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PHOTOGRAPHIC AND IDENTIFICATION PROTOCOL

Identifying individual ungulates from fecal DNA: a comparison of field collection methods to maximize efficiency, ease, and success

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Abstract

Non-invasive genetic sampling can facilitate the identification of individual animals across a landscape, with applications to management and conservation. Fecal material is a readily available source of DNA, and various methods exist for collecting fecal samples for DNA preservation. In particular, swab methods offer considerable promise, but their utility in real-world field contexts remains relatively untested. We systematically compared multiple genetic fecal sampling methods across all stages of data collection and analysis, including sampling in the field, DNA extraction in the lab, and identification of individuals using microsatellite genotyping. We collected 112 fecal samples from black-tailed deer (*Odocoileus hemionus columbianus*) in the field in Mendocino County, California, across a range of sample conditions of unknown age. We systematically compared the efficiency, ease, and genotyping success of three methods for field collection and storage of ungulate fecal samples: whole pellets in ethanol, whole dry pellets in paper envelopes, and cotton swabs in buffer. Storage method, sample condition, and their interaction predicted genotyping success in the top binomial GLMMs. We found that swabbing pellets resulted in the greatest percentage of individually identifiable genotypes (81%, compared to 60% for dry samples and 56% for ethanol), despite lower DNA concentrations. While swabbing pellets requires a greater time investment in the field, the samples are easier and safer to store and transport, and subsequent labwork is more efficient as compared to whole-pellet collection methods. We, therefore, recommend the swab method for most contexts. We provide additional recommendations and field protocols based on subsequent collection of 2284 swab samples for a larger monitoring study of the deer population, given that this large number of samples spanned a range of sample conditions and time spent in storage.

Keywords Environmental DNA · Fecal swab · Microsatellite · Multiplex PCR · Non-invasive sampling · Population genomics

Introduction

Non-invasive genetic sampling is an important tool for individual identification of animals, with applications to wildlife management, conservation, and population ecology (Taberlet and Luikart 1999; DeSalle and Amato 2004). The ability to detect an individual organism's unique genetic signature can facilitate an understanding of individual space use, population demography and structure, and landscape connectivity (Piggott and Taylor 2003; Waits and Paetkau 2005). By collecting sources of DNA that animals leave behind, such as hair and fecal material, researchers can access an individual's genetic material without physical contact (Newediuk and Vander Wal 2022). Researchers can thus avoid exposing animals to stressful and dangerous trapping effort, reduce field costs and permit requirements, and increase collection

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opportunities in a variety of field conditions. Non-invasive methods of DNA collection can also more accurately capture a larger and more representative portion of a study population than trapping or other invasive methods (Piggott and Taylor 2003; Ferreira et al. 2018; though see Lampa et al. 2015), and are useful for species that are particularly elusive or rare (Canu et al. 2017; Quasim et al. 2018). Non-invasive genetic sampling can also supplement other individual identification and population monitoring methods, such as photographic identification or telemetry (Caroll et al. 2011; Furnas et al. 2018), and is becoming more feasible and affordable (DeSalle and Amato 2004).

Fecal material is a particularly promising source of non-invasive genetic samples (Kohn and Wayne 1997), and has provided insights into the biology of elusive animals (Bellemain et al. 2005), the conservation of endangered species (Gebremedhin et al. 2009), and the demography of harvested species (Lounsberry et al. 2015). Compared to hair, mucous, skin, or other sources of DNA, for many species fecal samples are readily obtainable in the field, conspicuous and easy to locate in a variety of conditions, and generated in high frequency by all individuals in a population. Furthermore, fecal samples often retain relatively high amounts of subject DNA as compared to substrates like soil, water, and vegetation, or materials such as hair or feathers (Ramón-Laca et al. 2018).

A variety of collection and extraction methods for fecal DNA samples currently exist, and they vary in their affordability, efficiency, and utility. The most widespread method for fecal DNA collection is pellet storage in ethanol (e.g., Valière et al. 2007; Lounsberry et al. 2015; Kierepka et al. 2016). This method has a track record of success, but it also presents several logistical challenges in the field and lab. Field collection of samples can be unwieldy when space and weight are a premium because fresh ethanol needs to be carried into the field, and safe ethanol procurement and storage can introduce logistical complications. Furthermore, for questions involving the biology of the host animal rather than its microbiota or diet, DNA isolation involves a time-consuming step where a researcher must scrape, wash, or homogenize the fecal matter and remove any remaining solid materials and inhibitors from the lysate (Deuter et al. 1995; Quasim et al. 2018). Host endogenous DNA is only a minor constituent of feces (Fernando et al. 2003), forming a thin membrane from the epithelia lost onto gut contents as they pass through the digestive system. In addition, feces contain compounds that are not readily separated from DNA in regular isolation techniques, yet also inhibit downstream applications such as the polymerase chain reaction (PCR; Deuter et al. 1995). Taken together, these challenges have limited efficiency, ease of processing, and success rates in the lab.

To address these concerns with ethanol storage and overcome known issues with DNA degradation of fecal samples (Taberlet and Luikart 1999), several investigators have explored alternative field collection methods (Frantzen et al. 1998; Waits and Paetkau 2005). Some studies have had success directly collecting fecal matter and storing it dry in coin envelopes, which eliminates the need for ethanol (Poole et al. 2011; Woodruff et al. 2014). In-situ swab methods have also been proposed (Cullingham et al. 2010; Ball et al. 2006) and formally validated with fresh fecal samples (Ramón-Laca et al. 2015). Previous work has established that the outer surface of fecal material provides the best source of host DNA, particularly for ungulates (Flagstad et al. 1999; Huber et al. 2002; Wehausen et al. 2004). In-situ swabbing involves directly swabbing the surface of fecal samples in the field and breaking off the swab head in a small tube containing a lysis buffer. The samples can be stored in this buffer until DNA isolation can be performed in the lab at a later date, in the same tube used for collection and storage (Ramón-Laca et al. 2015).

Given the growing importance of fecal DNA for individual identification of wild animals, there is a need to compare methods for collection and processing and to establish best practices for the field and lab. Many studies that test the merit of fecal DNA collection and extraction methods have done so in controlled conditions with samples of known age. For example, Ramón-Laca et al. (2015) compared swabs of fecal samples with ethanol-stored samples, all fresh (< 24 h old) and taken directly from the rectum of harvested animals without any exposure to weathering, and found that swab method resulted in higher DNA yield and lower inhibition. However, degradation of fecal DNA of samples on the ground due to rainfall and sun exposure presents a significant barrier to obtaining large sample sizes, and may result in a waste of effort and supplies if samples cannot be genotyped (Piggott 2004; Brinkman et al. 2010). It is, therefore, important to understand how different collection methods perform in field settings, given that field conditions can dramatically impact the success of fecal DNA. Furthermore, it is unclear how weathering affects DNA yield of fecal samples, so that samples with a higher likelihood of genotyping success can be prioritized during field collection. Finally, it is important to understand the ease and cost of each method, in addition to its success rate, as these factors are relevant considerations in study design.

In this study, we present a systematic comparison of the dry, ethanol, and swab methods for collecting genetic material from fecal pellets of Columbian black-tailed deer (*Odocoileus hemionus columbianus*) of various conditions, collected in the field in northern California. Our primary objective was to evaluate and compare performance of each of the three storage methods in terms of the sample's DNA yield and its ability to be associated with an individual animal through microsatellite genotyping. We also examined how samples of different conditions (as a result of weathering in the field) performed in genotyping. Furthermore, we

qualitatively compared the ease of collection and processing of the dry, ethanol, and swab methods in both the field and the lab and provide recommendations for future studies.

Methods

We opportunistically collected fecal samples from the ground across a range of pellet conditions, and collected material from each sample in triplicate for direct comparison of the three storage methods (Fig. 1).

Field collection

We collected all samples at the Hopland Research and Extension Center (HREC) in Mendocino County, California (39.989–39.039, − 123.056 to − 123.102). Mean daily temperatures range from 44°F in the winter to 92°F in the summer, and annual precipitation ranges from 37 inches (at the lowest elevation) to 45 inches (at the highest elevation). With over 5300 acres, HREC covers an array of natural habitat types, including oak woodland, grassland, chaparral, and riparian zones, and it is surrounded by multiple land-use types, including intensive agriculture and sheep production. Columbian black-tailed deer, a subspecies of mule deer sensu lato, occur on site at high densities. Mule deer, widespread in northwestern North America (Latch et al. 2014), are typically a dominant species in the ecosystems they inhabit, and are one of the most economically important game species in the United States. As such, understanding their ecology and population sizes is critical for management, and non-invasive genetic sampling is a promising method for monitoring and research.

We collected fecal samples opportunistically from December 2016 to April 2017. We scored the condition of each pile of fecal pellets as 1 (slimy and fresh), 2 (wet), 3 (shiny) or 4 (dry, cracked, and weathered) in 0.5 increments, adapting protocols from the California Department of Fish and Wildlife (Supplementary Material Protocol 1). Condition is a rough proxy for age, although some newer samples can be more weathered, or vice versa (Piggott 2004). We collected each sample in triplicate for the three storage methods, only collecting piles with a sufficient number of pellets (> 10). We split the pile into three equally sized samples for each of the three storage methods, ensuring that the individual pellets used for each storage method were comparable in size and condition. The order in which we sampled for each storage condition varied by sample, and thus was unlikely to systematically bias the results.

For the ethanol collection, we placed the pellets in 95–100% lab-grade ethanol. For the dry collection, we placed the pellets into a paper coin envelope. When samples were wet at collection, we attempted to dry them in the sun or with silicone bead desiccant. For the swab method, we dipped polyester-tipped medical swabs in Longmire buffer (Longmire et al. 1997), and applied pressure to the outside of the pellet with the swab, rubbing the swab over at least 3 pellets to slough off epithelial cells. We stored the tips of the swabs in 1.5 mL plastic snap-top centrifuge tubes with Longmire buffer. Complete field protocols can be found in the supplementary material (Supplementary Material Protocol 1).

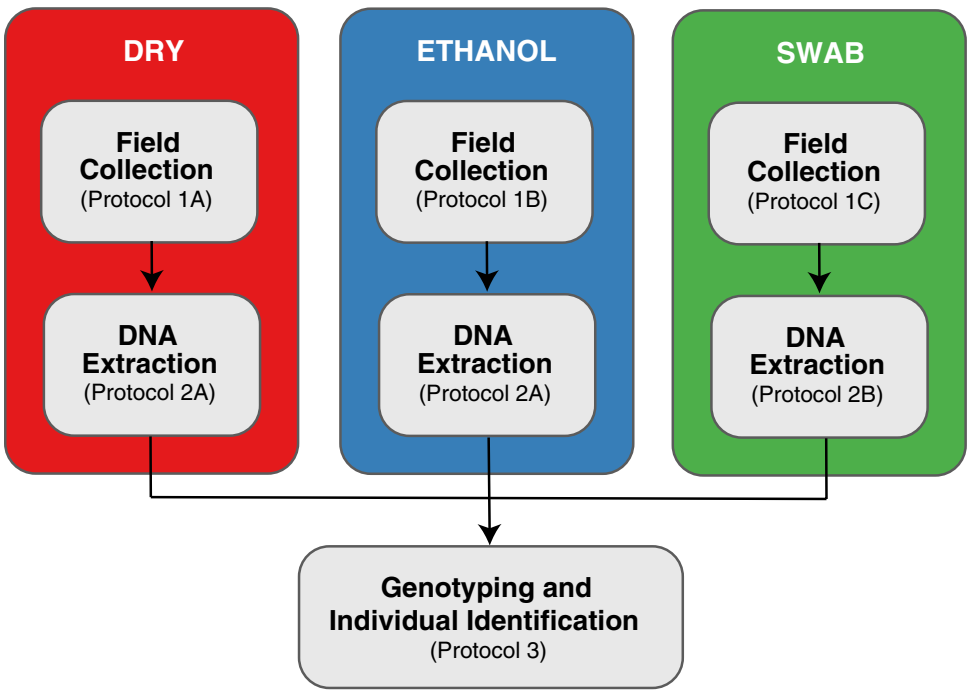

Fig. 1 Workflow for collecting fecal samples, extracting DNA, and genotyping for individual identification. In this study, we systematically compared dry, ethanol, and swab storage methods and quantitatively and qualitatively assessed their performance in the field and laboratory. Detailed protocols for each stage of the workflow, as referenced in the figure, can be found in the supplementary information

Following this systematic comparison of storage methods, we also collected an additional 2,284 samples from 2017–2018 using only the swab method, as part of a larger genotyping study for population monitoring. These additional samples allowed us to further explore success rates of swab samples across sample conditions and, importantly, varying time in storage, given that many of these samples were not extracted immediately after collection.

DNA isolation from fecal pellets

We extracted DNA from each of the samples using silica columns, following slightly different protocols for each of the storage methods (Boom et al. 1990). Ethanol and dry samples required an initial lysis and incubation phase. To dry ethanol samples prior to extraction, we placed 3–6 pellets in a new plastic 15 mL vial (Nalgene 5005–0015, Rochester, New York) with the lid open, agitating periodically until the outside surface appeared dry. We added Qiagen Buffer ATL to each vial (375 µL for ethanol samples and 975 µL for dry samples), closing the lid and regularly agitating vigorously and adding 200 µL of additional buffer if the pellets had soaked up the initial buffer. We then transferred the lysate to a microcentrifuge tube, added 20 µL of Proteinase K (20 g/L) to each sample, and incubated at 55 °C. After 10 min, we added at least 200 µL of Qiagen Buffer AL to each tube and up to the initial volume of the lysate if a gelatinous layer formed. For swab samples, no initial lysis phase was required because they were already collected and stored in a lysis buffer. We started the DNA isolation procedure by adding 20 µL of Proteinase K (20 g/L) and 340 µL of a buffer containing chaotropic salt (e.g., Qiagen Buffer AL or Omega Buffer BL) directly into each sample tube (with swab heads still present).

Regardless of method, we then incubated the lysate at 55 °C. After incubation, we added 200–300 µL of ethanol and loaded a silica spin column or plate (Qiagen DNeasy Blood and Tissue Kit, Omega E.Z.N.A. Tissue DNA kit, or Epoch EconoSpin). We then completed the isolation procedure using a modification of the manufacturer's guidelines. Regardless of method, for the final elution, we eluted with a mixture of 10 µL of Buffer LTE (10 mM Tris–HCl pH 8; 0.1 mM EDTA) and 190 µL of ultrapure water. To maximize DNA concentration, we performed two elutions, evaporated them down to 10 µL each, and then combined them. Complete laboratory protocols can be found in the supplementary material (Supplementary Material Protocol 2).

Microsatellite genotyping for individual identification

Given that our ultimate goal was to identify individual deer using microsatellite markers, we were interested in comparing the performance of different storage methods in terms of producing usable samples for those analyses. We genotyped each sample using a modified version of the "Deer10Y" multiplex microsatellite PCR assay, a set of ten microsatellite markers and the sex marker *SRY*, as described in Lounsberry et al. (2015; see supplementary material for the specific modifications/protocol). Full genotyping procedures are described in Supplementary Protocol 3. To reduce the chance of allelic dropout or false alleles, we performed two PCR replicates for each triplicate sample (for the 2017–2018 swab samples, we performed up to four additional PCR replicates if the first two did not yield usable genotypes). Consensus genotypes were composed from individual runs. We considered a sample to be usable if it amplified for at least eight of the microsatellite markers (Lounsberry et al. 2015). We then used the *alleleMatch* package in R (Galpern et al. 2012) to determine individual identity from each usable sample, following the protocols of Lounsberry et al. (2015), and then determined the total number of unique individuals in our sample.

We used binomial Generalized Linear Mixed Models (GLMM) with the *lme4* package in R (Bates et al. 2015) to explore how storage method and sample condition influenced performance in the microsatellite assay, in terms of yielding usable genotypes for individual identification. The dependent variable was binary, representing whether or not the sample yielded a usable genotype (at least 8/10 markers amplifying). We determined this cut-off for individual identification based on a previous study of black-tailed deer in the region (Lounsberry et al. 2015). We evaluated all combinations of the following fixed effects: storage method (ethanol, dry, or swab), sample condition (1.0–4.0, continuous variable), and interaction between storage method and condition. We included sample ID as a random effect in the model, given that samples were collected in triplicate across storage methods. To compare and evaluate models, we used AICc (a modification of the Akaike Information Criterion to adjust for small sample sizes). To assess goodness of fit of the top models, we bootstrapped a calculation of the area under the receiver operating characteristics curve (AUC). We determined the AUC using the "performance" function in the *ROCR* package in R (Sing et al. 2005), and calculated mean and standard deviation over 100 bootstrapped iterations, splitting data 80% training and 20% testing. We also explored the potential of incorporating sample condition as a categorical variable, but it resulted in models with higher AICc and lower AUC, so we treated condition as a continuous variable (Table A1).

For the 2017–2018 swab-only samples, we used binomial Generalized Linear Models to evaluate the effect of sample condition and time in storage (days elapsed from field collection to DNA extraction in the lab; ranged from 4 to 59 days) on genotyping success (whether or not a sample

was individually identifiable). We used AIC to compare and evaluate models, and AUC to determine goodness of fit.

Assessing DNA concentration and inhibition

We used quantitative PCR (qPCR) to calculate the concentration of host DNA in the fecal extractions, described in detail in Supplementary Materials Protocol 4. We developed primers against the *PRKCI* (protein kinase C iota) marker, and constructed a standard curve from a purified total DNA extraction from ear-punch tissue to convert the quantification cycle (C_q) values of the fecal extracts to units of log ng/μL. For all analyses, we kept qPCR concentrations on a log scale to meet assumptions of normality.

We then conducted a repeated-measures Analysis of Variance (ANOVA) to compare DNA concentration, as determined through qPCR, across the three storage methods, using log-transformed qPCR concentration to meet assumptions of normal distributions. To evaluate potential correlations between DNA concentration and genotyping success, we used t-tests to compare DNA concentration between samples that produced individually identifiable genotypes (8 of 10 markers amplifying) and those that did not, for each of the three storage methods.

To check whether the extracted samples retained compounds from the feces that would inhibit downstream assays such as PCR, we ran qPCR as described above against the *PRKCI* target for a selection of samples. This inhibition assay proceeded similarly to the qPCR quantification assay, except that we also included in each reaction a positive control of 2.5 nanograms of DNA extract from a tissue sample (Ramón-Laca et al. 2018). We expect that even if the template were degraded and failed to recover DNA, the control would still amplify as long as inhibitors were not present; therefore, we considered a sample inhibited if the apparent DNA amount was less than 1.768 nanograms (i.e., if its C_q value was > 0.5 compared to control). We found that every sample tested across all storage methods met this criterion and thus showed evidence of inhibition.

Results

We collected and extracted DNA from 112 unique fecal samples in triplicate storage methods (dry, ethanol, and swab). Of these 112 samples, 99 were individually identifiable, and represented a total of 68 unique individual deer. Of these 68 deer, 52 deer were associated with a single fecal sample, while only one deer was associated with > 5 samples (8 samples in total for this deer). The mean sample condition (on a 1–4 scale) was 2.75 ± 0.73 (mean ± SD), with 11 slimy samples (1.0 or 1.5), 21 wet samples (2.0 or 2.5), 73 shiny samples (3.0 or 3.5), 2 dull samples (4.0), and 5 of unrecorded condition.

Table 1 Model selection for genotyping performance of genetic samples for individual ungulate identification across fecal pellet storage methods and condition

Model	AICc	deltaAICc
Storage method + condition	**361.5**	**0**
Storage method * condition	**363.1**	**1.6**
Storage method	379.0	17.5
Condition	386.6	25.1
Intercept (null model)	405.8	44.3

The dependent variable was binary, representing whether or not the sample yielded an individually identifiable genotype (at least eight alleles amplifying). The top models (deltaAICc < 2) are bolded. Sample ID was incorporated as a random effect in all models

Individual genotyping was most successful when we used the swab storage method (Figs. 2, A1). For the swab samples, 8.5 ± 3.0 (mean ± SD) of 10 markers amplified successfully, compared to 6.5 ± 4.4 (mean ± SD) markers for dry samples and 6.3 ± 4.4 (mean ± SD) markers for ethanol samples. The swab method yielded the highest percentage of usable samples (81%), with at least 8 of the 10 markers amplifying. Of the 112 swab samples, 91 yielded individually identifiable genotypes, from 63 individual deer. Dry samples were the next best in terms of microsatellite amplification (60% usable, 67 samples from 47 individuals), followed by ethanol samples (56% usable, 63 samples from 44 individuals).

In the top-ranked GLMMs based on AICc, storage method and sample condition were both predictors of whether a sample yielded a usable genotype for individual identification (Tables 1, 2). The interaction between storage method and condition was present in the second-best model, which is also a top model (within 2 AICc of the model without the interaction term). The bootstrapped AUC values indicated a strong fit for both the top model (0.77 mean ± 0.06 SD) and second-best model (0.76 mean ± 0.06 SD).

For the larger set of 2,284 swab samples collected later in 2017–2018 (after refining our swab method protocols), we found that both sample condition and time in storage,

Table 2 Results of the top model for genotyping performance of genetic samples for individual ungulate identification

Coefficient	Estimate	SE	*p* value
Intercept	1.53	1.04	0.139
Storage method = ethanol	– 0.26	0.36	0.471
Storage method = swab	1.80	0.45	< 0.001
Condition	– 0.25	0.35	0.474

The dependent variable was binary, representing whether or not the sample yielded an individually identifiable genotype (at least eight markers amplifying). The reference level for storage method was "Dry". Sample ID was incorporated as a random effect

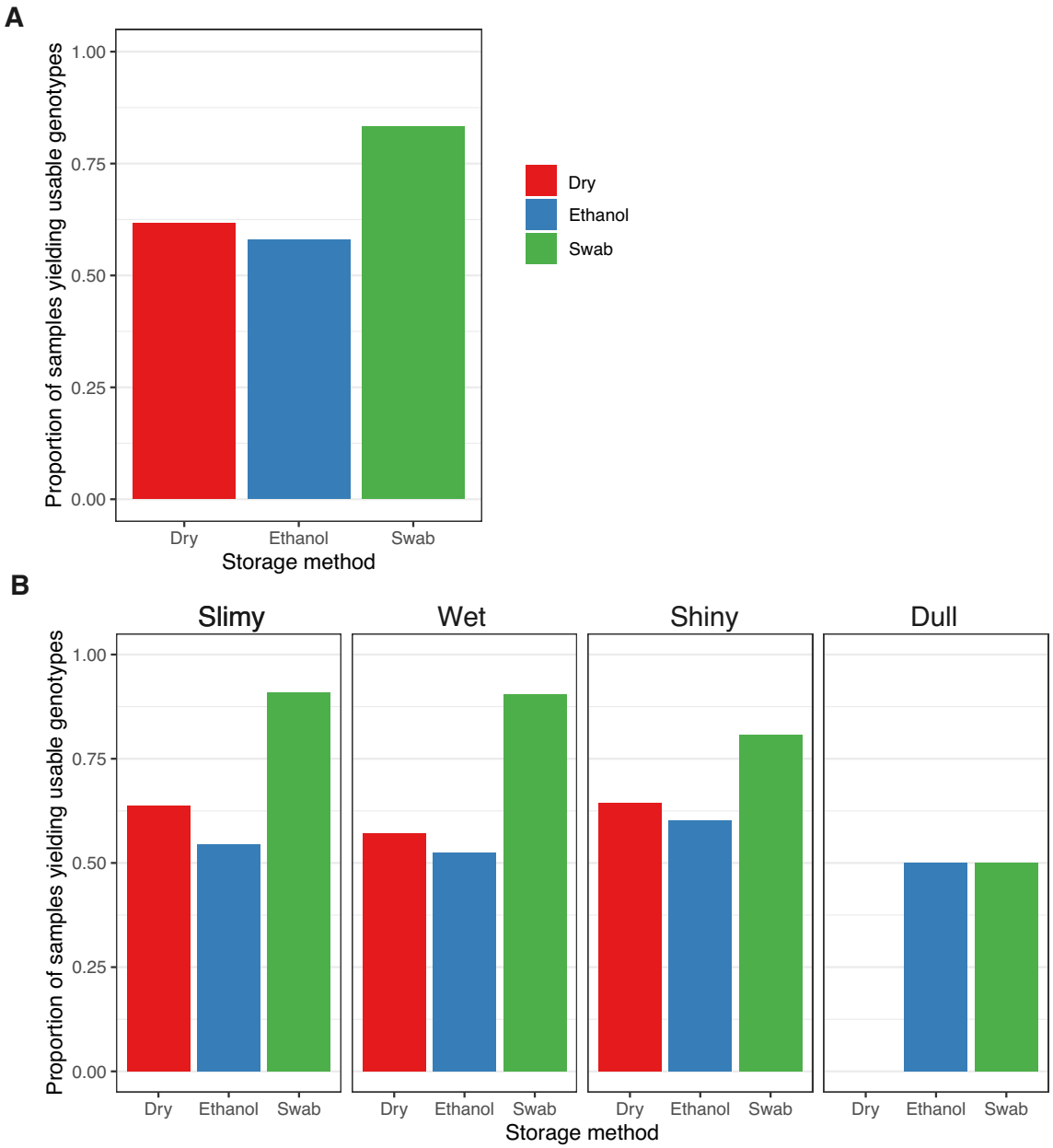

Fig. 2 Genotyping success across storage methods. The proportion of samples yielding usable genotypes for individual identification (at least 8 of 10 microsatellite markers amplifying) for (**A**) all samples combined, and (**B**) samples split by condition. "Slimy" represents the freshest samples, and "Dull" represents the most weathered

and the interaction between the two, were important predictors of genotyping success (Tables 3, 4). Samples with a fresher condition score performed better, although samples of all conditions yielded usable genotypes. In this larger study, we were able to individually identify deer for 97% of slimy samples (the freshest category; $n = 30$), 77% of wet samples ($n = 177$), 75% of shiny samples ($n = 1{,}321$), and 53% of dull samples ($n = 756$). We also found that genotyping success declined slightly with time in storage for all sample conditions, with an odds ratio of 0.89 (Table 4; Fig. A3). The bootstrapped AUC values indicated a moderate fit for the top model of genotyping success among the 2017–2018 swab samples (0.67 mean ± 0.03 SD).

DNA concentration, as determined through qPCR, differed across the three storage methods (Fig. A2; ANOVA: $F = 3.75$, $p = 0.03$, $df = 2, 280$). However, there was high variance in recovered yields overall. DNA concentration was highest for dry samples (median = 4.73 ng/µl; IQR = 0.78–13.5), followed by ethanol (median = 3.39 ng/

Table 3 Model selection for genotyping performance of genetic samples for individual ungulate identification across fecal pellet condition and time in storage, for swab samples collected 2017–2018

Model	AIC	deltaAIC
Time in storage * condition	**2656.7**	**0**
Time in storage + condition	2674.4	17.7
Condition	2709.1	52.4
Time in storage	2830.1	174.0
Intercept (null model)	2835.7	179.0

The dependent variable was binary, representing whether or not the sample yielded an individually identifiable genotype (at least eight markers amplifying). The top models (deltaAIC < 2) are bolded

Table 4 Results of the top model for genotyping performance of genetic samples for individual ungulate identification, for swab samples collected 2017–2018

Coefficient	Estimate	SE	*p* value
Intercept	8.69	0.91	< 0.001
Time in storage	− 0.11	0.02	< 0.001
Condition	− 2.06	0.25	< 0.001
Time in storage * condition	0.03	0.01	< 0.001

The dependent variable was binary, representing whether or not the sample yielded an individually identifiable genotype (at least eight markers amplifying)

µl; IQR = 1.29–8.05), and then swab (median = 1.94 ng/µl; IQR = 0.51–6.37). For each of the three storage methods, DNA concentration was higher for the samples that yielded usable genotypes than those that did not (Dry: $t = 4.02$, $df = 51.6$, $p < 0.001$; Ethanol: $t = 2.18$, $df = 65.3$, $p = 0.033$; Swab: $t = 2.56$, $df = 32.3$, $p = 0.015$) (Fig. 2).

Discussion

The collection of fecal DNA presents a valuable opportunity for non-invasive monitoring of wild animals. To be effective and efficient, such monitoring requires sampling methods that maximize the amount and quality of DNA. We found that identification of individual deer was most effective when using the swab method to sample fecal DNA, in comparison to ethanol and dry sampling methods. When considering the entire data generation process from field collection to individual identification, swabbing fecal pellets in situ provides a blend of ease, efficiency, and effectiveness (Table 5). The swab method targets the epithelial cells on the exterior of fecal samples, reducing laboratory processing time associated with whole-pellet collection and resulting in higher-quality data. Given the high success rates and relatively lower costs, as discussed further below, the swab method has potential to facilitate future research and monitoring that relies on individual identification from fecal samples of varying conditions encountered in the field.

Our findings suggest that it is worthwhile to sample all fecal pellets of all conditions that are not wholly degraded or destroyed, especially when using the swab method. While we were unable to determine the precise age of the samples, we assessed sample condition and weathering based on visual characteristics of the pellets (Piggott 2004). Weathering negatively affected the ability to determine an individual identification for a sample, likely because DNA degrades over time as a result of exposure to sunlight, heat, and moisture. However, our results suggest that all sample conditions yield usable genotypes most of the time when swabbed. Given that samples of inferior condition (i.e., "shiny" or "dull") are far more common than very fresh samples (i.e., "slimy"), broader inclusion criteria will greatly increase sample sizes. Notably, we did not collect the most degraded pellets in which the exterior surface was no longer intact, and preliminary exploration suggested that these pellets are less likely to yield usable genotypes.

Ease of fieldwork and labwork

Dry storage: Dry collected samples are smaller and weigh less than swabs or samples in ethanol, making it more convenient to transport samples from remote field sites. However, even with silica drying packets and refrigerated storage, samples stored dry without a preserving buffer still retain moisture, and we found that they tend to mold within several weeks. Dry collection of samples is viable for immediate extraction, but it is not advisable for samples that may sit for longer periods of time before extraction. Additionally, the processing of dry samples for extraction is much more arduous in the laboratory. Because whole pellets are being handled, fecal particles commonly clog columns during DNA extraction, and it is difficult to prevent additional non-DNA material from entering what should be a DNA-only solution. Dried fecal pellets also absorbed the lysis buffer used during the extraction process, requiring larger volumes of reagents and resulting in a more expensive extraction process.

Ethanol storage: Samples in ethanol, a common method for collecting DNA from fecal material, do not have the same long-term storage issues as dry samples, but produce their own suite of issues during both sample collection and lab processing. During collection, ethanol must be brought into the field, necessitating significant additional weight and materials. Handling ethanol in the field also creates opportunities for spills and sample contamination, and procurement and storage of ethanol in field settings can present logistical barriers. Additionally, carrying ethanol in large quantities poses safety concerns and requires special storage and handling considerations. Once brought to the lab, samples stored in ethanol are more manageable than the

Table 5 Pros and cons of methods for collecting and storing ungulate fecal DNA for individual identification

Storage method	Pros	Cons
Ethanol	*Storage* Able to sit in storage longest No mold issues *Labwork* Requires less ATL buffer than dry samples during DNA extraction	*Field collection* Difficulty storing ethanol in field Samples can be heavy (weight of ethanol and pellets) *Storage* Least compact of storage methods Potential for ethanol to leak and evaporate Highly flammable and toxic *Labwork* Requires time to evaporate ethanol before DNA extraction Some clogged columns during DNA extraction Fresher condition samples may sequence at lower success rate than expected due to clumping together in ethanol Relatively more expensive due to material and labor costs
Dry	*Field collection* Easy collection in the field, only requires envelopes *Storage* Relatively compact storage Low flammability and toxicity	*Storage* Can mold in storage, especially when samples are collected wet Higher degradation of DNA in long-term storage *Labwork* Higher instance of clogged columns and troubleshooting needed Lowest success rate Longest amount of time needed for extractions Relatively more expensive due to material and labor costs
Swab	*Field collection* No need to collect fecal pellets *Storage* No mold issues Able to sit in storage longer than dry samples Storage is compact Low flammability and toxicity *Labwork* Greatest success in microsatellite analysis No dealing with fecal material in lab Fastest of extraction methods Does not require costly ATL buffer during extraction Lowest cost in terms of materials and labor	*Field collection* Requires more materials (swab, tube, buffer, tool to hold pellets in place) *Labwork* Requires more time in incubator prior to DNA extraction Cannot redo extractions from raw material (current methods use entire sample during extraction) Certain swab and tube combinations create difficulties with pipetting (best to have smaller swab, or more rounded tube bottom)

dry-collected samples, as they are not prone to desiccation or molding. However, like the dry samples, processing whole fecal pellets—even those stored in ethanol—means that the final DNA extraction is at greater risk of downstream inhibition. We frequently observed fragments of fecal matter in post-extraction eluate from both dry and in ethanol-stored samples. Like dry-stored samples, ethanol-stored samples also experienced more complications through the process of DNA extraction, such as clogging of DNA extraction spin-columns and precipitation of the lysis solution.

Swab storage: The swab method requires few additional field materials compared to dry collection. While it is more time-intensive in the field than either dry or ethanol methods, there are fewer safety, storage, or contamination concerns compared with the other two methods, as the swab heads are stored in lysis buffer. Because the host cells are lysed directly in the storage buffer, there is less need to worry about degradation from vibration, sunlight, heat, or moisture after collection (Longmire et al. 1997; Ramón-Laca et al. 2015). Furthermore, swab samples do not need to be immediately returned to the lab for processing, in contrast to dry samples. It is also possible to collect a greater number of swab samples than ethanol samples during a single field excursion, given that they are smaller and lighter to transport.

In the lab, swabbed samples require fewer reagents compared to the dry and ethanol samples, and they are less prone to the logistical problems we observe when extracting from whole pellets. The swab method is by far the least intensive in terms of lab time, because it is most amenable to batch processing of many dozens of individual samples at once (c.f., Quasim et al. 2018). Because cell lysis occurs directly in the sample tube prepared in the field, the extraction procedure can be drastically sped up using tools such as a repeater pipette, an adjustable-width multichannel pipette, and plate-based silica-column extractions. Using these three tools together, it is possible to complete the entire isolation procedure for 96 samples in as little as three hours of labor time. In comparison, it would take about two to three times as long to extract dry and ethanol samples, due to less

efficient processing and incubation of fecal pellets. Other potential avenues for efficiency gains include magnetic-bead extractions in lieu of silica plates, vacuum manifolds, and extraction robots.

In addition to saving on labor, the swab method can provide significant savings in material cost—the dry and ethanol methods were around 3 and 4 times as expensive as the swab method, respectively. We purchased many DNA extraction materials (e.g., swabs, tubes, pipette tips) in bulk and prepared many reagents in our lab (e.g., Tris buffer), and materials for the swab method cost as low as $0.92 per sample, compared to $3.83 for ethanol samples and $2.72 for dry samples, excluding labor. Ethanol samples were more expensive than dry samples due to the cost of the 15 mL Nalgene vials used for field collection, and swab samples were the least expensive as they did not require the costly ATL buffer or a 15 mL vial for lysis. See Supplementary Material Protocol 2 for more detailed cost information.

Additional considerations

Time in storage: In our expanded study with swab samples, we kept samples in storage for a range of 4–59 days, and found very slight declines in genotyping performance with a longer time elapsed between field collection and DNA extraction in the laboratory. We, therefore, recommend that samples be extracted as quickly as possible after collection, as it seems that DNA may degrade with time in storage. However, success rates remain very high even for samples that sat for two months. Small sample sizes precluded similar analyses for ethanol and dry samples, although anecdotally, we observed that dry samples often retained a small amount of moisture and molded in storage, so we recommend desiccation and/or rapid DNA extraction for dry samples.

Inhibition: Despite our high success rate of genotype recovery, we found that even with swabbed samples, many of our deer DNA extracts were inhibited, as with other methods, and as was found for other herbivore fecal samples (Wehausen et al. 2004). Our extraction method does not include any special inhibitor-removal step, but if empirical tests require inhibitor removal, we suggest diluting DNA 1:64 and adding 6 or more extra PCR cycles to compensate, or performing a kit-based cleanup incorporating an inhibitor-removal reagent (Schrader et al. 2012).

Scaling for research applications: For our purposes of methods comparison, we only ran each sample twice for genotyping. In typical microsatellite analyses from fecal samples, there would be additional repeat PCRs for each sample to obtain more complete genotypes, as we did for the 2017–2018 swab samples. Subsequent reruns (in addition to the two runs that we conducted) would likely improve results for all three methods, with higher success rates that we found in the present study. However, because improvement from subsequent reruns is proportionate among the collection methods, the swab method is the recommended method if highest sample quality is the desired outcome. Over multiple field seasons or with many samples, a target-capture or host-enriched approach to genome-scale sequencing may prove to be more time- and cost-effective than the multiplex microsatellite method that we have pursued (Aylward et al. 2018; Chiou and Bergey 2018).

DNA concentration vs. DNA quality: In a qPCR assay, swab samples yielded a lower concentration of target DNA than the ethanol or dry samples. It is nonetheless likely that the quality of target host DNA from swab samples is higher, given that this sampling method resulted in improved performance in genotyping. Our findings suggest that a multilocus PCR assay panel may be a more functional and reliable indicator of host DNA recovery compared to a qPCR assay of one or two loci, and are also in line with prior work that has suggested DNA quantification alone may not predict whether a sample work in a multilocus functional assay (Fernando et al. 2003; Bourgeois et al. 2019). In addition, our qPCR variances were quite large, suggesting that the assays we designed and ran could be further optimized, and that using additional markers could improve performance in a future study, though this can be quite time- and cost-prohibitive. We note that qPCR is probably as likely to experience allele dropout as endpoint microsatellite PCR. Non-target DNA likely also overestimates the amount of host DNA as determined by fluorescence or spectrophotometry, in addition interfering with qPCR. As evidence to both points, despite developing deer-specific qPCR primers using sequences from the deer genome, we had to eliminate the results from many fecal samples due to erratic behavior with the melt curve, while tissue samples had no such issue with the melting analysis.

Conclusions

After comparing three methods for storage of fecal DNA samples (dry pellets, pellets in ethanol, and swabs), we found that all three methods were generally successful, but the swab method was most suitable for real-world sampling in imperfect field conditions. We recognize that optimal approaches may vary across field conditions and species, however, and our study highlights the value of systematic pilot studies when initiating a large-scale collection effort (Taberlet and Luikart 1999; Waits and

Paetkau 2005; Lampa et al. 2015). We subsequently used swabbing exclusively to sample deer systematically for two field seasons, with high recovery of individual identification. The storage and DNA extraction techniques that we explored can be used to individually identify animals using a multiplex microsatellite method, as we have done here. However, these methods are also amenable to a wide array of other genetic analyses using fecal DNA, a readily available source of genetic material that can shed light on population size and structure and inform management and conservation of a wide range of mammal species.

Appendix

See Figs. A1, A2, A3; Table A1.

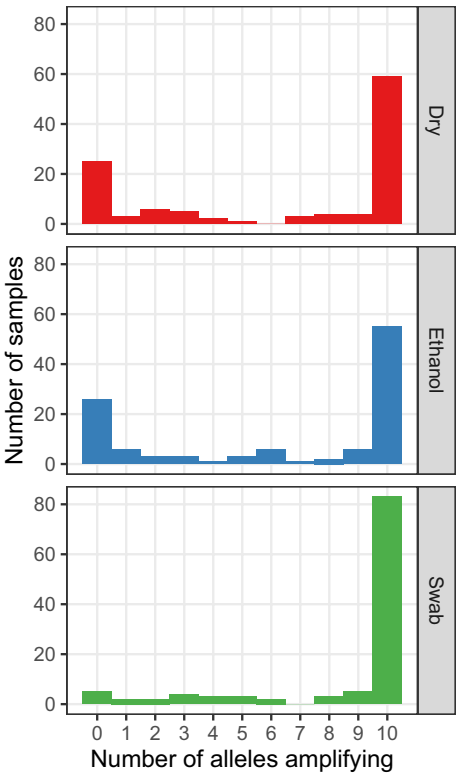

Fig. A1 Genotyping results for each of the three storage methods. Height of bars corresponds to the number of samples with a given number of microsatellite markers amplifying during genotyping (out of 10 total) for each of the three storage methods (dry, ethanol, and swab)

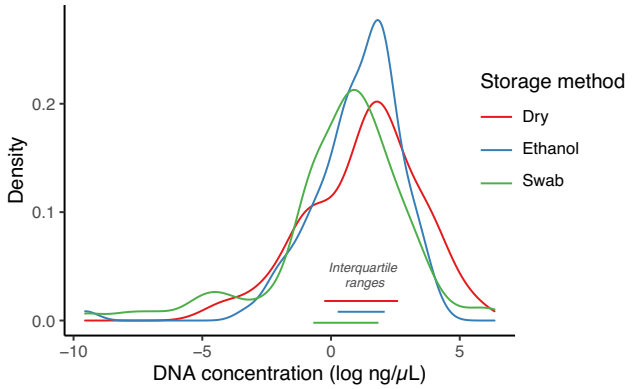

Fig. A2 DNA concentration of samples across storage methods (dry, ethanol, and swab), as determined through qPCR. There was high variance in recovered yields overall, and small differences in mean DNA yields across storage methods. Horizontal lines indicate interquartile ranges

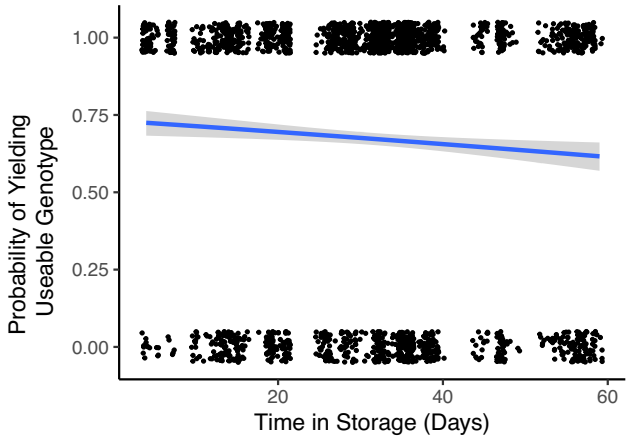

Fig. A3 Probability of a swab sample yielding a usable genotype as a function of time spent in storage, as predicted by a binomial regression model. Genotyping success declined slightly, but significantly, over time. Points represent swab samples, jittered for visibility

Table A1 Comparison of univariate models for genotyping performance of genetic samples for individual ungulate identification. These three models include different measures of fecal pellet sample condition

Condition measure	AICc	AUC (mean ± SD)
Continuous	386.6	0.714 ± 0.065
Categorical (4 levels)	387.1	0.699 ± 0.064
Categorical (7 levels)	389.7	0.708 ± 0.060

The model that included condition as a continuous variable had the lowest AICc and highest Area Under ROC Curve (AUC, a measure of goodness of fit)

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Author contributions BB, AQ, and KG contributed equally to this work, and led the writing. JB, KG, BB, KC, and AQ designed the study. BB, AQ, and KC conducted labwork. KG supervised field collection and labwork and conducted data analyses. All authors contributed to writing and revisions.

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Data availability All data will be published on Dryad upon acceptance of the manuscript.

Code availability All code will be published on Dryad upon acceptance of the manuscript.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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