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# Use of Feces as a Source of DNA for Individual Identification of Wolves of Denali National Park and Preserve

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## Abstract

To determine movements of wolves within and surrounding the Denali National Park and Preserve (DPP), Alaska, and better understand the impact of harvest on wolves there, the NPS seeks to use DNA extracted from feces to determine individual identity of wolves. We conducted a successful exploratory analysis of DNA extracted from a small number of fecal swab samples (n = 22, comprising 11 paired fecal samples) collected from locales in DPP during January 2015. Following that exploratory analysis, we leveraged extant wolf genetic datasets and multilocus microsatellite marker suites in a pilot study to determine whether DNA extracted from feces collected during summer months would yield reliable data for determining individual identity. The primary goals of the pilot study were to 1) determine if the marker suites used in the exploratory analysis were sufficiently reliable and provided adequate resolution for use in individual identity of wolves using DNA extracted from fecal samples, as suggested by the exploratory analysis; and 2) test the use of the method on fecal samples collected outside the winter season. We also tested a novel sexing marker, developed for a mustelid species, for utility in sexing wolves using DNA extracted from fecal samples.

Results of this pilot study, which used a 6-locus suite of microsatellite marker and data from a carnivore-specific sexing marker, suggest that sufficiently robust microsatellite data for use in genetic tagging efforts can be obtained from feces sampled by swabbing during winter months (January through March) in DPP. However, we found that sampling during summer months (July and August) may not provide sufficient (or sufficiently reliable) data for use in determining identity. We recommend that if genetic markers are to be used as individual tags from samples collected non-invasively during summer months, an alternative method, such as use of DNA extracted from guard hairs, be applied. We also recommend substituting at least one marker used in the 6-locus suite with another that performs better when used to genotype using DNA extracted from fecal samples.

## Introduction

The USGS Alaska Science Center has conducted genetic research on wolves of Alaska, starting in 1998 and continuing through the present (Weckworth et al. 2005, 2010, 2011, 2015; Talbot et al. 2011a), and this has included the collection of genetic data from wolves of Denali National Park and Preserve (DPP). The primary goal of those studies has been to assess levels and partitioning of genetic diversity in wolves occupying different locales in Alaska. However, we have demonstrated that a subset of the same markers used to assess phylogeographic relationships can also be used to determine individual identity in carnivore species, including wolves, even from non-invasively collected low quality DNA sources, such as hairs, saliva surrounding bite marks, and feces (Talbot et al. 2011a, Farley et al. 2014).

Non-invasively collected samples have been used for many years in ecological and demographic surveys intending to estimate population parameters using individual identity determined from DNA, including DNA extracted from feces (Broquet et al. 2006, Eggert et al. 2005, Ramón-Laca et al. 2015, Schwartz and Monfort 2008). Determining genetic identity from non-invasively collected samples is often called "genetic tagging" (Palsbøll et al. 1997). Among the suite of low-quality and low-quantity DNA sources for use in genetic tagging, such as hairs and feathers, feces counts as one of the lowest-quality sources. This is due not only to low target DNA concentrations in fecal samples, but also to the DNA degradation and co-purification of polymerase chain reaction (PCR) inhibitors from the digestive system, the diet, and from soil. Combined, these limitations can exacerbate the generation of PCR artifacts — including allelic dropout and false alleles (Opel et al. 2010) — when using DNA extracts from fecal samples. Our prior research (see Farley et al. 2014) suggests that the quality of DNA extracted from low-quality DNA sources increases when swabs are used to collect samples, and when those swabs are stored in Longmire Buffer (Longmire et al. 1988) immediately following collection. This was recently tested by other researchers from DNA extracted from swabs used to collect intestinal epithelial cells on the external surfaces of feces of various mammalian species (Ramón-Laca et al. 2015).

To determine movements of wolves within and surrounding the Denali National Park and Preserve (DPP), Alaska, and better understand the impact of harvest on wolves there, the NPS seeks to use DNA extracted from feces to determine individual identity of wolves. In support of that goal, we conducted an exploratory genotyping analysis using DNA extracted from a small number of fecal swab samples (n = 22, comprising 11 paired fecal samples) collected from locales in DPP during January 2015. That exploratory analysis suggested the approach might be feasible, but the samples used were collected only during the winter and may not have reflected success rates based on samples collected during warmer months. We therefore leveraged our extant wolf genetic datasets and multilocus microsatellite marker suites to conduct a pilot study to determine whether DNA extracted from feces collected during different months, including summer months, would yield reliable data for use in individual identity. The goals of the pilot study were to 1) determine if the marker suites used in the exploratory analysis were sufficiently reliable and provided adequate resolution for use in individual identity of wolves using DNA extracted from fecal samples, as suggested by the exploratory analysis; and 2) test the use of the method on fecal samples collected outside the winter season. We also 3) tested a novel sexing marker, developed for a mustelid species, for

utility in sexing wolves using DNA extracted from fecal samples, and 4) provided recommendations for sampling based on the results of the initial exploratory analysis.

# Methods

## Sampling recommendations

Based on our experience with the use of epithelial cells as a source of DNA (Handel et al., 2006, Farley et al. 2014, Roffler et al. 2014, Hollenbeck et al. 2017), we provided recommendations for collection of swabs, as follows:

1) Collect two swab samples for each specimen (feces pile), and store them in separate microcentrifuge tubes containing 1mL of a field preservation buffer [100mM TRIS pH 8.0, 100mM EDTA, 10mM NaCl, 0.5% SDS (w/v); Longmire et al., 1988].
2) Collect samples as soon after defecation as possible, and if sampling cannot occur immediately, then store the frozen feces in a freezer (-80°C recommended; -20°C adequate) for subsequent sampling.
3) Conduct sampling on frozen feces, to the extent possible.
4) In the collection of swab samples, wipe the external surfaces of a fecal sample with a sterile foam-tipped applicator (swabs) for a total of 5-10 swipes per feces pile.
5) Use commercially-available sterile swabs (REF 25-1506 1PF: Puritan), which are packaged in individually wrapped sterile paper sleeves.
6) Store any hairs dry in envelopes, preferably in proximity to silica gel.

## DNA extraction protocols

All fecal swab samples (n = 352) stored in Longmire Buffer, and a set of hair samples (n = 72) stored in envelopes along with silica gel powder, were forwarded by NPS collaborators to the USGS Alaska Science Center Molecular Ecology Laboratory (MEL) for processing. Prior to extraction of DNA from swab samples, we rolled the swab around the inner diameter of the storage microcentrifuge tube to dislodge epithelial cells into the Longmire buffer. After mixing with a pipette, we transferred approximately 130µL of the buffer, presumably containing epithelial cells from the outside surfaces of the feces, to a clean, sterile microcentrifuge tube for extraction. Hair samples, which comprised mostly tufts of underfur, were examined to identify follicles and >10 individual hairs, including hairs with follicles (when present) per tuft were placed in microcentrifuge tubes for extraction. Extraction procedures followed Medrano et al. (1990) using modifications reported in Sonsthagen et al. (2004), Talbot et al. (2011b), and Farley et al. (2014).

## Baseline datasets and marker selection

We referred to extant genotype data already available from 39 wolves sampled from the Denali area (SLT, GKS, and M. Flamme, NPS), referred to herein as the 'DPP baseline dataset,' to select an appropriate suite of markers for individual identification from among 15

autosomal loci from which data were collected using methods similar to those reported in Weckworth et al. (2005). To aid in selecting the marker suite, general level of error for each locus due to allelic dropout, null alleles, or scoring error, was assessed for the each of the 15 loci in the DPP baseline dataset using MICROCHECKER (van Oosterhout et al. 2004) and all loci were tested for deviation from Hardy Weinberg Equilibrium (HWE) using Genepop '007 (Raymond and Rousset 1995, Rousset 2008).

Genetic diversity values and locus-specific probability of identity ($P_{ID}$) values for the 15 loci assayed for the DPP baseline dataset are given in table 1. Based on our earlier exploratory analyses of 22 fecal samples collected during 2015, we selected 6 (C30, C203, C204, C213: see Weckworth et al. 2005; Ren144, Ren199, see McNay 2005) among the 15 loci for use in genetic identification in the pilot study. Although our locus selection criteria included the use of locus-specific and combined $P_{ID}$ values (both $P_{ID}$ and $P_{ID}$*sib*, Waits et al. 2001; see table 1), we also considered: 1) fragment size (Paetkau 2003); and 2) marker performance, based on previous research on wolves elsewhere in Alaska (Weckworth et al. 2005, SLT and GKS, unpublished data). Thus, for example, although locus AHT121 (see McNay 2005) had higher $P_{ID}$ values than all or most others selected (table 1), that locus presented a potential problem when used in non-invasively collected wolf samples. Primers for locus AHT121 yields a large fragment size in the target populations (allele size range was wide, ranging from 190-208bp), which may foster allelic dropout (Paetkau 2003, Sefc et al. 2003) and so was not selected for initial inclusion in the 6-locus marker suite. Other loci showed periodic failure to amplify robustly in non-invasively collected samples or demonstrated low levels of genetic variation (table 1) although several (C173, C250, C109 and C123) appear to be robust and can be used to augment the marker suite if deemed necessary. We observed that C213 deviated from HWE in the DPP baseline dataset (table 1), and analysis using MICROCHECKER suggested this may be due to the presence of null alleles. However, we have not consistently observed this pattern for locus C213 in other high latitude wolf populations (Weckworth et al. 2005; SLT and GKS, unpublished data) and suggest the deviation from HWE at that locus is likely due to population-specific demography rather than to the presence of null alleles. Analysis by MICROCHECKER did not find any evidence of null alleles at any other locus.

The selected 6-locus suite generated $P_{ID}$ and $P_{ID}$*sib* values within bounds recommended by Waits et al. (2001), suggesting this marker suite can distinguish one individual from among more than 1.3 million other wolves (given a randomly breeding population), and from among more than 230 other wolves selected from among first-order relatives ($P_{ID} = 7.5383 \times 10^{-7}$; $P_{ID}$*sib* $= 4.31 \times 10^{-3}$). We caution that $P_{ID}$ values provide useful guidelines for initial selection of markers, but the multilocus panel may still need to be modified or augmented for future use, based on results of the pilot study and/or during the course of subsequent data collection. All loci had a minimum expected heterozygosity ($H_E$) value of 0.69, as recommended by Paetkau (2003) for a 6-locus suite, except for one locus (C204; see table 1); the average $H_E$ for the entire 6-locus suite was 0.74.

## Microsatellite genotyping

Primers targeting the 6 variable nuclear dinucleotide repeat microsatellites for amplification were used to generate multilocus genotypes using methods similar to those described in Weckworth et al. (2005). Because we generated data from 2 samples per fecal pile, quality control based on duplicate, independently extracted and processed samples was 100%; nevertheless, we also extracted DNA from an additional 1% of the samples.

**Table 1**. Parameters of genetic diversity and information content of 15 microsatellite loci estimated from wolves of Denali (SLT, GKS and M. Flamme, unpublished data). Loci are shown in descending order of single locus unbiased $P_{ID}$ values. Loci in rows shaded green were selected for analyses (see text for a discussion of selection criteria). Observed heterozygosity values at locus C213 deviated significantly from Hardy Weinberg equilibrium in DPP wolves, due to heterozygosity deficit, and MICROCHECKER suggested this may be due to the presence of null alleles ($P < 0.05$)

| LOCUS | NUMBER OF ALLELES (A) | OBSERVED HETEROZYGOSITY ($H_O$) | EXPECTED HETEROZYGOSITY ($H_E$) | PROBABLITY OF IDENTITY ($P_{ID}$)[a] | PROBABLITY OF IDENTITY ($P_{ID\,sib}$)[b] |
|---|---|---|---|---|---|
| **AHT121** | 9 | 0.85 | 0.86 | 2.69E-02 | 3.32E-01 |
| **C30** | 12 | 0.77 | 0.83 | 2.99E-02 | 3.44E-01 |
| **C213** | 11 | **0.28** | **0.83** | 4.05E-02 | 3.51E-01 |
| **C173** | 5 | 0.90 | 0.76 | 7.73E-02 | 3.90E-01 |
| **REN144** | 7 | 0.87 | 0.76 | 8.11E-02 | 3.94E-01 |
| **C203** | 7 | 0.62 | 0.72 | 9.90E-02 | 4.18E-01 |
| **REN199** | 5 | 0.79 | 0.73 | 1.05E-01 | 4.12E-01 |
| **C250** | 6 | 0.82 | 0.68 | 1.11E-01 | 4.42E-01 |
| **C109** | 5 | 0.67 | 0.70 | 1.30E-01 | 4.39E-01 |
| **REN112** | 4 | 0.56 | 0.65 | 1.62E-01 | 4.67E-01 |
| **C123** | 4 | 0.54 | 0.61 | 1.73E-01 | 4.92E-01 |
| **C225** | 4 | 0.54 | 0.60 | 1.96E-01 | 5.04E-01 |
| **C377** | 6 | 0.64 | 0.62 | 1.99E-01 | 4.91E-01 |
| **C204** | 4 | 0.51 | 0.58 | 2.51E-01 | 5.27E-01 |
| **C172** | 2 | 0.49 | 0.37 | 4.50E-01 | 6.82E-01 |
| | | | | | |
| **AVERAGE/ OVERALL** | **6.07** | **0.66** | **0.69** | **3.78E-15** | **4.20E-06** |

[a] $P_{ID}$ is the probability at which another individual with the same genotype would be observed, given the sample frequency of the alleles observed at those loci, within the population.

[b] $P_{ID\,sib}$ estimates the probability of observing identical multilocus genotypes between two individuals sampled from a population comprised of first-order relatives (e.g., between siblings or parent-offspring).

We used GeneCap (Wilberg and Dreher 2004) to identify matches as well as pairs of genotypes that mismatched at 1 or 2 alleles at each of the 6 loci (1MM- and 2MM-pairs, respectively). Results of the analysis are provided in the Appendix and various associated tabs therein. Data from feces collected during 2015, assayed as part of the exploratory analysis were included in the analysis along with samples collected in 2016. All 1MM- and 2MM-pairs were examined to identify cases where different samples from the same individual have non-identical genotypes, as a result of genotype error. The final dataset (see Appendix, *Data* tab) contained 28 1-MM pairs and 74 2-MM pairs (Appendix, *1 allele difference* and *2 allele difference* tabs). The 1MM-pairs involved genotypes found in 2 samples (1789 and 1790, collected in 2015) mismatching a genotype found in 14 other samples (Appendix, *1 allele difference* tab). The mismatch with the other samples occurred as a result of an allele (136) at locus C203, which was not present in the other 14 samples (Appendix, *1 allele difference* tab). Examination of the raw data image did not resolve this issue; we were unable to determine whether that allele was a false allele and samples 1789 and 1790 were the same individual as the individual represented by 2016 sample 1893 (Appendix, *1 allele difference* and *Matches* tabs) or whether 1789/1790 were first order relatives with the individual represented by 2016 sample 1893. We retained that pair (1789/1790) as a separate individual until further analysis with additional loci can be performed.

The 2MM-pairs were not pursued to the same degree, but each pair was reviewed to confirm that the genotypes were replicated either from multiple samples, or tagged for further analysis with other loci (Appendix, *2 allele difference* tabs). We also reanalyzed or discarded any mismatching markers that we suspected conformed to a pattern expected in the case of allelic dropout, which is the type of genotyping artifact that most often results in DNA samples from poor DNA sources (Paetkau 2003).

## Molecular sexing

To determine sex of the individual wolves, we tested a primer set we developed specifically for wolverine (*Gulo gulo*) as part of another study. That study leveraged sequence data obtained from wolverine and wolves (specifically, a female wolf from Chignik Bay, Alaska) by sequencing product using the primers LGL331 and LGL335 (Shaw et al. 2003), which target an intron within the mammalian zinc-finger x (*Zfx*) and the zinc-finger y (*Zfy*) genes (Kresja 2017). We used sequencing procedures reported elsewhere (Fischbach et al. 2008). That reaction amplifies a >950 bp product that requires fragment separation via electrophoresis on agarose gels, which we deemed too large to reliably determine sex using DNA extracted from low quality source materials, such as feces. We used the sequence data to develop a second forward primer (MustelaZF) that, together with LGL335 yielded a smaller fragment size than that reported in Shaw et al. (2003); details of the development are provided in Krejsa (2017).

The primer MustelaZF (5' – GMAATCATTCATGAATAT – 3') along with LGL335 targets (bp) products from the *Zfx* and *Zfy* gametologs of wolves (in both males and females) that differ in size by approximately 36 base pairs (bp). This estimate is based on sequence data from the *Zfx* gametolog of the female wolf from Chignik, AK (GenBank Accession No. pending) and the *Zfy* gametolog of a male wolf sampled from China (GenBank Accession No. KT448252). However, based on comparison of sizes against a fluorescently-labeled M13 sequence ladder of known size, the size difference between the *Zfx* and *Zfy* fragments generated using the MustelaZF and LGL335 primers, applied to DNA extracted wolves of Alaska, is estimated at 16 bp. While this not as large as predicted based on the *Zfy* sequence data from a male wolf in China, it is sufficiently large to facilitate the determination of sex of wolves sampled from Alaska, even using agarose gel as an electrophoresis medium, although we electrophoresed sexing reactions on one of our automated genotyping platforms (typically LICOR IR$^2$, LI-COR, Lincoln, NB). We tested the primers against known-sex wolves of the

DPP area to ensure they indeed successfully and reliably determined sex in wolves there, then tested them using DNA extracted from feces samples collected by NPS researchers in 2015. We assigned sex based on the absence (female: 204/204) or presence (male: 188/204) of the band for the Y-chromosome (assigned sizes do not include the size of the added universal tail; see Krejsa 2017). Sex assignments by sample are provided in Appendix (Appendix, *Data* and *Matches* tabs).

## Results and Discussion

### Success rates

There are two regular correlates of success rate for use of DNA from low-quality sources for individual identification: collection period, and sample quality. These two correlates are not independent; collection period may impact sample quality. For example, we predicted that DNA extracted from feces sampled during winter months, when feces freeze following defecation and remain frozen, would yield a higher proportion of useable genotypes than DNA from feces defecated during summer months. Thus, one goal of this pilot study was to determine whether there were seasonal differences in our ability to leverage DNA collected from feces samples.

Following examination of each locus for each individual, PCR products were classified into three categories:

1) Reliable: the genotype at that locus is considered to be an accurate representation of the actual genotype. This is based on a combination of factors; for example, the presence of two alleles (heterozygotes) at a locus typically negates the likelihood of allelic dropout, as do consistent allele scores for paired samples. We acknowledge that researcher experience facilitates the assessment of PCR product reliability (Paetkau 2003).

2) Unreliable: the genotype at that locus is considered to be an inaccurate representation of the actual genotype, due to the likely presence of allelic dropout or false alleles. Allelic dropout can only result in a homozygous genotype, and was assessed when a locus was called a homozygote in only one of two paired samples. False alleles were assumed in cases where the pattern of the putative allele differed from what is typically observed for that allele. We note that false alleles are easily detectable using our direct gel-image automated genotyping platform, but acknowledge that researcher experience further facilitates the detection of false alleles (Paetkau 2003).

3) No product: the PCR reaction did not yield a product.

Based on this classification by locus and sample, we observed distinct differences between seasons in the current project (fig. 1); the greatest proportion of reliable data was produced from samples collected during the winter months (January, February, March). Of the 352 fecal swab samples collected from 176 fecal piles between 8 January and August 7, 2016, 93 samples produced data deemed suitable for individual identification. Most of these were collected during winter months, with 81.3% of these samples yielding a reliable multilocus genotype. (January, n = 7/18 swabs [9 fecal piles]; February, n = 43/104 swabs [52 fecal piles]; March, n = 37/92 swabs [46 fecal piles]). Two additional samples judged to be useable were not accompanied by a collection date. Of the 128 fecal swab samples collected from 64 fecal piles during July-August, those collected from only 6 fecal piles (9.4%) produced data suitable for individual identification.

Hair samples collected in July and August, 2016, also provided scant data. Follicles (1-7) were observed in 23 among the 72 (~32%) hair samples received. Among the 72 hair samples, none produced a 6-locus genotype and only 3 (H36, H40 and H59, Riley Creek Sanctuary, Den 16) produced multilocus genotypes (4-5 loci only) deemed sufficiently reliable to identify an individual (Appendix, *Data* tab). The three individuals identified from Riley Creek Sanctuary, Den 16, did not appear to be first order relatives, as they did not share at least one allele per locus. The envelope that stored sample H36 also contained what appeared to be blood stains, and the underfur from sample H40 contained at least one follicle; this may have facilitated our ability to collect DNA from those samples. However, sample H59 was only from underfur (no follicles observed). We note here that locus Ren199 failed for these hair samples, and that locus also routinely failed for all hair samples, and is the locus that routinely failed in fecal swab samples (data not shown).

We observed two cases of allelic dropout in the sexing marker (see Appendix*, Matches* tab). Allelic dropout was determined based on its observation in cases where multiple samples had the same multilocus genotype (for example, the individual represented by sample 1771-1, determined to be a male across 11 samples, and a female in 1 sample; see lines 18-24 in Appendix, *Matches* tab). Since sex is determined based on the presence of two alleles for males, and one allele for a female, allelic dropout is expected to impact only samples from males, resulting in a male erroneously being called a female. Allelic dropout in females would be put into the 'no product' category.

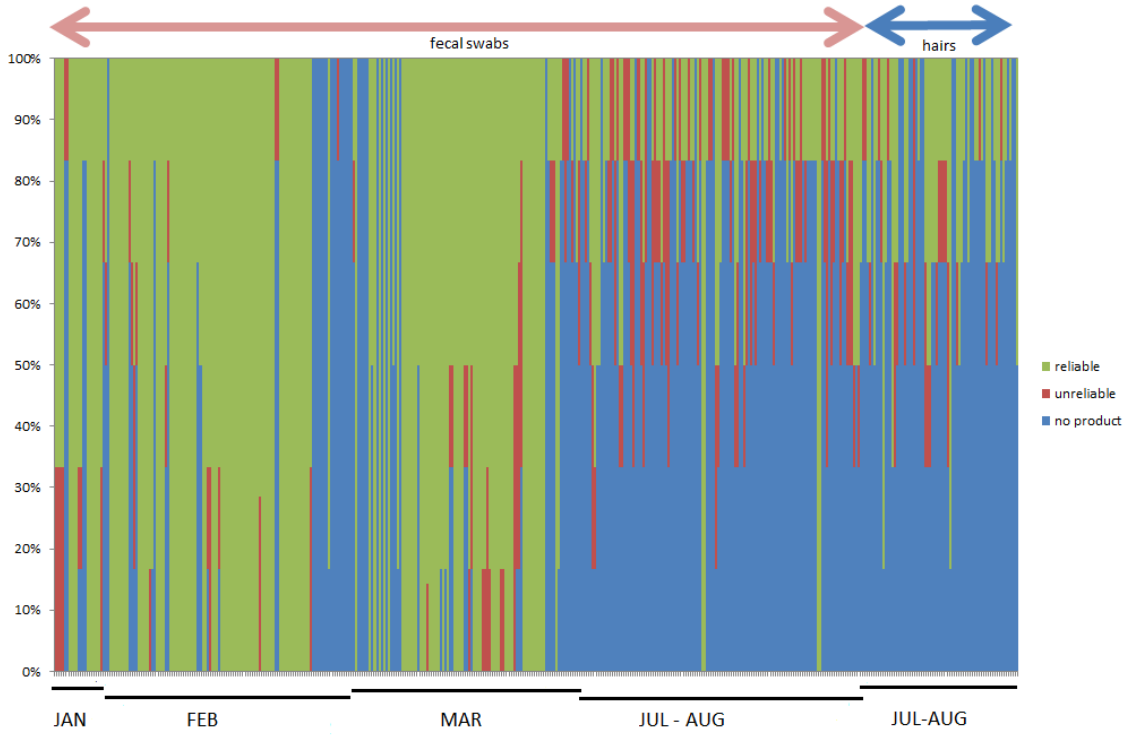



**Figure 1**. Classification of fecal swab samples by month with regard to results of genotyping a 6-locus panel of microsatellite loci. Samples are classified as follows: reliable = genotypes obtained from sample provided scores that were judged to be adequate for use in individual identity; unreliable = genotypes that were judged to be inadequate for use in individual identity; and no product = no PCR product observed. See text (Success rates section) for details.

## Individual identification and recaptures

Once genotypes were completed and checked for errors, individuals were defined for each unique genotype in the dataset. Identification numbers were taken from the first sample assigned to each individual (see Appendix, *Matches* tab, column B). Starting with 106 samples judged as sufficiently reliable to be included as potential individuals (Appendix, *Data* tab), we identified 33 individuals, although given uncertainty associated with decisions for 1-MM and 2-MM samples, the number of individuals in the dataset may be as few as 26 and as many as 33 (see Appendix, *Command* tab). Among the 33 putative individuals in the dataset were 10 that were genotyped from more than one fecal pile locale (see Appendix, Matches Tab), including three instances (two females, 1769 and 1893; and one male, 1913) in which their unique multilocus genotype recovered in 2016 was observed in samples collected in 2015 and analyzed as part of the initial exploratory analysis.

One pair of samples collected in 2015 (1789 and 1790) were deemed to represent a single individual, but this is the pair that may include a false allele (136) for locus C203 and may be the same individual as 2016 sample 1893. An alternative explanation is that there is allelic dropout at locus C203 for the 1893 matching group (for which the multilocus genotype was present in a total of 15 samples). The latter explanation would require that allelic dropout at locus C203 to have occurred 15 times. Data replication (multiple samples yielding the same genotype) provide strong support for the accuracy of the multilocus genotype shared by sample 1893 and the remaining 14 samples, and some support for the accuracy of the multilocus genotype shared by samples 1789 and 1790, since the most common sources of errors — allelic dropout and amplification of false alleles — are not expected to be reproducible (Waits and Paetkau 2005). A third explanation is that these two multilocus genotypes (1789/1790 and 1893) are first-order relatives.

## Species determination

We assumed that all samples derived from feces left by wolves, but coyotes (*C. latrans*) do occur in Alaska and their range includes locales in and around DPP (MacDonald and Cook 2009). As well, there are likely to be domestic dogs (*C. familiaris*) in the area. Thus, it is possible that some of the samples were from canids other than wolves. While we can distinguish coyotes from wolves using nucleotide data from the mitochondrial genome (Talbot et al. 2011a), we have not obtained sufficient samples from coyotes (preferably 30 samples) to test for species-specific alleles/genotypes in the microsatellite panel we are currently utilizing. Our microsatellite dataset for domestic dogs is sparse, comprising data from only 10 dogs.

We used factorial correspondence analysis (FCA) option in GENETIX (4.0.5; Belkhir et al. 1996-2004), to plot multilocus genotypes of the different samples on a two-dimensional grid and visualize relationships among the fecal swab/hair samples. FCA identifies relationships between variables in the absence of *a priori* expectations as to the underlying nature of the relationships, and unlike principal components analyses is a factorial decomposition of contingency tables with a primary goal of providing a graphic display of tabulated numerical information (Greenacre 1984). FCA analysis was based on the 6 microsatellite marker suite and includes data (from only 4 of the 6 markers) from a single coyote — an individual killed on the Kenai Peninsula during the late 1990s — and from all six loci from the 10 domestic dogs. The analysis suggests most swab samples derived from wolves (fig. 2).

However, two of the 2016 feces swab samples (1417 and 1545, fig. 2) do not fall into the DPP wolf cluster, but rather into the dispersed cluster that represents dogs and the single coyote assayed. This suggests those two samples may have derived from canids other than wolves. These two samples differ by 2 alleles (Appendix, *2 allele difference* tab), due to a missing genotype for locus Ren199 for 1545. However, 1545 is also in a 2-MM match with samples from

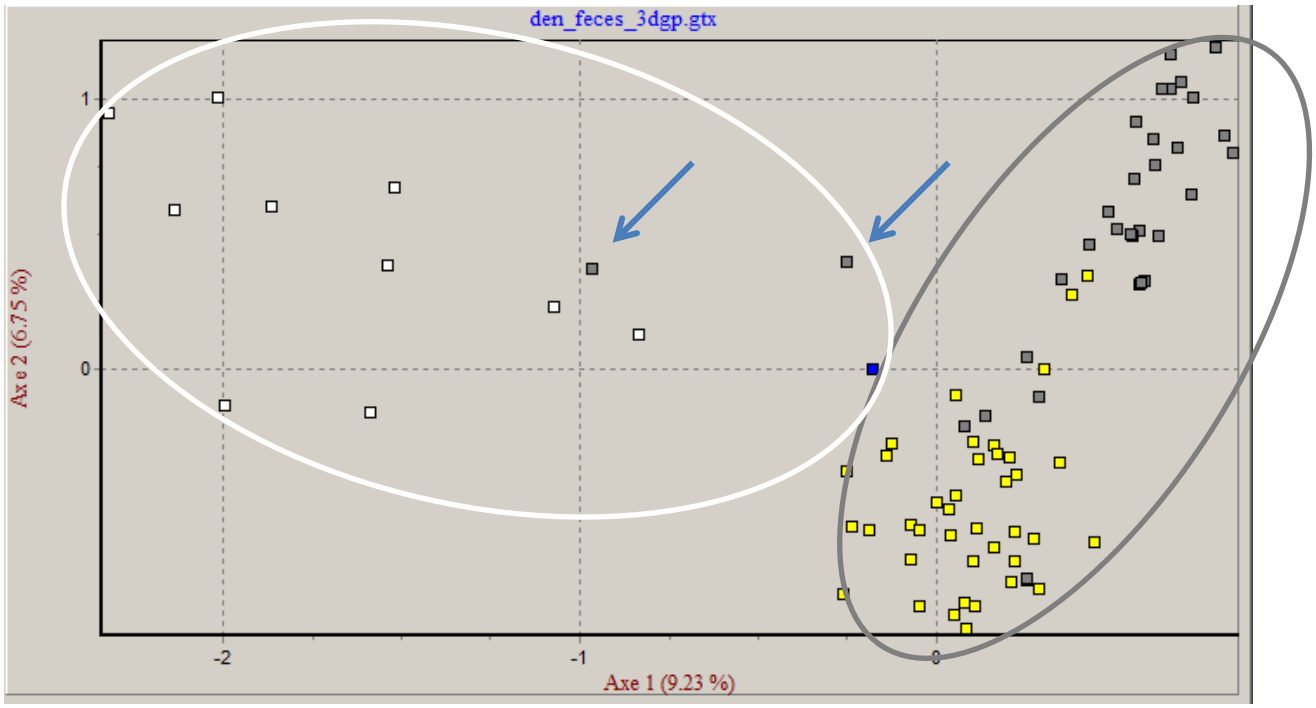



**Figure 2**. Clustering of individuals based on FCA, a PCA-style analysis of microsatellite markers (GENETIX, AFC 2D option). Yellow squares represent alleles found in the DPP baseline dataset used to generate genetic diversity values used for this study, and gray squares represent alleles from the 33 individuals estimated from the 106 feces samples. White squares represent alleles from dogs; the blue square represents alleles from a single coyote harvested on the Kenai Peninsula. The gray oval encompasses wolf alleles; the white encompasses dogs and coyotes. Arrows point to outlier feces swab samples 1545 (left) and 1417 (right).

other wolves, due to the missing genotype at Ren199 (Appendix, *2 allele difference* tab), and those wolves fall well within the 'wolf cluster' (fig. 2). As such, we cannot determine whether these samples are from wolves, or other canids, but we suspect they are from wolves. Regardless, additional sampling of both coyotes and dogs will be needed to determine whether genotypes from feces swab samples derive from wolves or other canids.

### Power of markers to detect individuals

Examination of the $P_{ID}$ and $P_{ID}$*sib* values associated with matches (Appendix, *Matches* tab) provide high confidence that the samples came from the same individuals. Match-specific $P_{ID}$ values ranged from $2.1745 \times 10^{-5}$ to $2.5121 \times 10^{-10}$. This suggests that, given a randomly breeding population, the probability that another individual possessed the same genotype of each individual within the match ranged from greater than 1/45,980 to greater than 3 billion. $P_{ID}$*sib* values ranged from 0.0118 to 0.0079. This suggest that, given the population is comprised solely of first order relatives, the probability that another individual possessed the same genotype within the match ranged from greater than 1/84 to greater than 1/126.

Among individuals, we observed 355 3MM-pairs, 74 2MM-pairs, and 28 1MM-pairs. Thus, the trend does not conform to a pattern of 10-fold reductions with increasing degrees of similarity observed in many datasets (Paetkau 2003); rather, we observed an approximately 4-fold reduction. As well, given that trend, we would expect the number of 0MM-pairs to be approximately 7, but we observed 383 0MM-pairs. Unlike those datasets, however, the sample design for this pilot study was not designed to be a random sampling of the DPP population but rather to determine whether we could recover individual identity from fecal samples. Given that wolf packs often comprise a number of highly-related individuals and show high fidelity to territories, we would expect that the multiple fecal piles sampled within each particular locale are likely to have been left by a single individual or a group of related individuals; this would result in an individual or a close relative being sampled more often during a single sampling effort.

## Summary

Results of this pilot study suggest that sufficiently robust microsatellite data for use in genetic tagging efforts can be obtained by swabbing external surfaces of feces piles, at least during winter months (January through March) in DPP. However, sampling during summer months (represented here by July and August) may not provide sufficient (or sufficiently reliable) data for use in genetic tagging. We recommend that if genetic tagging is desired during summer months, an alternative method, such as using DNA extracted from hairs, be applied. We note, nevertheless, that the majority of hair samples provided as part of this pilot study were underfur, not guard hairs; guard hairs with follicles typically yield greater quality and quantity of DNA (Tredick et al. 2007; GKS, personal observation). Thus, although we are cognizant that sampling is likely to be opportunistic, we recommend that hair sampling target guard hairs whenever possible.

To facilitate species determination for future research, sufficient samples from coyotes (preferably, 30 unrelated individuals from the sampling locale) and additional samples from domestic dogs be genotyped to identify diagnostic microsatellite markers, if any, among the suite used. Alternatively, a short portion of the mitochondrial DNA control region can be assayed to determine species differences, as there are diagnostic site substitutions among the species in that molecule (Talbot et al. 2011a).

We also recommend we drop locus Ren199 from future efforts involving non-invasively collected samples (including hairs and swabs from feces), substituting locus C173 in its place. Ren199 performed well in the exploratory analysis, but not in the pilot study. Additional loci that can be leveraged in the place of Ren199, or to otherwise augment the

marker suite as needed to resolve individuals, include loci C250 and C109, or even AHT121, which produces a large product but appears to be reliable in a study that involved low-quality DNA samples (Talbot et al. 2011a, GKS and SLT, unpublished data). We caution, however, that the addition of each locus to a marker suite increases the likelihood of adding error to the dataset (Paetkau 2003), so augmentation of the dataset needs to be considered carefully.

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