

Techniques for application of faecal DNA methods to field studies of Ursids

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Abstract

We describe methods for the preservation, extraction and amplification of DNA from faeces that facilitate field applications of faecal DNA technology. Mitochondrial, protein encoding and microsatellite nuclear DNA extracted and amplified from faeces of Malayan sun bears and North American black bears is shown to be identical to that extracted and amplified from the same individual's tissue or blood. A simple drying agent, silica beads, is shown to be a particularly effective preservative, allowing easy and safe transport of samples from the field. Methods are also developed to eliminate the risk of faecal DNA contamination from hair present in faeces.

Keywords: faeces, DNA, Ursids, field, preservation, contamination, PCR

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Introduction

DNA techniques that distinguish between species, populations and individuals are of value to a variety of disciplines. However, the reliability and power of these measures depends not only on the genetic variability in the population(s), but also on having a sample size that is large enough to characterize this variation accurately. In fact, the difficulty of acquiring an adequate sample size using tissue or blood samples has significantly limited the application of molecular technology to field studies. Hair sampling has been shown to be an effective means of acquiring DNA from free-ranging species (Morin *et al.* 1992, 1993; Taberlet & Bouvet 1992; Taberlet *et al.* 1993; Takasaki & Takenaka 1991); however, accessibility and expense may limit the use of hair under some circumstances. DNA acquired from faeces potentially provides an ideal sampling alternative (Sidransky *et al.* 1992; Höss *et al.* 1992; Constable *et al.* 1995; Gerloff *et al.* 1995; Kohn *et al.* 1995; Tikel *et al.* 1995; van der Kuyl *et al.* 1996). Faeces is abundant; its collection can be totally noninvasive; and a single gram of faeces contains large quantities of the host's DNA from millions of sloughed intestinal mucosal cells (Albaugh *et al.* 1992).

The utility of faecal DNA technology for application to the field depends upon the following key issues: (a) the length and copy number of DNA that can be extracted and amplified from faeces; (b) confirmation that the amplified DNA from faeces is identical to that obtained from the same individual's tissue or blood; (c) elimination of sample contamination (e.g. from ingested tissue or hair); (d) prevention of sample degradation in the laboratory or field; and (e) removal of dietary inhibitors. This paper addresses each of the above issues.

Materials and methods

Four captive Malayan sun bears and at least 35 North American black bears free-ranging in Washington state were sampled in this study. Whole blood was collected, stored at 4 °C and extracted within 1–15 days. Tissue samples were collected from the inner flank on sun bears and from the ear of American black bears.

Faeces were collected from captive sun bears within 1 h post-defecation. The entire sample was thoroughly mixed using a sterile tongue depressor and a 10-g aliquot stored frozen (–20 °C) in zip lock bags until freeze dried, prior to DNA extraction. Fresh faeces (0–6 h post-defecation based on temperature and moisture content) were collected *ad libitum* off the road or forest floor from free-ranging North

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American black bears. The entire sample was mixed as above and a 2-g portion stored in 25-mL vials containing ethanol (5 mL ETOH/g faeces) or silica gel (4 g silica/g faeces, silica gel beads Type II, 1/8" Sigma Chemical Company, St Louis, MO, USA) until processed (see below).

Sample extraction and purification

Sun bear blood and tissue DNA extractions were performed using a QIAamp Kit (Qiagen, Chatsworth, CA, USA) with the supplied reagents and procedures. DNA from faeces was extracted by three different methods.

Method 1 added 600 μ L of Qiagen Load Buffer (QLB: 500 mM Tris-HCl, 16 mM EDTA, 100 mM NaCl, pH 6.0) to 100 mg of freeze-dried faeces. Samples were vortexed briefly and then centrifuged at 13 000 rpm for 6 min. Four-hundred microlitres of supernatant was transferred to a fresh tube, 400 μ L of AL buffer and 50 μ L of Proteinase K were added, and the mixture incubated for 16 h at 37 °C. After adding 420 μ L of ethanol, lysates were loaded in two centrifugations on to the spin columns, washed and eluted in 100 μ L of 10 mM Tris HCl, pH 9.0.

In methods 2 (GuSCN-DE; Gerloff *et al.* 1995) and 3 (GuSCN-Sil; Boom *et al.* 1990 and Kohn *et al.* 1995), 100 mg of lyophilized faecal powder was purified and then eluted with 100 μ L of 10 mM Tris-HCl, pH 8.0.

DNA amplification

All PCR reactions included mock extraction blanks and positive and negative DNA controls.

PCR amplifications of a portion of the mitochondrial control region (D-loop) used the following primers: HSF21 and LTPROBB13 (246 bp; S. Fain, unpublished data); H623 and L636 (398 bp; Kohn *et al.* 1995); and H16498 and L15774 (700 bp; Kocher *et al.* 1989).

Sex determination of sun bears and black bears from extracted faecal and tissue DNA was accomplished by a hot-start PCR amplification procedure (Bloch 1992), using the primers: ZFY/X-P1-5EZ and ZFY/X-P2-3EZ (442 bp; Aasen & Medrano 1990) and SRY-Y53-3C and SRY-Y53-3D (224 bp; S. F. Fain and J. P. LeMay unpublished). Primer pairs for microsatellite loci included: G10C-(GT) and G10C-(CA) (97/113 bp; Paetkau *et al.* 1995); G10H-(GT) and G10H-(CA) (252/262 bp; D. Paetkau, unpublished data); and G10M-(GT) and G10M-(CA) (211/217 bp; Paetkau *et al.* 1995).

Restriction fragment length polymorphism (RFLP) analyses

The amplified 246-bp mtDNA PCR products from blood, tissue and faeces of four individual sun bears were digest-

ed with the restriction enzymes *AluI* and *RsaI* according to the manufacturer's instructions (New England Biolabs, Beverly, MA, USA), electrophoresed on 4–20% TBE-acrylamide gradient gels (Novex, San Diego, CA, USA) and ethidium bromide stained.

DNA sequencing

Direct double-stranded sequencing of the 246 bp mtDNA PCR product from faeces and tissue of two different sun bears was performed using the AmpliCycle sequencing kit (Perkin Elmer; Branchburg, NJ) and the Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.; Foster City, CA).

Experimental designs

1 Comparisons of extraction techniques and sample types. All comparisons of extraction techniques or of sample types (i.e. matched tissue, blood and faeces) were always analysed in parallel (extraction, amplification and electrophoresis).

2 Sexing individuals using SRY and ZFX/Y protein encoding genes. The length of the ZFX/Y genes (present on the sex chromosomes in either females or males, respectively) is approximately twice that of the SRY gene (present only on the Y chromosome in males); this makes it unlikely that ZFX/Y will amplify without SRY also amplifying when present. Thus, female assignment can be made reliably if the ZFX gene/locus amplified alone. By contrast, the male sex could be assigned if both the SRY and ZFX/Y loci products amplified or if the SRY gene amplified alone. (Fain and LeMay, unpublished).

DNA isolated from blood was used to assign sex to 35 individual wild North American black bears. All assignments were performed prior to receiving knowledge of the bears known sex. These same analyses were then conducted using faeces from 15 individuals of known sex.

3 Hair contamination. A sun bear's faeces was directly contaminated with hair from another, genetically distinct, sun bear to determine if the aggressiveness of extraction or preservation procedures used impact the likelihood of DNA from hair contaminating faeces. The blood RFLP pattern of the 246-bp control region of mtDNA from animal B contained an extra band compared with that of animal A. Faeces from animal A was, accordingly, spiked with 0, 5 or 10 hairs from animal B. Faecal samples were then extracted and analysed concurrently for the appearance of the extra band using each of the three different DNA extraction techniques described above.

4 Sample preservation. Preservation methods were identified that would be simple, cost-effective, and require

minimal equipment in the field (e.g. no liquid nitrogen or low-temperature freezers). Five-hundred milligram amounts of fresh faeces from an individual sun bear were preserved on the same day in triplicate, in each of the following ways: (a) freeze-dried; (b) 2 : 1 ratio of the CaSO₄ desiccant, Drierite (W. A. Hammond Drierite Company, Xenia, OH, USA); or (c) 4 : 1 ratio (by weight) of desiccating silica beads (Sigma, St Louis, MO, USA) to 500 mg faeces; (d) 100% ethanol; (e) 70% ethanol; (f) 70% ethanol with sodium azide; (g) Queens Lysis Buffer (R. Wayne, personal communication); (h) LST Buffer (Muralidharan & Wemmer 1994); (i) Gerloff Lysis Buffer (Gerloff *et al.* 1995); (j) L6 Buffer (Kohn *et al.* 1995); (k) Qiagen Load Buffer (QLB; this paper); (l) freezing at -70 °C; (m) freezing at -20 °C; (n) refrigerating at 4 °C; (o) no treatment control at room temperature (see also Table 2).

Samples were preserved for 1–6 months. Owing to the large number of techniques tried, not all preservation methods were examined in all cases; some methods were added or deleted as results accumulated. All preserved samples were analysed for mtDNA, microsatellite DNA and the gender identifying, protein encoding nuclear DNA using the Qiagen extraction method.

5 Detection and removal of DNA amplification inhibitors in faeces. Amplification of the 246 bp mtDNA product was examined after mixing blood that readily amplified with extract from faecal samples that (a) failed to amplify or (b) successfully amplified. The former fecal extracts were then purified using glass milk (Geneclean Spin Kit, Bio 101, Inc., Vista, CA), and the amplification procedure repeated.

Results

DNA extraction and amplification from matched faeces, tissue and blood

Mitochondrial DNA (mtDNA) products ranging in size from 246 to 700 bp were successfully amplified in tissue, blood and faeces. Two RFLP types were found in the sun bears when the 246 bp mtDNA was digested with the restriction enzymes *AluI* and *RsaI*. The RFLP types found in faeces were identical to the amplification products found in the same individuals tissue or blood. The sequences of the 246-bp gene product amplified from the same individual's faeces and tissue were also identical ($n =$ two sun bears). These two RFLP types differed by a total of 10 bp.

The SRY and ZFX/Y protein encoding genes were successfully amplified from DNA isolated from tissue, blood and faeces. Amplification of these genes enabled us to correctly assign a sex to 100% of American black bear samples examined ($n =$ 35 blood samples and 15 matched faecal samples), including two individuals whose blood samples were initially mislabelled by field personnel. Figure 1

illustrates our ability to extract and amplify the SRY and ZFX/Y genes from faecal DNA of 17 out of 20 wild North American black bears, even after the faecal samples had been preserved in silica and frozen (-20°C) for over 1 year.

Microsatellite alleles ranging from 97 to 262 bp were successfully amplified from bear tissue and faecal extracts. The microsatellite alleles amplified from tissue and faeces of the same individual clearly matched one another at each of the three loci examined (Figure 2).

Comparisons of extraction techniques

Qiagen and the two guanidine thiocyanate techniques (GuSCN-Sil, Kohn *et al.* 1995; and GuSCN-DE, Gerloff *et al.* 1995) extracted genomic DNA in the size range from 250 to 3000 bp (data not shown). The Qiagen and GuSCN-DE techniques proved comparable when tested for their ability to extract amplifiable products of the mitochondrial, nuclear protein encoding and microsatellite loci being measured (see previous section). The GuSCN-Sil technique also produced amplifiable mitochondrial and protein encoding nuclear DNA, but not as reliably as the other two techniques (data not shown). For this reason, we abandoned this procedure before testing it for amplifiability of microsatellite alleles.

Hair contamination

The extra band from the RFLP type 2 DNA pattern of animal B was observed in the faecal DNA of animal A after the latter was spiked with five or 10 hairs from animal B and analysed by the GuSCN-DE technique. The extra band was absent in the unspiked faecal sample of animal A. By contrast, the extra band only appeared in faeces of animal A spiked with 10H when analysed by the more moderate GuSCN-Sil technique, and was absent in all spiked samples analysed by the Qiagen extraction technique dM the least harsh of the three procedures tested.

Preservation studies

The quality of faecal DNA, as indicated by PCR amplifiability, was preserved to varying degrees depending on the preservation technique used (Table 1). Silica (sil) consistently fared best among the drying preservation methods, followed by freeze drying (FD) and Drierite (Dri) for all types of DNA examined, at all temperatures. Dri and FD were not tested at 6 months because Dri failed to amplify the 398- and 700-bp mtDNA products at 4 weeks and FD failed to amplify the 700-bp mtDNA product, or the three microsatellite loci when stored below room temperature, at 4 weeks. Lower ratios of silica to faeces were also tried but performed less well; higher ratios (8 : 1) performed comparably to the 4 : 1 ratio described in Table 1.

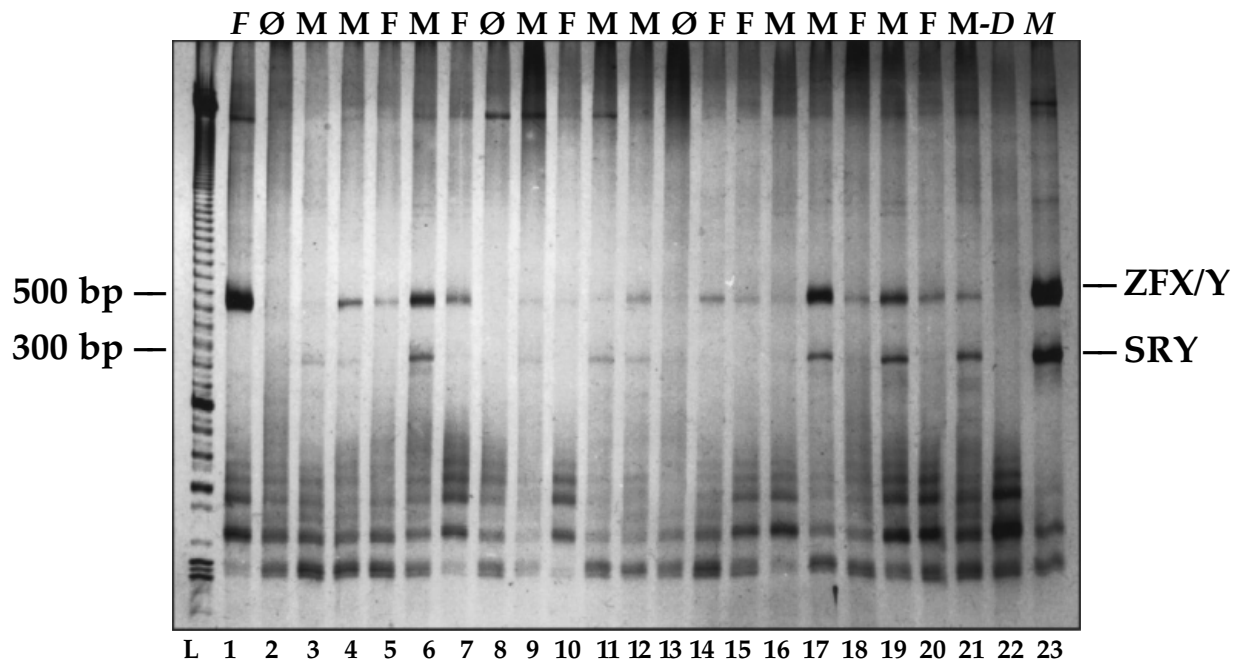


Fig. 1 Gender determination in wild American black bears by amplification of the SRY and ZFX/Y genes in faeces, preserved in silica and frozen for 1 year. DNA from male bear faeces amplifies both SRY (224 bp) and ZFX/Y (442 bp) genes; females show the ZFX gene only. Lane L = 20 plus 50 bp ladder size standards; lanes 1 and 23 = female and male positive controls, respectively; lanes 5, 7, 10, 14, 15, 18, 20 = females; lanes 3, 4, 6, 9, 11–12, 16, 17, 19 and 21 = males; lanes 2, 8, 13 = sex undetermined; lane 22 = -DNA control

Similarly, increasing the container surface area (up to 4.5 cm in width) improved the quality of the silica preservation technique. Separating the silica from faeces with filter paper also provided a higher quality amplification product than simply mixing silica and faeces together (data not shown). Combining drying techniques (e.g. FD plus sil) did not notably improve DNA preservation.

All buffer preservation techniques performed comparably, working best when stored at -20 °C. None of the buffer techniques worked better than the silica drying

technique, and of all methods, silica worked best when stored at room temperature.

Samples stored at different temperatures only (bottom four rows, Table 1) were examined for mtDNA amplification up to 398 bp. Samples were best preserved when stored at -70 °C or -20 °C compared to 4 °C or room temperature.

A subset of preservation techniques (i.e. freeze dried, silica, 100% ethanol, Gerloff buffer, L6 buffer, QLB buffer; Table 1) were also examined after extraction with GuSCN-



Fig. 2 Comparison of three microsatellite loci amplified in duplicate from faecal (F) and tissue (T) DNA from two Malayan Sun Bears, Sibuh (S) and Chamah (C). Matched F and T are identical in all cases. The two bears differ at the G10C and G10H loci but share the same pattern at G10M. Numbers refer to allele size in bp. DNA = a negative control reaction that contained no DNA.

Table 1 Effectiveness of different methods used to preserve mtDNA (246, 398, and 700 bp), single copy nuclear (scnDNA; SRY and ZFX/Y genes) and microsatellite DNA (G10M locus). FD = freeze dried; rt = room temperature ; az=sodium azide (see text); ** = clear, distinct amplification of DNA bands; • = amplification of DNA bands apparent, - = no amplification; blank = not tested

DNA type Time	246 bp ¹ mtDNA			398 bp ¹ mtDNA				700 bp ¹ mtDNA	scnDNA ¹		Microsatellite ¹	
	Week 1	Week 4	Week 7	Week 1	Week 4	Week 7	6 Mos.	Week 4	Week 4	6 Mos.	Week 4	6 Mos.
Preservation Method¹												
FD -20°C		••			••			-	•		-	
FD 4°C		•			•			-	•		-	
FD rt	••	••	••	••	••	••		-	•		•	
Drierite -20°C		•			-			-	•			
Drierite 4°C		•			-			-	•			
Drierite rt	••	•	••	••	-	••		-	•			
Silica -20°C		••			••			•	•	••	••	•
Silica 4°C		•			•			-	•			
Silica rt	••	•	••	••	•	••	•	•	•	•	•	•
FD+sil -20°C		•			•			-	•		•	
FD+sil 4°C		•			•			-	•			
FD+sil rt		•			•			-	•			
FD+Dri -20°C		-			-							
FD+Dri 4°C		-			-							
FD+Dri rt		-			-							
100% ethanol -20°C					••				•	•	•	•
100% ethanol 4°C					•				•		•	•
100% ethanol rt					•		•		•	•	•	•
70% ethanol rt	•		-	•		-						
70% ethanol +az rt	•		-	•		-						
Queen's Lysis Buffer rt	••		•	••		•						
LST Buffer rt	•		-	•		-						
Gerloff Buffer -20°C		•			••			•	•		••	
Gerloff Buffer 4°C		•			•			-	•		•	
Gerloff Buffer rt		•			•			-	•	•	•	•
L6 Buffer -20°C		•			••			-	•		••	
L6 Buffer 4°C		•			•			-	•		•	
L6 Buffer rt		•			•			-	•		•	
QLB Buffer -20°C		•			••			•	•		••	
QLB Buffer 4°C		•			•			-	•			
QLB Buffer rt	•	•	-	•	-	-		-	•			
Frozen -70°C	••		•	••		•						
Frozen -20°C	••		•	••		•						
Frozen 4°C	•		-	•		-						
room temp	•	•	-	•	-	-		-	-		-	

¹ = see methods

DE. Results were consistent with those using the Qiagen extraction, although the GuSCN-DE did not perform as well in amplifying the SRY and ZFX/Y genes (data not shown).

Amplification success and removal of DNA amplification inhibitors

Amplification of the 246- and 398-bp mtDNA product occurred in 90% (45 of 50 samples) of the faecal samples from captive individuals compared with 80% (16/20) in wild individuals; 65% (32/50) of the 700-bp mtDNA product amplified in faecal samples from captive compared with 60% (12/20) from wild individuals. Amplification of

the SRY and ZFX/Y genes was comparable (67%; 33/50) from captive vs. 85% (17/20) from wild individuals. However, only the wild sample gender amplification products were examined on high-resolution gels (Gene Gel Clean 15/24; Pharmacia Ltd, Uppsala, Sweden). Amplification of the G10H microsatellite locus was 80% (40/50) from captive vs. 67% (12/18) from wild individuals. Previously amplified DNA from blood failed to amplify when mixed with DNA extracts from unamplifiable faecal samples of wild individuals ($n = 2$). Purification of these extracts with glass milk restored amplification of both the previously inhibited faecal DNA sample and the faecal-blood DNA mixture.

Discussion

A wide size range of host mitochondrial, microsatellite and even protein encoding nuclear DNA loci can be amplified from faecal extracts. These results are reproducible; however, removal of amplification inhibitors is occasionally required, particularly from samples collected in the wild. The potential for sample contamination from ingested hair can be readily controlled with the appropriate extraction methodology. Faecal samples can also be easily preserved in the field without relying on chemicals that may prove hazardous for safe air transport.

Kohn *et al.* (1995) noted that the reproducibility of DNA analyses appeared to be reduced by the uneven distribution of intestinal cells in faeces. We resolved the uneven distribution problem by thoroughly mixing the entire faecal sample at the time of collection. Once dried, the collected portion was crushed and thoroughly remixed prior to subsample removal for extraction (Wasser *et al.* 1988; 1996).

Researchers should consider the natural history of the organism being studied when selecting collection, storage and extraction procedures to insure that initial storage methods do not compromise their ability to reliably amplify host DNA. For example, we have yet to find DNA contaminants associated with ingested tissue from other animals (i.e. from prey). Ingested hair, on the other hand, could be a significant DNA contaminant. Hair is more difficult to break down than tissue, typically surviving passage through the corrosive gastrointestinal tract. This form of contamination could be particularly problematic in species that ingest hair of conspecifics (e.g. from social grooming); species-specific DNA primers that can be used to distinguish DNA from nonhost species are, by definition, useless for distinguishing between conspecifics in these cases. Our studies only examined the worst case scenario by placing hair directly in faeces prior to extraction. Yet, contamination could still be eliminated by using a relatively mild extraction procedure that does not digest hair (e.g. Qiagen). For the same reasons, use of extraction buffers as sample preservatives in the field may be inadvisable because of their likelihood of digesting ingested hair, exacerbating the potential for DNA contamination (see also below).

We found that silica beads provided ideal alternatives to freeze drying or extraction buffers as sample preservatives (Table 1). Silica beads are also most conducive to field applications; they have no associated leakage problems or restrictions when transporting samples as air cargo. The better performance of silica stored at $-20\text{ }^{\circ}\text{C}$ vs. room temperature may have resulted from accumulation of moisture over time. This could be alleviated by vacuum sealing vials containing samples stored in silica.

In conclusion, appropriately processed faecal DNA can

now make feasible molecular based field studies that were previously inaccessible. We look forward to the application of this important new technology to a wide array of conservation, management, biomedical and biological studies in the future.

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