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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

Samuel K. Wasser
University of Washington, wassers@u.washington.edu

Jerry A. Higbee
Amplicon Express, jerry@genomex.com

See next page for additional authors

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An evaluation of long-term preservation methods for brown bear (*Ursus arctos*) faecal DNA samples

Melanie A. Murphy[1], Lisette P. Waits[1]*, Katherine C. Kendall[2], Samuel K. Wasser[3], Jerry A. Higbee[4] & Robert Bogden[4]

[1]*Department of Fish and Wildlife Resources, University of Idaho, Moscow, ID 83843, USA;* [2]*USGS Northern Rocky Mountain Science Center, Glacier Field Station, Glacier National Park, West Glacier, MT 59936-0128, USA;* [3]*Center for Conservation Biology, Department of Zoology, University of Washington, Box 351800, Seattle, WA 98195, USA;* [4]*Amplicon Express, 1610 NE Eastgate Blvd., Pullman, WA 99163, USA (*Author for correspondence, E-mail: lwaits@uidaho.edu)*

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Abstract

Relatively few large-scale faecal DNA studies have been initiated due to difficulties in amplifying low quality and quantity DNA template. To improve brown bear faecal DNA PCR amplification success rates and to determine post collection sample longevity, five preservation methods were evaluated: 90% ethanol, DETs buffer, silica-dried, oven-dried stored at room temperature, and oven-dried stored at –20 °C. Preservation effectiveness was evaluated for 50 faecal samples by PCR amplification of a mitochondrial DNA (mtDNA) locus (∼146 bp) and a nuclear DNA (nDNA) locus (∼200 bp) at time points of one week, one month, three months and six months. Preservation method and storage time significantly impacted mtDNA and nDNA amplification success rates. For mtDNA, all preservation methods had ≥ 75% success at one week, but storage time had a significant impact on the effectiveness of the silica preservation method. Ethanol preserved samples had the highest success rates for both mtDNA (86.5%) and nDNA (84%). Nuclear DNA amplification success rates ranged from 26–88%, and storage time had a significant impact on all methods but ethanol. Preservation method and storage time should be important considerations for researchers planning projects utilizing faecal DNA. We recommend preservation of faecal samples in 90% ethanol when feasible, although when collecting in remote field conditions or for both DNA and hormone assays a dry collection method may be advantageous.

Introduction

In small or elusive populations, non-invasive genetic sampling sources such as faecal DNA may be the only feasible method for obtaining genetic and baseline population data (Kohn and Wayne 1997). However, faecal DNA is technically difficult to amplify and contamination risk is high due to low template concentration (Gerloff et al. 1995). Genotyping errors often occur at microsatellite loci requiring several amplifications for accurate data (Gerloff et al. 1995; Taberlet et al. 1996, 1999). Identification of optimal preservation methods can improve PCR amplification success rates and increase the feasibility of using faecal DNA.

In this study, we compared the effectiveness of dry and liquid storage methods for preserving brown bear (*Ursus arctos*) faecal DNA over a six-month period. The objectives were: (1) to find a faecal DNA field preservation method with nDNA PCR amplification success rates higher than 75% and (2) to determine if PCR amplification success rates decline during six months of storage. Five preservation methods were compared and tested at one week, one month, three months and six months after collection: (1) 90% ethanol (Wasser et al. 1997), (2) DETs buffer

(Frantzen et al. 1998), (3) silica dried and stored at room temperature (Wasser et al. 1997), (4) oven-dried (Murphy et al. 2000) and stored at room temperature, and (5) oven dried (Murphy et al. 2000) and stored at –20 °C. The study is an important component of a research project evaluating the usefulness of faecal DNA for monitoring bear populations in Glacier National Park, Montana, USA.

Materials and methods

Faecal samples (n = 50) were collected from eight captive brown bears at Washington State University. Bear diet was restricted to alfalfa pellets, and each faeces was collected less than 24 hours after defecation. The entire faecal sample was homogenized to prevent non-uniform intestinal cell distribution from influencing results. Twenty subsamples were collected from each faeces, four subsamples per preservation method. The preservation methods included: 90% ethanol (Wasser et al. 1997), DETs buffer (Frantzen et al. 1998), silica-dried (Wasser et al. 1997), oven-dried (Murphy et al. 2000) stored on silica and oven dried stored at –20 °C.

Ethanol samples were collected at a 4 (ethanol):1 (faeces) ratio by volume (Wasser et al. 1997), mixed and stored at room temperature. The DETs buffer is composed of DMSO, EDTA, Tris and salt (Frantzen et al. 1998), and faeces were added at a 4 (DETs):1 (faeces) ratio. Silica-dried samples were placed in plastic freezer bags separated by filter paper from indicating silica at a 4 (silica):1 (faeces) ratio (Wasser et al. 1997; Murphy et al. 2000) and transferred to new silica when the indicant showed saturation. The indicant was monitored for moist air entering the bag and none was observed. Oven samples were dried according to Murphy et al. (2000). One set of oven-dried samples was stored at room temperature in airtight tubes with silica. The second set of oven-dried samples was stored frozen at –20 °C.

DNA extractions were carried out at one week, one month, three months, and six months in a laboratory dedicated to faeces, hair, and bone extractions (Murphy et al. 2000). DETs preserved samples were washed twice with 500 μl 1× phosphate-buffered saline before extraction to prevent interference with proteinase K digestion. Ethanol and DETs samples were extracted wet (0.2 ml faeces per extraction) and oven and silica samples were extracted dry (0.1–0.2 ml faeces per extraction) using a QIAmp tissue kit (Qiagen) with a modified protocol (Murphy et al. 2000). To evaluate effects of extracting samples wet, a subset of 21 matched samples (one week time point) were freeze-dried according to Murphy et al. (2000) and extracted wet and dry to evaluate differences in DNA amplification success. All extracts were concentrated and purified using a modified silica pellet method (Geneclean II kit Bio101, Murphy et al. 2000). All DNA extractions, DNA purification, and PCRs contained 1–3 negative controls (reagents only).

Preservation effectiveness was evaluated by two PCR amplification reactions: 146 bp mtDNA (IDL and H16145, Murphy et al. 2000) and 180–200 bp nDNA (microsatellite G1A, Paetkau et al. 1995) locus. PCR conditions are described in Murphy et al. (2000). The PCR products were separated on a 1.5% agarose gel and visualized using ethidium bromide staining. All lanes were evaluated product/no product according to Murphy et al. (2000). Genotyping errors were evaluated for all positive PCR products at time points one week and six months using a ABI Prism 377 sequencer. Gels were analyzed using Genescan 2.0 and Genotyper 2.5 (PE Applied Biosystems) software packages, and all samples were scored blind.

Faecal genotypes were established based on the multiple tubes approach (three repeats for heterozygotes and seven for homozygotes; Taberlet et al. 1996). Genotypes with sufficient replication are classified as the correct consensus genotype (CCG, Goosens et al. 2000). Faecal samples with insufficient replication or inconsistent patterns were classified as unknown (UK). For error rate analysis, total number of amplifications includes only faeces where the CCG was established. Genotype data that deviated from the CCG were classified into three categories: (1) false homozygote (FH), (2) false allele (FA), or (3) multiple alleles (MA). When the CCG was a heterozygote and only one of the alleles was represented in the PCR product, the error was referred to as a false homozygote. When the genotype contained allele(s) not present in the CCG but contained ≤ 2 alleles, the genotyping error was referred to as a false allele. When the PCR product contained more than two alleles or the pattern was too noisy to call, the genotyping error was classified as multiple alleles.

Significance of the faecal DNA PCR amplification results was evaluated using repeated measures within categorical modeling (CATMOD) in SAS (CATMOD; SAS Institute Inc. 1999) at $\alpha = 0.05$. CATMOD bases significance on a chi-squared statistic. MtDNA and nDNA results were tested for significance of preserva-

tion method, time, and preservation∗time interaction. Overall comparisons of success and error rates were tested using a chi-squared contingency test at $\alpha = 0.05$. For error rate analysis, the number of samples was reduced to include only those with a CCG and all error types were collapsed.

Results

Brown bear faecal DNA successfully amplified in 1371/2000 PCR reactions, no extraction or PCR negative controls amplified. For mtDNA (Table 1), the effects of preservation method (χ^2_4 = 50.00, P <0.0001) and an interaction between time and silica preservation method (χ^2_3 = 23.93, P <0.0001) were both significant. MtDNA PCR amplification success was significantly lower for the silica preservation method after one week (χ^2_4 = 20.93–51.04, P = 0.0003 – < 0.0001), but preservation effectiveness did not continue to decline (χ^2_2 = 1.12, P = 0.5706). For nDNA (Table 2), the overall effects of preservation method (χ^2_4 = 141.79, P < 0.0001) and time (χ^2_4 = 57.15, P <0.0001) were both significant. The interaction between time and preservation method was also significant for all preservation methods except ethanol: DETs buffer (χ^2_3 = 21.06, P = 0.0002), silica desiccated (χ^2_3 = 11.03, P = 0.0116), oven-dried stored on silica (χ^2_3 = 24.84, P < 0.0001), and oven-dried stored at –20 °C (χ^2_3 = 11.51, P = 0.0093).

When nDNA success rates were observed over time, the ethanol preservation method was not significantly different from the DETs buffer, oven-dried stored on silica, or oven-dried stored at –20 °C preservation methods at one week (χ^2_3 = 3.52, P = 0.3171). After one week, PCR amplification success rates have a significant interaction with time for the DETs buffer, oven-dried stored on silica, and oven-dried stored at –20 °C preservation methods (χ^2_3 = 16.97 – 20.42, P = 0.0007 – 0.0001, Table 2). The performance of the latter three preservation methods was not significantly different (χ^2_3 = 0.11 – 0.77, P = 0.4289 – 0.5217). Silica-dried samples had the lowest PCR amplification success rate out of all preservation methods (36.5%), and had a significant interaction with time (P < 0.0001). Three month time point success rates were slightly lower than six month success rates for some preservation methods, but this difference is believed to be spurious and is only statistically significant for oven dried samples stored on silica.

Extracting ethanol and DETs preservation samples wet did not negatively impact PCR amplification success rates. When 21 matched samples were compared at one week for mtDNA PCR amplification success, there was no significant difference between samples extracted wet or freeze-dried for ethanol (χ^2_1 = 2.10, P = 0.1472) or DETs buffer (χ^2_1 = 1.10, P = 0.2931). For nDNA, the success rates for wet versus freeze-dried are not significantly different for ethanol (χ^2_1 = 2.04, P = 0.1528), but PCR amplification success rates for freeze-dried DETs buffer samples were significantly lower (χ^2_1 = 18.70, P < 0.0001).

Eighty percent (262/326) of the samples had sufficient replicates to establish a CCG (Table 3) and all positive PCRs contained microsatellite product in the expected size range. Across all preservation methods, 70% (183/262) of the PCR products contained a genotype consistent with the CCG. The most common error was multiple alleles (18%), followed by false homozygotes (7%) and false alleles (6%). Silica dried samples had significantly higher error rates than the other preservation methods at both one week (χ^2_4 = 42.12, P < 0.0001) and six months (χ^2_4 = 10.68, P = 0.0319). The genotyping error rate for silica dried samples is influenced by a high frequency of MA (Table 3), but the increase over time was not significant.

Discussion

Three other studies have examined faecal DNA preservation effectiveness (Wasser et al. 1997; Frantzen et al. 1998; Murphy et al. 2000). Wasser et al. (1997) qualitatively evaluated freezing, freeze-drying, silica drying, 100% ethanol, and 30 other methods for preserving bear faecal DNA over six months and recommended silica-drying samples because it was the most practical for field application. In this study, silica preservation performed poorly after one week for mtDNA amplification and had the lowest success rates for nDNA amplification at all time points (Tables 1, 2). Silica preserved samples also had higher genotyping error rates than all other methods (Table 3). There are several potential reasons for the discrepancy in results. First, samples were freeze-dried by Wasser et al. (1997) before extraction. Freeze-drying silica preserved samples before extraction could remove any remaining moisture and potentially improve PCR amplification success rates. Second, Wasser et al. (1997) used airtight vials to desiccate and store silica preserved samples. Plastic freezer bags were tested

Table 1. PCR amplification success of brown bear faecal DNA extracts for a short (∼146 bp) mtDNA locus by preservation method and time point. Values inside the parentheses are number positive/number samples. Significance of the overall time and preservation method effects are indicated in the heading for the effect. Overall significance of a preservation method is indicated in the cell with the overall success rate for that treatment. Significance for column totals indicates a time effect for that treatment

	Ethanol	DETs	Silica	Oven/Silica	Oven/–20 ° C	Time effect*
One Week	92% (46/50)	88% (44/50)	78% (39/50)	80% (40/50)	82% (41/50)	84% (210/250)
One Month	78% (39/50)	78% (39/50)	52% (26/50)***	90% (45/50)	78% (39/50)	75% (188/250)
3 Months	80% (40/50)	96% (48/50)	42% (21/50)****	86% (43/50)	86% (43/50)	78% (195/250)
6 Months	96% (48/50)	92% (46/50)	50% (25/50)****	72% (36/50)	86% (43/50)	79% (198/250)
Preservation method effect****	87% (173/200)**	87% (177/200)	56% (111/200)****	82% (164/200)	83% (166/200)	79% (791/1000)

****P < 0.0001, ***P < 0.001, **P < 0.01, *P <0.05.

Table 2. PCR amplification success of brown bear faecal DNA extracts for a nDNA locus (180–200 bp) by preservation method and time point. Values inside the parentheses are number positive/number samples. Significance of the overall time and preservation method effects are indicated in the heading for the effect. Overall significance of a preservation method is indicated in the cell with the overall success rate for that treatment. Significance for columns indicates a time effect for that treatment

	Ethanol	DETs	Silica	Oven/Silica	Oven/–20 ° C	Time effect*
One Week	88% (44/50)**	80% (40/50)	56% (28/50)*	76% (38/50)	74% (37/50)	74.8% (187/250)
One Month	86% (43/50)***	50% (25/50)	30% (15/50)*	54% (27/50)	58% (29/50)	55.6% (139/250)
3 Months	74% (37/50)***	52% (26/50)	26% (13/50)*	32% (16/50)	46% (23/50)	46.0% (115/250)
6 Months	88% (44/50)***	52% (26/50)	34% (17/50)	52% (26/50)	52% (26/50)	55.6% (139/250)
Preservation method effect***	84% (168/200)	58.5% (117/200)***	36.5% (73/200)*	53.5% (107/200)***	57.5% (115/200)**	58% (580/1000)

***P < 0.0001, **P < 0.01, *P <0.05.

Table 3. PCR amplification error rates at microsatellite locus GIA for all faecal DNA preservation methods at one week and six months. Headers are as follows: CCG – correct consensus genotype (number CCG/number known genotypes), FA – false allele (number false alleles/number known genotypes), FH – false homozygote (number false homozygotes/number of known genotypes), and MA- Multiple alleles (number of multiple alleles/number of known genotypes). See methods for details on error type classification

		CCG	FA	FH	MA
Ethanol	1 week	81% (30/37)	2% (1/37)	5% (2/37)	11% (4/37)
	6 months	79% (26/33)	3% (1/33)	6% (2/33)	12% (4/33)
DETs	1 week	75% (24/32)	6% (2/32)	6% (2/32)	13% (4/32)
	6 months	71% (17/24)	8% (2/24)	4% (1/24)	17% (4/24)
Silica	1 week**	43% (9/21)	5% (1/21)	14% (3/21)	38% (8/21)
	6 months*	27% (3/11)	9% (1/11)	9% (1/11)	55% (6/11)
Oven/Silica	1 week	71% (22/31)	6% (2/31)	6% (2/31)	16% (5/31)
	6 months	60% (12/20)	10% (2/20)	10% (2/20)	20% (4/20)
Oven/–20 °C	1 week	76% (22/29)	7% (2/29)	3% (1/29)	14% (4/29)
	6 months	71% (17/24)	4% (1/24)	8% (2/24)	17% (4/24)
		70% (183/262)	6% (15/262)	7% (18/262)	18% (46/262)

**P < 0.0001, *P <0.05.

here because they are lighter and easier to carry into remote field conditions than vials. The bags were monitored for seal leakage and none was observed. Third, samples preserved with silica in this study molded and took up to 10 days to dry. Although molding may not occur under all field conditions, it has been observed in samples collected during the wet season (Kendall et al., unpublished data; Waits et al., unpublished data). When samples are collected with high moisture content, silica may not desiccate faeces quickly enough to prevent further DNA degradation. When samples are naturally desiccated in the field, preservation method may be of lesser importance (for example, see Kohn et al. 1999).

Frantzen et al. (1998) found baboon fecal samples preserved in DETs buffer had the highest PCR amplification success rates in a quantitative comparison including air-drying, freezing, and 70% ethanol. Our results suggest the DETs buffer performed equal with 90% ethanol for the first week, but time had a significant impact on nDNA PCR amplification success. Frentzen et al. (1998) also used a different extraction method which may lead to differences in results. In an evaluation of brown bear fecal desiccation methods, Murphy et al. (2000) concluded oven-drying was the most effective and practical method compared to silica desiccation and microwave drying; however, this study did not investigate liquid preservation methods or storage time.

Large-scale collections of bear faecal DNA samples are performed for two main purposes: (1) species identification using mtDNA and (2) individual identification using multilocus nDNA genotyping. Species identification from faecal DNA is practical using all preservation methods based on the low laboratory supply cost ($\sim$\$8 US) and relatively high success rates (78–92%, Table 1). For individual identification in the Glacier National Park brown bear population with probability of identity (PID) sibs $\leq$0.05 (Woods et al. 1999), data from 4–6 microsatellite loci are needed. The high nDNA amplification success for faecal samples preserved in 90% ethanol ($\geq$74%, Table 2) makes individual identification of brown bears in large-scale, high throughput studies ($>$500 samples) more feasible compared to other tested preservation methods. Success rates from field collected samples are expected to be lower since all faecal samples were fresh when collected and the most reliable nDNA locus was tested.

Preliminary field data support 90% ethanol as an effective faecal DNA preservation method. Faecal samples were collected from the Glacier National Park trail system in 1998 and 1999 and subjected to different preservation methods. In 1998, faecal samples were collected $\geq$3 times over the season from all trail systems on silica (4:1) and silica dried ($>$10:1) at the field station (four hours–seven days after collection) until no moisture remained in the samples. Some faecal samples from the field molded and took up to 10 days to dry, especially during the rainy season of May and June. In 1999, faecal samples were collected every two weeks from all trail systems in 90% ethanol and stored at room temperature until DNA extraction (six–15 months after collection). The mtDNA PCR amplification success rate for samples dried on silica in 1998 was 66% (351/529), and the multi-locus nDNA microsatellite genotyping success rate was 29% (6/21). PCR products were qualitatively "noisy" and difficult to score, supporting the observed multiple alleles from silica preserved samples (Table 3). The mtDNA PCR amplification success rate for samples collected in 1999 was 87% (443/507) and multi-locus nDNA microsatellite genotyping success rate was 42% (9/21). PCR products were qualitatively much easier to score than samples from 1998 and fluorescent intensity increased $\sim$50%.

Conclusions

Low success rates and high error rates at nDNA microsatellite loci have two main implications for application of faecal analysis to large-scale studies. First, fewer samples can be identified to individual. Second, the number of PCR reactions needed to obtain sufficient replication for a CCG will increase, escalating analysis time and cost. It is critical to maximize success rates and minimize error rates by choosing the most effective faecal DNA preservation method. We recommend collecting faecal DNA samples in 90% ethanol at $\geq$4:1 ratio by volume (12 ml ethanol: 2–3 ml faeces). Collection of faecal DNA samples in ethanol has potential limitations. Buffer tubes can leak; high-grade ethanol can be difficult to obtain; ethanol transportation by air may be restricted; and ethanol preservation may not be optimal for hormone analyses (Hunt and Wasser unpublished data). If a dry collection method is required, samples should be collected on silica and desiccated quickly by some other method.

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