2003

The influence of diet on faecal DNA amplification and sex identification in brown bears (*Ursus arctos*)

Melanie A. Murphy
University of Idaho, melanie.murphy@uwyo.edu

Lisette P. Waits
University of Idaho, lwaits@uidaho.edu

Katherine C. Kendall
USGS-NRMSC Science Center, kkendall@usgs.gov

Molecular Ecology (2003) **12**, 2261–2265 doi: 10.1046/j.1365-294X.2003.01863.x

SHORT COMMUNICATION

The influence of diet on faecal DNA amplification and sex identification in brown bears (*Ursus arctos*)

MELANIE A. MURPHY,* LISETTE P. WAITS* and KATHERINE C. KENDALL†
Department of Fish and Wildlife Resources, University of Idaho, Moscow, ID, 83843, USA, †USGS Northern Rocky Mountain Science Center, Glacier Field Station, Glacier National Park, West Glacier, MT 59936–0128, USA

Abstract

To evaluate the influence of diet on faecal DNA amplification, 11 captive brown bears (*Ursus arctos*) were placed on six restricted diets: grass (*Trifolium* spp., *Haplopappus hirtus* and *Poa pratensis*), alfalfa (*Lupinus* spp.), carrots (*Daucus* spp.), white-tailed deer (*Odocoileus virginianus*), blueberries (*Vaccinium* spp.) and salmon (*Salmo* spp.). DNA was extracted from 50 faecal samples of each restricted diet, and amplification of brown bear DNA was attempted for a mitochondrial DNA (mtDNA) locus and nuclear DNA (nDNA) locus. For mtDNA, no significant differences were observed in amplification success rates across diets. For nDNA, amplification success rates for salmon diet extracts were significantly lower than all other diet extracts ($P < 0.001$). To evaluate the accuracy of faecal DNA sex identification when female carnivores consume male mammalian prey, female bears were fed male white-tailed deer. Four of 10 extracts amplified, and all extracts were incorrectly scored as male due to amplification of X and Y-chromosome fragments. The potential biases highlighted in this study have broad implications for researchers using faecal DNA for individual and sex identification, and should be evaluated in other species.

Keywords: diet, faecal DNA, noninvasive genetic sampling, PCR, sex identification, *Ursus arctos*

Received 29 January 2003; revision received 11 March 2003; accepted 11 March 2003

Introduction

Successful amplification of faecal DNA has been reported for a variety of mammalian species including herbivores (Flagstad *et al*. 1999), omnivores (Kohn *et al*. 1995) and carnivores (Reed *et al*. 1997; Kohn *et al*. 1999; Ernest *et al*. 2000). Faecal DNA amplification success rates vary considerably among species and individuals (Goossens *et al*. 2000), but the reasons for such differences are unclear. Some researchers have hypothesized that differences in diet may influence DNA extraction or amplification success rates (Reed *et al*. 1997; Farrell *et al*. 2000; Goossens *et al*. 2000; Huber *et al*. 2002). If this hypothesis is correct, there are a number of important implications for faecal DNA studies. For example, faecal DNA amplification success rates could vary seasonally as species utilize different food resources. In addition, individual differences in diet could make some individuals less likely to be sampled, leading to biases in mark–recapture estimates of population size.

Another potential complication in DNA analysis from carnivore faeces is amplification of nontarget prey DNA (Ernest *et al*. 2000; Lucchini *et al*. 2002). In most cases, nontarget DNA will not impact individual identification since microsatellite primers are specific to an individual species and its close relatives. However, due to the high degree of sequence conservation in the X and Y-chromosomes (Aasen & Medrano 1990; Griffiths & Tiwari 1993) and the use of conserved mammalian primers (Woods *et al*. 1999; Ernest *et al*. 2000; Lucchini *et al*. 2002), sex identification from faecal samples could be error prone when carnivores consume mammalian prey.

The objectives of our study are: (i) to quantify the influence of primary brown bear diet components on brown bear faecal DNA amplification success; (ii) to evaluate the impact of consumed male mammalian meat on female sex identification; and (iii) to determine the implications of our results for faecal DNA analysis of brown bears in our study area.

Correspondence: Lisette P. Waits. Fax: (208) 885–9080; E-mail: lwaits@uidaho.edu

Materials and methods

Diet conditions and sample collection

Eleven captive brown bears (three adult males, two adult females, one subadult male, one subadult female, two female yearlings and two female cubs) at Washington State University, Pullman, WA, USA were fed six restricted diets: grass (*Trifolium* spp., *Haplopappus hirtus* and *Poa pratensis*), alfalfa (*Lupinus* spp.), carrots (*Daucus* spp.), blueberries (*Vassinium* spp.), white-tailed deer and salmon (*Salmo* spp.). Each diet was fed to all bears and initiated at least 24 h before the first faecal collection, giving sufficient time for gut passage (Pritchard & Robbins 1990). Each restricted diet continued until 50 faecal samples were collected (3–5 days) and collection of samples was distributed evenly among individuals. For the grass diet, bears were allowed to free-graze on vegetation found in the facility's outdoor enclosure, which represented an analogue for the leaves and stems of forbs. Commercial alfalfa feed pellets (135 kg) were fed for the alfalfa diet. Carrots (160 kg) were fed uncleaned with the vegetative tops attached as a surrogate for tubers eaten in the wild. Blueberries, a huckleberry substitute (205 kg), were fed including twigs and leaves (10–15% by weight). Frozen white-tailed deer (170 kg) was thawed and cut into large unaltered pieces with all skin, bones and organs. Frozen whole salmon were thawed and five to eight intact fish were fed to each bear per day. Each faecal sample was less than 24 h old at the time of collection, and was collected, freeze-dried and ground according to Murphy *et al*. (2000).

DNA extraction and PCR amplification

DNA extractions were conducted in a laboratory dedicated to noninvasive and ancient DNA samples and spatially separated from polymerase chain reaction (PCR) products, blood, tissue and concentrated DNA to reduce the risk of contamination. A small amount of ground faeces was extracted (0.1–0.2 mL) using a QIAamp™ tissue kit (Qiagen™) protocol followed by a silica pellet method (Geneclean II) from Bio101 as described in Murphy *et al*. (2000). Each group of DNA extractions and PCRs contained one to three negative controls (reagents only) to monitor for contamination. All extracts were amplified using two loci: a 146 bp mtDNA segment used for species identification (Murphy *et al*. 2000) and one approximately 200 bp nDNA microsatellite locus commonly used for individual identification (G1A; Paetkau & Strobeck 1994). For mtDNA, PCR conditions were as follows: 20 μL reactions containing a final concentration of 1× Amplitaq buffer, 2.5 mM $MgCl_2$, 1.35 mg/mL bovine serum albumin (BSA; Sigma), 0.2 μM each primer, 0.05 mM each dNTP, 1.5 μL template and 0.5 U Amplitaq gold DNA polymerase (Murphy *et al*. 2000). For nDNA microsatellite locus (G1A), PCR conditions were as follows: 15 μL reactions containing 1× Amplitaq buffer, 2.5 mM $MgCl_2$, 1.35 mg/mL BSA (Sigma), 0.2 μM each primer, 0.1 mM each dNTP, 1.5 μL template and 1.5 U Amplitaq gold DNA polymerase (Murphy *et al*. 2000). An MJ Research thermal cycler was used with the following profile: initial 10 min at 95 °C, then cycles (45 for mtDNA and 55 for nDNA) of 30 s at 95 °C, 30 s at 44 °C (mtDNA) or 56 °C (G1A), and 40 s at 72 °C (Murphy *et al*. 2000). Each group of PCRs contained one to three positive controls (black bear, *Ursus americanus*, DNA extracted from blood or tissue) to monitor reaction efficiency.

Salmon faecal DNA extracts were tested for PCR inhibitors using two methods. First, the reaction volume was doubled to 30 μl, maintaining the reagent concentrations but adding only 1.5 μl DNA template, effectively diluting any PCR inhibitors. Second, PCR amplification was repeated under the original 15 μl conditions with the addition of 150 ng brown bear DNA. For all PCR amplifications, products were visualized under UV radiation on an ethidium bromide-stained 1.5% agarose gel with a 50 bp ladder. The products were scored as positive (product) or negative (no product). For all PCR amplifications, samples were considered successful when a band was observed in the expected size range; band intensity and PCR amplification errors were not evaluated. To minimize the impact of stochastic pipetting error, a second amplification was attempted on samples that did not produce a positive PCR product on the first attempt.

Ten additional faecal samples were collected from female bears who had consumed meat of a male white-tailed deer. Sex identification was performed on these samples using a multiplex PCR with the primers P15EZ/P13EZ (X-chromosome, about 130 bp, Woods *et al*. 1999) and 41F/121R (Y-chromosome, about 120 bp) (Griffiths & Tiwari 1993; Woods *et al*. 1999). For sex identification, PCR was attempted in 30 μl reactions containing a final concentration of 1× Amplitaq gold buffer, 2.5 mM each dNTP, 1.35 mg/mL BSA (Sigma), 0.2 μM each X-chromosome primer, 0.35 μM each Y-chromosome primer, 0.1 mM each dNTP, 3.0 μl template and 1.5 U Amplitaq gold DNA polymerase (Perkin-Elmer). PCR was performed on an MJ Research thermocycler with the following conditions: initial 10 min at 95 °C, then 55 replicates of the following cycle: 30 s at 95 °C, 30 s at 58 °C, 40 s at 72 °C, and a 2 min extension at 72 °C after cycles were complete. PCR products were fluorescently labelled (TET for X and 6-FAM for Y) and 1.0 μl of undiluted product was added to a mix containing 0.25 μl GS350 Tamra standard (PE Applied Biosystems), 0.30 μl loading dye and 1.45 μl formamide. Loading mixture was denatured at 96 °C for 2 min, loaded in a 6% long-ranger acrylamide gel on an ABI Prism 377 DNA sequencer (PE Applied Biosystems) and run at 2400 V for

1 h on a 12 cm gel. Gels were analysed with GENESCAN 2.0 and GENOTYPER 2.5 software (Applied Biosystems). Y-chromosome amplification was recorded when (i) the X and Y-chromosome loci amplified, (ii) Y-chromosome amplification was ≥ 50% the fluorescent intensity of the X-chromosome amplification, and (iii) amplification was replicated at least three times. DNA extraction and PCR set up was performed by females to avoid any human Y chromosome contamination.

Data analysis

All PCR amplification gel lanes (including negatives) were scored blind. Statistical differences between the six diets in PCR amplification success were evaluated by a chi-squared contingency table test of independence with $\alpha = 0.05$ and d.f. = (number of diets – 1). When a result was significant, the diet with the lowest success rate was removed and the test of independence was repeated with the remaining diets until no significance was observed at $\alpha = 0.05$ (Ott 1993).

Results

PCR amplification success rates

In total, brown bear faecal DNA amplified successfully in 76% (457/600) of PCR attempts. None of the negative controls (reagents only) for faecal DNA extraction or PCR amplification produced positive product, and all positive controls amplified the target locus. As expected, PCR amplification success was greater overall for mtDNA (88%) than for nDNA (65%, *P*-value < 0.001, Table 1). Mitochondrial DNA PCR amplification success rates ranged from a maximum of 92% (46/50) for grass and salmon diets to a minimum of 78% (39/50) for the blueberry diet (Table 1). Mitochondrial DNA PCR amplification success rates for each diet were not significantly different ($\chi^2_5 = 6.44$, *P*-value = 0.27).

Table 1 PCR amplification success rates for faecal samples collected from brown bears on restricted diets. The number of successful amplifications is shown in parentheses

Diet	mtDNA ($n = 50$)	nDNA ($n = 50$)
Grass	92% (46)	76% (38)
Alfalfa	88% (44)	72% (36)
Salmon	92% (46)	26% (13)*
Carrots	86% (43)	74% (37)
Deer	90% (45)	68% (34)
Blueberries	78% (39)	72% (36)
Total	88% (263/300)	65% (194/300)

*Significantly lower than results from all other diets (*P*-value < 0.001).

For the nDNA microsatellite locus, amplification success rates ranged from a maximum of 76% (38/50) for the grass diet to a minimum of 26% (13/50) for the salmon diet. PCR amplification success for the salmon diet was significantly lower than the other five diets (Table 1, $\chi^2_5 = 40.03$, *P*-value < 0.001). Faecal nDNA amplification success rates for the grass, alfalfa, carrot, deer and blueberry diets were not significantly different ($\chi^2_4 = 0.66$, *P*-value = 0.9715).

A subset of 22 salmon diet DNA extracts was tested for PCR inhibitors (see methods). When DNA extracts were diluted in 30 µl reactions, 23% (5/22) amplified the nDNA locus and all were samples that amplified the target locus in the original 15 µl reactions. When 150 ng of brown bear DNA were added to 15 µl reactions, 91% (20/22) of the samples successfully amplified nDNA with intensities comparable to the blood positive control.

Sex identification

Amplification of sex chromosome fragments was observed in four of the 10 faecal samples collected from female bears who had consumed male deer. Both the X and Y-chromosome fragments amplified in all four faecal samples, and all samples were scored as male. All Y-chromosome amplifications were 50–130% the fluorescent intensity of the X-chromosome amplification. The results were replicated four times for one sample and three times for the other three samples.

Discussion

Researchers have hypothesized that diet may impact faecal DNA amplification success rates (Reed *et al*. 1997; Farrell *et al*. 2000; Goossens *et al*. 2000; Huber *et al*. 2002). Our study is the first to directly quantify the influence of different diets on faecal DNA amplification and provides convincing evidence that some diets can lead to a significant decrease in nDNA amplification success rates. These results may help explain some of the variation in amplification success rates among species and also highlight a potential source of bias in faecal DNA surveys. If researchers are performing species ID of faecal samples to evaluate abundance, relative densities or habitat use for multiple species (Kohn & Wayne 1997), species-specific dietary differences that influence PCR success rates could lead to the over or under representation of some species. In addition, population and sex ratio estimation using faecal samples could be biased if some segment of the population is feeding extensively on foods that decrease amplification success rates.

While nDNA amplification of salmon faecal extracts was strikingly low compared with other diet extracts, no significant differences in mtDNA amplification success rates

were observed for all six of the restricted diets, and nDNA amplification success rates did not differ significantly among faecal extracts from the grass, alfalpha, carrot, blueberry or deer diet. Previous studies have suggested that plant secondary compounds may inhibit PCR (Huber *et al*. 2002), but we found no significant declines in success rates among the four plant diets, or when comparing the plant diets with meat diets. Differential amplification of faecal samples due to dietary differences of individuals or species may only occur for a small percentage of diet items. Additional work on other omnivores will be necessary to determine the universality of our findings.

The reason for low nDNA amplification success rates of salmon diet extracts is unclear. The high amplification success rates for the mtDNA fragment and our inhibition experiments suggest that PCR inhibitors do not explain our results. The high lipid and low fibre content in salmonids may lead to nDNA amplification difficulties due to a lower intestinal cell slough rate, or salmonid by-products may interfere with the extraction protocol chemistry. Faecal samples from other mammalian species that rely heavily on fish may also have low nDNA amplification success rates (Dallas *et al*. 2000; but see Reed *et al*. 1997). In populations where brown bears rely heavily on salmonids, individual identification may not be feasible without additional optimization of DNA extraction or PCR amplification methods since nDNA amplification success from the salmon diet faeces was so low (26%, Table 1). Also, avoiding microsatellite genotyping errors may require replication up to seven times per locus (Taberlet *et al*. 1996), making individual identification prohibitively expensive when success rates are low. Our study did not evaluate microsatellite genotyping errors, but some diets may cause increases in genotyping error rates and additional research is needed to evaluate this possibility.

The results of our experiments also demonstrate that sex determination in faecal DNA studies can be inaccurate when female carnivores or omnivores are preying on male mammalian meat. Errors in sex identification from faecal DNA analysis would bias sex ratios and all population parameters derived from sex ratio data. Thus, researchers who are using conserved mammalian primers for sex identification of carnivore faecal samples should address this potential problem. An important first step is evaluating the accuracy of sex identification using faecal samples collected from known individuals. For example, Ernest *et al*. (2000) analysed samples from cougars of known sex and discovered that both the X and Y-chromosome fragments amplified for three of four females, potentially due to the co-amplification of prey DNA present in the faeces. Lucchinni *et al*. (2002) addressed the potential inaccuracy of faecal DNA sex identification by sequencing the Y-chromosome fragment in a subset of their wolf faecal samples and demonstrated that all Y-chromosome products were wolf DNA and not prey DNA. Another potential solution is to develop species-specific primers. However, this is challenging as the Y-chromosome is highly conserved (Aasen & Medrano 1990; Griffiths & Tiwari 1993) and faecal DNA is degraded, so target loci must be short (< 250 bp) (Murphy *et al*. 2000).

The results of this study have two major implications for study design and implementation of faecal DNA sampling of brown bears in our study area, Glacier National Park, Montana, USA. First, the availability and use of diet components fluctuate seasonally for brown and black bear populations. Consumption of berries varies most widely, with a low of 1% of diet volume in the spring and a maximum of 81% of the diet volume in late summer (Fig. 1, Martinka & Kendall 1986). Our results suggest that high berry content in faeces will not impact mtDNA or nDNA amplification success. Bears in Glacier National Park rarely consume fish, so the reduced success rates from a salmonid diet are not a concern. However, insects (especially ants and moths) and white bark pine (*Pinus albacans*) seeds are important diet components for some bears in Glacier National Park. They were not included in the study due to the difficulty and expense of obtaining large quantities. Potential errors in sex identification from faecal DNA samples are a major concern for the Glacier National Park study, and sex ratio analyses should not be based on faecal samples alone. While mammalian meat comprises only 2–21% of the brown bear diet (Martinka & Kendall 1986), consumed male mammalian meat may still impact sex identification accuracy at low concentrations.

Faecal DNA analysis has the potential to be highly valuable for both conservation and management of elusive or endangered species. A better understanding of the factors that impact faecal DNA amplification success and accuracy is needed. The influence of diet on faecal DNA amplification success and accuracy should be tested in additional species with different feeding strategies, food

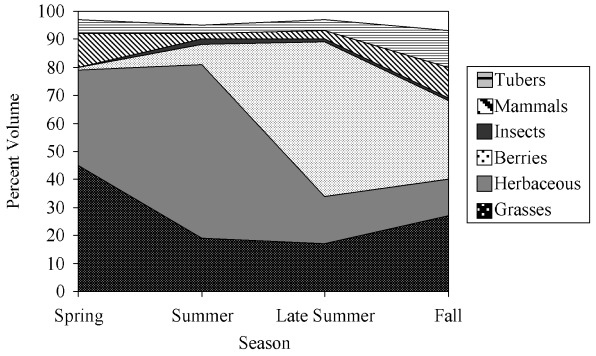

Fig. 1 Percent volume of brown bear diet components over time in Glacier National Park, Montana, USA. (Adapted from Martinka and Kendall 1986).

habits and physiology, and should be considered when designing faecal DNA sampling studies.

Acknowledgements

Major funding for the study came from the US Geological Survey Biological Resources Division, National Park Service, Canon Corporation, EarthWatch Institute and the National Fish and Wildlife Foundation. We would like to thank: Dr Sam Wasser and Christine Clarke for assisting in protocol optimization, Dr Charles Robbins for extensive use of the captive brown bear facility, Dr Rodney Mead and Dr Steve Brunsfeld for use of laboratory equipment, Christine Cegelski, Carol Perugini, Jeff Stetz and Eric Penn for laboratory assistance.

References

Aasen E, Medrano JF (1990) Amplification of the ZFX and ZFY genes for sex identification in humans, cattle, sheep and goats. *Biotechnology*, **8**, 1279–1281.

Dallas JF, Carss DN, Marshal F *et al.* (2000) Sex identification of the Eurasian otter *Lutra lutra* by PCR typing of spraints. *Conservation Genetics*, **1**, 181–183.

Ernest HB, Penedo MCT, May BP, Syvanen M, Boyce WM (2000) Molecular tracking of mountain lions in the Yosemite Valley region in California: genetic analysis using microsatellites and faecal DNA. *Molecular Ecology*, **9**, 433–442.

Farrell L, Roamn J, Sunquist ME (2000) Dietary separation of sympatric carnivores identified by molecular analysis of scats. *Molecular Ecology*, **9**, 1583–1590.

Flagstad O, Roed K, Stacy JE, Jakobsen SK (1999) Reliable noninvasive genotyping based on excremental PCR of nuclear DNA purified with magnetic bead protocol. *Molecular Ecology*, **8**, 879–884.

Goossens B, Chikhi L, Utami SS, Ruiter JD, Bruford MW (2000) A multi-samples, multi-extracts approach for microsatellite analysis of faecal samples in an arboreal ape. Conservation Genetics, 1, 157–162.

Griffiths R, Tiwari B (1993) Primers for the differential amplification of the sex-determining region Y gene in a range of mammal species. *Molecular Ecology*, **2**, 405–406.

Huber S, Bruns U, Arnold W (2002) Sex determination of red deer using polymerase chain reaction of DNA from feces. *Wildlife Society Bulletin*, **30**, 208–212.

Kohn M, Knauer F, Stoffella A, Schröder W, Pääbo S (1995) Conservation genetics of the European brown bear — a study using excremental PCR of nuclear and mitochondrial sequences. *Molecular Ecology*, **4**, 95–103.

Kohn MH, Wayne RK (1997) Facts from feces revisited. *Trends in Ecology and Evolution*, **1**, 223–227.

Kohn MH, York EC, Kamradt DA *et al.* (1999) Estimating population size by genotyping faeces. *Proceedings of the Royal Society of London*, **266**, 1–7.

Lucchini V, Fabbri E, Marucco F *et al.* (2002) Noninvasive molecular tracking of colonizing wolf (*Canis lupus*) packs in the Western Italian Alps. *Molecular Ecology*, **11**, 857–868.

Martinka CJ, Kendall KC (1986) Grizzly bear habitat and research in Glacier National Park, Montana. In: *Proceedings of the Grizzly Bear Habitat Symposium*, General Technical Report, US Department of Agriculture, Utah Int207, pp. 19–32.

Murphy MA, Waits LP, Kendall KC (2000) Quantitative evaluation of fecal drying methods for brown bear DNA analysis. *Wildlife Society Bulletin*, **28**, 951–957.

Ott L (1993) *An introduction to Statistical Methods and Data Analysis*. Wadsworth, Belmont, CA, USA.

Paetkau D, Strobeck C (1994) Microsatellite analysis of genetic variation in black bear populations. *Molecular Ecology*, **6**, 347–354.

Pritchard GT, Robbins CT (1990) Digestive and metabolic efficiencies of grizzly and black bears. *Canadian Journal of Zoology*, **68**, 1645–1651.

Reed Z, Tollit DJ, Thompson PM, Amos W (1997) Molecular scatology: the use of molecular genetic analysis to assign species, sex and individual identity to seal faeces. *Molecular Ecology*, **6**, 225–234.

Taberlet P, Griffin S, Goossens B *et al.* (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research*, **24**, 3189–3194.

Woods JG, Paetkau D, Lewis D, McLellan BN (1999) Genetic tagging of free-ranging black and brown bears. *Wildlife Society Bulletin*, **27**, 616–627.

Melanie Murphy received her M.S. at the University of Idaho working on noninvasive genetic sampling in brown bears. Lisette P. Waits is an associate professor at the University of Idaho. Her research group focuses on carnivore conservation genetics and molecular ecology. Katherine Kendall is an ecologist with the US Geological Survey Biological Resources Division at the Glacier Field Station. Her research interests include bear ecology and methods for monitoring population status.
