

TECHNICAL ADVANCES

Impacts of sampling location within a faeces on DNA quality in two carnivore species

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Abstract

We investigated the influence of sampling location within a faeces on DNA quality by sampling from both the outside and inside of 25 brown bear (*Ursus arctos*) scats and the side and the tip of 30 grey wolf (*Canis lupus*) scats. The outside of the bear scat and side of the wolf scat had significantly lower nuclear DNA microsatellite allelic dropout error rates (*U. arctos*: $P = 0.017$; *C. lupus*: $P = 0.025$) and significantly higher finalized genotyping success rates (*U. arctos*: $P = 0.017$; *C. lupus*: $P = 0.012$) than the tip and inside of the scat. A review of the faecal DNA literature indicated that <45% of studies report the sampling location within a faeces indicating that this methodological consideration is currently underappreciated. Based on our results, we recommend sampling from the side of canid scats and the outside portion of ursid scats to obtain higher quality DNA samples. The sampling location within a faeces should be carefully considered and reported as it can directly influence laboratory costs and efficiency, as well as the ability to obtain reliable genotypes.

Keywords: *Canis lupus*, faecal DNA sampling, genotyping errors, genotyping success, *Ursus arctos*

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Introduction

Faecal samples have small amounts of low-quality DNA because of the instability of DNA when separated from normal cellular processes and exposed to the elements (Lindahl 1993; Deagle *et al.* 2006; Broquet *et al.* 2007). DNA success and error rates from faecal samples are influenced by storage method (Murphy *et al.* 2002; Nsubuga *et al.* 2004), season of collection (Lucchini *et al.* 2002; Piggott 2004; Hájková *et al.* 2006), diet (Murphy *et al.* 2003) and age of scat (Fernando *et al.* 2003; Piggott 2004; Murphy *et al.* 2007; Santini *et al.* 2007). Researchers have hypothesized that DNA of the focal species may not be uniformly distributed in a faecal sample because of physiological reasons (Fernando *et al.* 2003) or small-scale environmental influences leading to differences in degradation from decomposers in the soil (Santini *et al.* 2007) and exposure to UV light (Friedberg 2003). To address this, researchers have chosen to swab the surface of the scat (Oka & Takenaka 2001; Frantz *et al.* 2003; Kalz

et al. 2006; Ball *et al.* 2007; Lampa *et al.* 2008), scrape the surface (Reed *et al.* 1997; Kohn *et al.* 1999; Fernando *et al.* 2000; Davison *et al.* 2002; Hung *et al.* 2004; Nagata *et al.* 2005; Prugh & Ritland 2005; Livia *et al.* 2007), wash the surface (Banks *et al.* 2002; Maudet *et al.* 2004; Piggott 2004; Bhagavatula & Singh 2006; Perez *et al.* 2006), sample from the outside/end (Garnier *et al.* 2001; Bidlack *et al.* 2007; Ferrando *et al.* 2008), or homogenize the scat sample (Wasser *et al.* 1997; Frantzen *et al.* 1998; Murphy *et al.* 2000; Clarke *et al.* 2001; Eggert *et al.* 2003; Kovach *et al.* 2003; Deagle *et al.* 2005; Pilot *et al.* 2007; Puechmaille *et al.* 2007) prior to DNA extraction.

We reviewed 123 wildlife molecular scatology studies from 1992 to present to evaluate how many of them specified the portion of the faeces collected and/or extracted and whether any of these studies empirically tested for differences. To our knowledge, only four studies have experimentally tested for differences in DNA quality because of the use of material taken from different parts of the scat, and in all cases, a surface wash or outside sampling led to higher amplification success than homogenized faeces (Flagstad *et al.* 1999; Palomares *et al.* 2002; Piggott & Taylor 2003; Wehausen *et al.* 2004). Most of this research has been conducted on herbivores

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(Flagstad *et al.* 1999; Piggott & Taylor 2003; Wehausen *et al.* 2004), although Palomares *et al.* (2002) tested surface washing compared with homogenization of Iberian lynx (*Lynx pardinus*) faeces and Piggott & Taylor (2003) tested the difference between surface washing, surface scraping and homogenization of faeces from red fox (*Vulpes vulpes*) and two marsupial carnivores (*Dasyurus maculatus*; *Dasyurus viverrinus*). As success and error rates are often species-specific and vary with environmental conditions (Waits 2004), it is critical to employ DNA quality tests for the use of material taken from different sections of the faeces for other species. In addition, for species producing larger faeces (i.e. wolf and bear), it is generally not necessary to collect the whole scat and hence subsamples are often taken in the field. Therefore, it is important that samples are obtained from the location on the scat that maximizes DNA quality. To address this question for carnivores, we empirically evaluated the impact of sampling location within a faeces on DNA quality. Specifically, we tested if the tip or the side of Northern Rocky Mountain grey wolf scats (*Canis lupus*) or the outside or inside of brown bear (*Ursus arctos*) scats collected in Italy led to significant differences in success and error rates for microsatellite genotyping.

Materials and methods

Methods for wolf and bear samples were slightly different because these experiments were conducted as part of independent studies with different standard field and laboratory protocols.

Sample collection and DNA extraction

In June through August of 2007, ~0.5 mL of side and tip samples were collected with flamed tweezers at den and rendezvous sites from 30 grey wolf scats in Idaho and Montana and stored in of 1.5 mL DETs buffer (Frantzen *et al.* 1998). Between May and November 2007, a ~10 mL sample from the inside and outside of 25 brown bear scats was collected with disposable latex gloves or wooden sticks in northern Italy and preserved in 40 mL of 95% ethanol (Murphy *et al.* 2002). Scats were of varying quality and age, which could not be determined. DNA was extracted in a laboratory designed specifically for genetic analysis of noninvasive genetic samples using the Qiagen QIAamp DNA stool mini kit (Qiagen Inc.). One extraction negative was included in each group of extractions to check for contamination.

Microsatellite analysis of grey wolf samples

Eight dye-labelled tetranucleotide microsatellite loci with a polymerase chain reaction (PCR) product size of

<250 bp were used (FH2001, FH2137, Mellersh *et al.* 1997; FH2054, FH2611, Eichmann *et al.* 2004; FH2088, Salim *et al.* 2007; FH2670, FH3725, Guyon *et al.* 2003; Pez15, Neff *et al.* 1999). The loci were combined into two PCR reactions. The 7- μ L PCR reaction for multiplex 1 consisted of 0.1 μ M FH2670 and FH2611, 0.05 μ M FH2088 and FH2054, 1x concentrated Qiagen Master Mix (Qiagen Inc.), 0.5x concentrated Qiagen Q Solution (Qiagen Inc.) and 1 μ L DNA extract. The 7- μ L PCR reaction for multiplex 2 consisted of 0.1 μ M Pez15, 0.05 μ M FH3725, FH2137 and FH2001, 1x concentrated Master Mix, 0.5x concentrated Q Solution and 1 μ L DNA extract. The PCR profile for multiplex 1 had an initial denaturation step of 15 min at 94 °C followed by a touchdown with 13 cycles of 30 s at 94 °C, 90 s at 62 °C with a decrease in annealing temperature of 0.4 °C in each cycle, and 1 min at 72 °C, followed by 28 cycles of 30 s at 94 °C, 90 s at 57 °C and 1 min at 72 °C. Multiplex 2 had the same PCR profile, except for the annealing temperature, which began at 60 °C in the touchdown cycles and was 55 °C in the following cycles. A PCR negative was included in each group of reactions to test for contamination.

Microsatellite analysis of brown bear samples

Eight dye-labelled dinucleotide microsatellite loci with a PCR product size of <200 bp were used (G1D, Taberlet *et al.* 1997; G10P, Paetkau *et al.* 1998; Mu11, Mu15, Mu23, Taberlet *et al.* 1997; Mu50, Mu59, Bellemain & Taberlet 2004; CXX20, Ostrander *et al.* 1993). The loci were combined into two PCR reactions. PCR reaction conditions were as for wolf except the usage of 2 μ L DNA extract and 0.07 μ M Mu23, 0.15 μ M G10P and CXX20, 0.06 μ M Mu11 in multiplex 1, and 0.07 μ M Mu15, 0.04 μ M Mu50, 0.06 μ M Mu59 and 0.09 G1D in multiplex 2. The PCR profile had an initial denaturation step of 15 min at 94 °C followed by a touchdown with 12 cycles of 30 s at 94 °C, 90 s at 57.3 °C with a decrease in annealing temperature of 0.4 °C in each cycle, and 1 min at 72 °C, followed by 27 cycles of 30 s at 94 °C, 90 s at 52.5 °C and 1 min at 72 °C. A PCR negative was included in each group of reactions to test for contamination.

Genotype determination

The two multiplexes were combined and run in one capillary for wolf side and tip samples while the multiplexes were run in two separate capillaries for bear outside and inside samples. Alleles were sized using the Applied Biosystems 3130xl ABI capillary machine and viewed with GENEMAPPER 3.7 software. Initially, each wolf sample was amplified and run twice and each bear sample was amplified and run once. For samples with more than half

the loci working in the initial run(s), additional PCRs were performed to finalize the consensus genotype for each scat. An allele was not accepted in a consensus genotype until it was seen at least twice, or for wolves three times if a homozygote. For bears, we also had reference genotypes for all individuals in the study area and were able to confirm scat genotypes by comparing with references. After seven to eight loci were confirmed for a sample, the consensus genotype was run through Gimlet v.1.3.3 (Valiere 2002) with all the consensus genotypes to check for matches. Genotypes observed in only one sample were run through RELIOTYPE to determine if further repetitions were needed to obtain 95% certainty in the accuracy of genotype (Miller *et al.* 2002).

Data analysis

To assess DNA quality, we evaluated PCR amplification success rates, error rates because of allelic dropout and false alleles, and finalized genotyping success. All measures were calculated for side, tip, outside and inside samples separately, by locus, once the consensus genotype was reached. PCR amplification success rate was measured as the number of successful PCRs divided by the number of initial PCR runs across all samples. Genotyping errors, the difference between the individual PCR results and the consensus genotype for a sample, were calculated separately for allelic dropout and false alleles and were based on the two initial PCRs for the wolf dataset, and a matched number (1–5) of successful PCRs of the inside and outside sample of each scat for the bear dataset. As allelic dropout only pertains to heterozygous individuals, it was calculated as the number of allelic dropouts at a given locus over the number of successful amplifications of heterozygous genotypes at that locus (Broquet & Petit 2004). False alleles were calculated as the number of amplifications leading to one or more false alleles at a locus over the total number of successful amplifications at that locus (Broquet & Petit 2004). We added the measure of per locus finalized genotyping success (Piggott & Taylor 2003) to reflect the probability that a sample will produce a complete genotype. Finalized genotyping success was calculated using data from all PCRs, and was reached when the alleles of the consensus genotype (as defined above) were observed in at least two independent trials at a locus. If each allele was not seen twice or the sample failed entirely, the finalized genotype was not reached.

Statistical testing

Because our data were not normally distributed, we applied the Wilcoxon (1945) signed-rank test using SYSTAT v.11 software (SYSTAT Software Inc.) to compare results

from PCR amplification success rates, error rates because of allelic dropout and false alleles, and finalized genotype success by pairing the measures at each locus for the side and the tip of the scat samples in the wolf dataset and the outside and inside of the scat samples in the bear dataset. Results were considered statistically significant at $\alpha = 0.05$ level.

Results

Grey wolf—side vs. tip

The side samples had equal or slightly higher PCR amplification success rates than the tip samples at all but one locus ($Z = 1.89$, $P = 0.058$, Table 1). The side samples had lower allelic dropout rates ($Z = -2.24$, $P = 0.025$) at all but one locus and higher genotyping success ($Z = 2.52$, $P = 0.012$) at all loci compared with the tip samples. There was not a definitive trend towards the side or tip samples having higher error rates because of false alleles by locus ($Z = -0.94$, $P = 0.345$).

Brown bear—outside vs. inside

Polymerase chain reaction amplification success rate was equal for both the outside and inside samples ($Z = 0$,

Table 1 The PCR amplification success rates, error rates due to allelic dropout and false alleles, and finalized genotype success rate averaged by locus for the side and tip of 30 grey wolf (*Canis lupus*) scats

Locus	Sample section	PCR			
		success rate*	Allelic dropout†	False alleles	Genotyping success rate†
FH2001	Side	0.77	0.05	0	0.69
	Tip	0.62	0.21	0	0.58
FH2054	Side	0.63	0	0	0.58
	Tip	0.62	0.10	0.11	0.50
FH2088	Side	0.75	0.11	0.02	0.63
	Tip	0.70	0.17	0	0.52
FH2137	Side	0.67	0.24	0.05	0.64
	Tip	0.68	0.28	0.02	0.57
FH2611	Side	0.65	0.07	0	0.61
	Tip	0.58	0.36	0.09	0.39
FH2670	Side	0.65	0.21	0.08	0.60
	Tip	0.60	0.32	0.03	0.48
FH3725	Side	0.72	0.13	0	0.63
	Tip	0.72	0.08	0	0.56
Pez15	Side	0.60	0.29	0	0.48
	Tip	0.60	0.43	0.06	0.40
Average	Side	0.68	0.14	0.02	0.61
	Tip	0.64	0.24	0.04	0.50

* P -value < 0.1 for signed-rank test statistic.

† P -value < 0.05 for signed-rank test statistic.

Table 2 The PCR amplification success rates, error rates because of allelic dropout and false alleles, and finalized genotype success rate averaged by locus for the outside and inside of 25 brown bear (*Ursus arctos*) scats

Locus	Sample section	PCR success rate	Allelic dropout*	False alleles	Genotyping success rate*
G1D	Outside	0.64	0.20	0.08	0.60
	Inside	0.58	0.43	0.16	0.48
G10P	Outside	0.63	0.49	0.07	0.63
	Inside	0.67	0.56	0.02	0.48
Mu15	Outside	0.52	0.27	0.03	0.56
	Inside	0.58	0.49	0	0.39
Mu23	Outside	0.60	0.39	0.02	0.60
	Inside	0.56	0.45	0	0.44
Mu50	Outside	0.68	0.19	0.02	0.63
	Inside	0.75	0.31	0.02	0.5
Mu59	Outside	0.60	0.41	0	0.56
	Inside	0.54	0.38	0.13	0.57
cxd20	Outside	0.54	0.48	0	0.54
	Inside	0.46	0.52	0	0.50
Mu11	Outside	0.67	0.15	0	0.63
	Inside	0.71	0.33	0.06	0.50
Average	Outside	0.61	0.32	0.03	0.59
	Inside	0.61	0.43	0.05	0.48

* P -value < 0.05 for signed-rank test statistic.

$P = 1$, Table 2). The outside had lower allelic dropout error rates ($Z = -2.38$, $P = 0.017$) and greater consensus genotype success rates ($Z = 2.38$, $P = 0.017$) at all but one locus. There was no trend towards either the outside or inside having greater error rates because of false alleles at each locus ($Z = -0.94$, $P = 0.345$).

Discussion

We demonstrate that the sampling location within a faeces can have a significant effect on nuclear DNA quality and on our ability to obtain finalized genotypes for wolf and bear scats. Surprisingly, this effect has been largely ignored in the faecal DNA literature. We reviewed 123 wildlife molecular scatology studies from 1992 to the present, and found only 41% of all studies and 33% ($n = 45$) of canid and ursid studies specified the portion of the scat from which the sample was taken (see Appendix S1 in Supporting information). As DNA from the faeces is obtained from sloughed epithelial cells of the intestinal lining (Albaugh *et al.* 1992), most (76%) of the molecular scatology studies that mentioned sampling location within a faeces sampled exclusively from the outside of the scat. Our results are consistent with previous studies that evaluated faecal DNA extraction protocols and found that including inside faecal material,

alone (Wehausen *et al.* 2004) or through homogenization (Flagstad *et al.* 1999; Palomares *et al.* 2002; Piggott & Taylor 2003), decreased DNA quality and/or quantity. Variation in DNA quality based on different locations along the outside of the scat has not been previously assessed. Our results showing significantly higher DNA quality in faecal samples taken from the side of wolf scat compared with the tip indicate that cells are unequally distributed on the surface of scats. These results highlight the need for additional assessments of the impacts of the sampling location within a scat on DNA quality in other species.

Four different measures were used to quantify DNA quality, but only two of these measures were significantly different among sampling sections in both datasets. PCR amplification success and error rates because of false alleles were not significantly different by sampling section, but these two measures are less important indicators of differences in DNA quality as PCR amplification products alone do not necessarily reflect true genotypes and false alleles are more difficult to quantify and occur less frequently than allelic dropout errors (Broquet & Petit 2004). In contrast, the allelic dropout error rates and finalized genotyping success rates showed significant differences among samples taken from different locations within a faeces in both species demonstrating that the section sampled impacts the probability of obtaining a correct genotype. For example, choosing the optimal section in wolf and bear faeces increased the overall per sample finalized genotyping success by 10% and 16% respectively. Thus, in large-scale noninvasive genetic sampling projects biologists can increase the number of completed genotypes and maximize output per unit effort by identifying the optimal sampling location within a faeces.

To our knowledge, this is the first study to evaluate and detect differences in DNA quality based on field protocols that sample different sections on a single faeces. These results are particularly important when studying larger animals because it is cumbersome to collect the entire scat, and researchers often collect only a small portion. To maximize DNA quality and insure accurate genotyping success, we recommend sampling from the surface of ursid scats and the side of canid scats. Our literature review also provided support for sampling from the outside portion of a scat and emphasized the need for more attention to faecal sampling section in field protocols. We encourage other researchers to evaluate effects of faecal sampling location within faeces on genotyping success rates and highlight the fact that laboratory costs for this experiment were <\$600 (US) for each species. We anticipate that changing our field protocols to sample faecal sections with the highest DNA quality will increase our productivity and efficiency while decreasing our per sample costs.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Appendix S1 A literature review of 123 molecular scatology studies to determine if the location sampled within a scat was explicitly mentioned.

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