13(7)**:2089-2094.

Panasci, M., W. B. Ballard, S. Breck, D. Rodriguez, L. D. Densmore, D. B. Wester and R. J. Baker. 2011. Evaluation of fecal DNA preservation techniques and effects of sample age and diet on genotyping success. J. Wildlife Manage. **75(7)**:1616-1624.

Parsons, K. M., J. F. Dallas, D. E. Claridge, J. W. Durban, K. C. Balcomb Iii, P. M. Thompson and L. R. Noble. 1999. Amplifying dolphin mitochondrial DNA from faecal plumes. Mol. Ecol. **8(10)**:1766-1768.

Piggott, M. P., E. Bellemain, P. Taberlet and A. C. Taylor. 2004. A multiplex pre-amplification method that significantly improves microsatellite amplification and error rates for faecal DNA in limiting conditions. Conserv. Genet. **5(3)**:417-420.

Piggott, M. P. 2004. Effect of sample age and season of collection on the reliability of microsatellite genotyping of faecal DNA. Wildlife Res. **31(5)**:485-493.

Piggott, M. P. and A. C. Taylor. 2003. Remote collection of animal DNA and its applications in conservation management and understanding the population biology of rare and cryptic species. Wildlife Res. **30(1)**:1-13.

Renan, S., E. Speyer, N. Shahar, T. Gueta, A. R. Templeton and S. Bar-David. 2012. A factorial design experiment as a pilot study for noninvasive genetic sampling. Mol. Ecol. Resour. **12(6)**:1040-1047.

Roon, D. A., L. P. Waits and K. C. Kendall. 2003. A quantitative evaluation of two methods for preserving hair samples. Mol. Ecol. Notes **3(1)**:163-166.

Santini, A., V. Lucchini, E. Fabbri and E. Randi. 2007. Ageing and environmental factors affect PCR success in wolf (*Canis lupus*) excremental DNA samples. Mol. Ecol. Notes **7(6)**:955-961.

Schwartz, M. K., G. Luikart and R. S. Waples. 2007. Genetic monitoring as a promising tool for conservation and management. Trends Ecol. Evol. **22(1)**:25-33.

Shih, C.-C., C.-C. Huang, S.-H. Li, M.-H. Hwang and L.-L. Lee. 2009 Ten novel tetranucleotide microsatellite DNA markers from Asiatic black bear, *Ursus thibetanus*. Conserv. Genet. **10(6)**:1845-1847.

Sloane, M. A., P. Sunnucks, D. Alpers, L. B. Beheregaray and A. C. Taylor. 2000. Highly reliable genetic identification of individual northern hairy-nosed wombats from single remotely collected hairs: a feasible censusing method. Mol. Ecol. **9(9)**:1233-1240.

Soto-Calderon, I. D., Ntie S, P. Mickala, F. Maisels, E. J. Wickings and N. M. Anthony. 2009 Effects of storage type and time on DNA amplification success in tropical ungulate faeces. Mol. Ecol. Resour. **9(2)**:471-479.

Stenglein, J. L., M. De Barba, D. E. Ausband and L. P. Waits. 2010. Impacts of sampling location within a faeces on DNA quality in two carnivore species. Mol. Ecol. Resour. **10(1)**:109-114.

Taberlet, P., L. P. Waits and G. Luikart. 1999. Noninvasive genetic sampling: look before you leap. Trends Ecol. Evol. **14(8)**:323-327.

Valiere, N. and P. Taberlet. 2000. Urine collected in the field as a source of DNA for species and individual identification. Mol. Ecol. **9(12)**:2150-2152.

Vallet, D., E. Petit, S. Gatti, F. Levréro and N. Ménard. 2008. A new 2CTAB/PCI method improves DNA amplification success from faeces of Mediterranean (Barbary macaques) and tropical (lowland gorillas) primates. Conserv. Genet. **9(3)**:677-680.

Vigilant, L. 1999. An evaluation of techniques for the extraction and amplification of DNA from naturally shed hairs. Biol. Chem. **380(11)**:1329-1331.

Vigilant, L., M. Hofreiter, H. Siedel and C. Boesch. 2001. Paternity and relatedness in wild chimpanzee communities. Proc. Natl. Acad. Sci. **98(23)**:12890-12895.

Vynne, C., M. R. Baker, Z. K. Breuer, S. K. Wasser and D. Reed. 2012. Factors influencing degradation of DNA and hormones in maned wolf scat. Anim. Conserv. **15(2)**:184-194.

Waits, L. P. and D. Paetkau. 2005. Noninvasive genetic sampling tools for wildlife biologists: a review of applications and recommendations for accurate data collection. J. Wildlife Manage. **69(4)**:1419-1433.

Waits, L. P., J. Sullivan, S. J. O'Brien and R. H. Ward. 1999. Rapid Radiation Events in the Family Ursidae Indicated by Likelihood Phylogenetic Estimation from Multiple Fragments of mtDNA. Mol. Phylogenet. Evol. **13(1)**:82-92.

Wasser, S. K., C. S. Houston, G. M. Koehler, G. G. Cadd and S. R. Fain. 1997. Techniques for application of faecal DNA methods to field studies of Ursids. Mol. Ecol. **6(11)**:1091-1097.

Wasser, S. K., J. L. Keim, M. L. Taper and S. R. Lele. 2011 The influences of wolf predation, habitat loss, and human activity on caribou and moose in the Alberta oil sands. Front. Ecol. Environ. **9(10)**:546-551.

Woods, J. G., D. Paetkau, D. Lewis, B. N. McLellan, M. Proctor and C. Strobeck. 1999. Genetic tagging of free-ranging black and brown bears. Wildlife Soc. B **27**:616-627.

Wozencraft, W. C. 2005. Order Carnivora. In:Wilson, D. E. and D. M. Reeder (eds) Mammal Species of the World: A Taxonomic and Geographic Reference, 3 edn. John Hopkins University Press, Baltimore, pp. 532-628.