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SHORT COMMUNICATION

Effects of time and rainfall on PCR success using DNA extracted from deer fecal pellets

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Abstract Non-invasive wildlife research using DNA from feces has become increasingly popular. Recent studies have attempted to solve problems associated with recovering DNA from feces by investigating the influence of factors such as season, diet, collection method, preservation method, extraction protocol, and time. To our knowledge, studies of this nature have not addressed DNA degradation over time in wet environments, and have not been performed on fecal pellets of ungulates. Therefore, our objective was to determine the length of time a fecal pellet from a Sitka black-tailed deer (*Odocoileus hemionus sitkensis*) could remain in the field in a temperate rainforest environment before the DNA became too degraded for individual identification. Pellets were extracted from the rectum of recently killed deer and placed in an environment protected from rainfall and in an environment exposed to rainfall. Pellets from each treatment group were sampled at intervals of 2, 7, 14, 21, and 28 days after deer harvest. DNA was extracted from sampled pellets and individual samples were genotyped using microsatellite markers. Amplification failure and errors (dropout and false alleles) were recorded to determine extent of DNA degradation. Eighty percent of samples in the protected environment and 22% of samples in the exposed environment were successfully genotyped during the 28-day experiment. With no samples being successfully genotyped in the exposed environment after 7 days, our study showed that rainfall significantly increases degradation rates of DNA from ungulate pellets.

Keywords DNA degradation · Feces · Microsatellites · *Odocoileus hemionus sitkensis* · PCR · Precipitation · Sitka black-tailed deer

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Introduction

Non-invasive wildlife research using DNA from feces has become increasingly popular (Bellemain et al. 2005, Ulizio et al. 2006, Schwartz and Monfort 2008). With the availability of sufficient genetic markers, non-invasive genetic sampling of feces may be used to identify individuals, facilitating capture-mark-recapture estimators of population size by removing the need to physically capture and mark individuals. This approach is useful for large, hard-to-study, mammals such as ursids, canids, felids, and mustelids, and with endangered species (Waits and Paetkau 2005).

Non-invasive sampling of deer (Cervidae) populations is uncommon (Ball et al. 2007; van Vliet et al. 2008a), presumably because tissue samples are often available from hunters. Moreover, in many cases techniques exist for estimating abundance of deer that are superior to genetic mark-recapture in terms of cost, time, or precision. Nonetheless, deer that live in thickly forested habitats with limited road access, such as Sitka black-tailed deer (*Odocoileus hemionus sitkensis*) in the temperate rainforest of southeastern Alaska, cannot be counted by traditional means but are candidates for genetic mark-recapture estimators. The efficacy of generating consistently accurate

genotypes from fecal pellets deposited in a cool, wet environment is unknown, however.

Problems may occur when fecal samples yield low quantity and quality DNA, such as that available on the surface of fecal pellets exposed to the environment (Ball et al. 2007; Buchan et al. 2005; Waits and Paetkau 2005). Problems associated with fecal DNA include contamination by microorganisms or digested food items, sensitivity to seasonal weather, high PCR-inhibitor to DNA ratios, and relatively high amplification and genotyping errors (Buchan et al. 2005; Maudet et al. 2004; Murphy et al. 2003; Sefc et al. 2003). Molecular ecologists are keenly aware of those errors and their consequences (Bonin et al. 2004; Miller et al. 2002; Morin et al. 2001; Valiere et al. 2002). Small errors in identifying genotypes can lead to large overestimates in key population parameters such as abundance (Creel et al. 2003; McKelvey and Schwartz 2004; Schwartz et al. 2006).

Recent studies have attempted to solve problems associated with recovering DNA from feces by investigating the influence of factors such as season, diet, collection method, preservation method, extraction protocol, and time since collection (see Murphy et al. 2007 for a synopsis of publications). Whereas a few studies have provided data on the influence of time and weather on DNA amplification from pellet deposition to collection (Farrell et al. 2000; Lucchini et al. 2002; Piggot 2004), only Murphy et al. (2007) have quantified impacts on a daily temporal scale. To our knowledge, studies of this nature have not been conducted in wet environments, and have not been performed on fecal pellets of ungulates. Influence of moisture on decay time of fecal pellets has been researched in duikers (*Cephalophus* spp.); however, DNA degradation was not evaluated (van Vliet et al. 2008b). With relatively low levels of moisture having been identified as significantly affecting amplification success (Murphy et al. 2007), further studies are warranted in wet environments. Therefore, our objective was to determine the length of time a deer fecal pellet could remain in the field in a temperate rainforest environment before the DNA became too degraded for individual identification. Those data will provide ungulate researchers and other wildlife researchers working in wet climates with data on the appropriate length of time between sampling periods when collecting feces for DNA extraction. This information will help researchers avoid costly and time-consuming steps associated with collecting fecal samples yielding insufficient DNA.

Study area and methods

We conducted our study on Prince of Wales Island, located within the Alexander Archipelago of southeastern Alaska. On Prince of Wales, rugged mountains extend to 1,160 m in elevation with habitats at <600 m dominated by temperate coniferous rainforest consisting primarily of Sitka spruce (*Picea sitchensis*) and western hemlock (*Tsuga heterophylla*) (Alaback 1982). Annual precipitation (mostly in the form of rainfall) of up to 400 cm is possible and mean monthly temperatures range from 1°C in January to 15°C in August (Alaska Climate Research Center 2008).

We used fecal pellets of Sitka black-tailed deer for experimentation. During August 2005, we collected muscle tissue and pellets (directly from intestines) from five deer harvested by hunters. Some of the pellets (2–3) and muscle tissue were immediately placed in 90% ethanol (Nsubuga et al. 2004), and stored at room temperature until DNA extraction. Of the remaining pellets, half were placed outdoors on soil and exposed to natural weather conditions, while the other half were placed on soil in a wax-coated cardboard box sheltered from rainfall. Pellets from each treatment group were sampled at intervals of 2, 7, 14, 21, and 28 days after deer harvest. All samples were then sent to the Rocky Mountain Research Station's genetics laboratory in Missoula, Montana for genotyping.

We extracted genomic DNA from single pellets using the DNeasy Tissue Kit (Qiagen Inc. Valencia, CA), and a protocol described by Maudet et al. (2004) with the following modifications: we performed lysis in 25 ml scintillation vials on a rocker at room temperature for 20 min using 900 µl of lysis washing buffer to completely coat the pellet. We screened twenty microsatellite primers for variability ($n = 10$ tissue samples from Prince of Wales Island) and suitability for use with DNA from deer pellets.

Initial descriptive statistics of the genetic variability of the ten deer samples used in this pilot study were calculated using GENALEX (Peakall and Smouse 2006) including mean number of alleles per locus, observed and expected heterozygosity, probability of identity (PID), probability of identity given siblings (PIDSIB), and F_{IS}. The probability of identity is the probability of two randomly chosen deer in the Prince of Wales Island population having identical genotypes, while the probability of identity (siblings) is the probability of two siblings drawn from the Prince of Wales Island population having identical genotypes. In general, we want PID to be less than 0.001 and PIDSIB to be less than 0.05 (Schwartz and Monfort 2008).

Once we selected a suite of microsatellites with adequate power to discern individuals we determined the multilocus genotype of DNA extracted from a single pellet for each individual (5). Multiplying individuals × treatments (wet and dry) × time periods (2, 7, 14, 21, and 28 days exposure) gives 50 different samples, plus controls (5 tissue samples and 5 pellets immediately placed in alcohol, t_0), for a total of 60 different samples. Three samples disintegrated in the wet site after 7 (1 sample) and 14 days (2 samples), thus only data for 53 different

samples were available. Genotypes from all pellet samples (wet and dry) were compared to those from associated tissue samples to determine if genotyping error was present, and if so, the type of error was reported for both wet and dry treatments. Two different genotyping errors were noted: allelic dropout and false alleles. Allelic dropout occurs when one of two bands in a heterozygous individual does not amplify due to insufficient template DNA; false alleles occur when a spurious band is produced. To determine the effects of rainfall (exposed vs. protected) and time on amplification failure and error rates we conducted a series of non-parametric Mann–Whitney *U* and Chi-Square tests using SPSS (SPSS Inc., Chicago, Illinois).

Results

Seventeen microsatellite loci amplified and nine were variable without error (allelic drop-out/false alleles) in the five deer used in this study (Table 1). Not counting the 10 controls and the 7 samples that could not be tested during the latter time periods because of disintegration, 27 (63%) pellet samples were genotyped without error for all loci whereas 3 (7%) pellet samples failed at 1 locus, and 13 (30%) failed ≥1 locus.

Effects of rainfall

During sampling, our experimental site received 15.2 cm of rainfall, and mean temperature was 15.7°C. Rainfall was not measured on a daily basis, but precipitation (frequency and intensity) was steady throughout our field investigation. Combining all sampling periods, error rates by locus were similar within treatments (Dry: $\chi^2 = 2.1$, $P = 0.550$, Wet: $\chi^2 = 2.3$, $P = 0.801$), but different (Mann–Whitney $U = 0.0$, $P < 0.001$) between treatments (Table 1). Mean number of loci amplified per sample was different (Mann–Whitney $U = 57.5$, $P < 0.001$) between protected (mean = 8.9, SD = 0.44, $n = 25$) and exposed treatments (mean = 5.4, SD = 2.81, $n = 18$). When evaluating all samples, 1.3% ($n = 225$) loci had amplification failures in the dry environment and 39.5% ($n = 162$) in the wet environment. When evaluating all samples but excluding loci that failed to amplify, 1.4% of alleles ($n = 444$) had errors (dropout or false allele) in the dry environment and 15.6% of alleles ($n = 128$) in the wet environment. Pooling time periods,

Table 1 Descriptive statistics of the nine variable microsatellites found in this study

Locus	GenBank accession no.	*N*	*A*	PID	PID_{SIB}	Ho	He	F_{IS}	Error rate (wet) $n = 36$	Error rate (dry) $n = 50$
TGLA 57[a]	CM000177.3	10	3	3.08E-01	5.74E-01	0.500	0.505	0.010	0.22	0.04
T159S[b]	AF102245	10	3	2.31E-01	5.10E-01	0.600	0.595	−0.008	0.28	0.02
C273[c]	AF102246	10	2	5.88E-01	7.69E-01	0.300	0.255	−0.176	0.33	0.02
Texan-4[d]	L24781	10	2	4.01E-01	6.23E-01	0.300	0.455	0.341	0.19	0.0
RT24[e]	U90746	9	3	5.25E-01	7.36E-01	0.333	0.290	−0.149	0.39	0.04
C89[b]	AF102247	10	2	5.14E-01	7.18E-01	0.400	0.320	−0.250	0.22	0.0
SR-CRSP-1[f]	L22192.1	10	2	8.24E-01	9.08E-01	0.100	0.095	−0.053	0.08	0.0
BM1225[g]	D20S11	10	2	3.86E-01	6.06E-01	0.200	0.480	0.583	0.28	0.06
T7[b]	AF102240	10	2	4.25E-01	6.46E-01	0.400	0.420	0.048	0.22	0.0
Mean/ Product		10	2.3	0.000608	0.026433	0.348	0.379	0.038	0.25	0.02

N is the number of sample analyzed at each microsatellite, *A* is the number of alleles per locus, PID is the probability of identity assuming random individuals, and PID_{SIB} is the probability of identity assuming siblings, Ho is observed heterozygosity, He is expected heterozygosity, and F_{IS} is a fixation index (Fis), and error rate is the total number of amplification failures and errors (dropout, false alleles) divided by number of fecal pellet samples tested in each treatment over all timer periods. The last column is a mean for all indices of diversity except PID and PID_{SIB} for which it is a product

[a] Barendse et al. (1994)

[b] Jones et al. (2000)

[c] Meredith et al. (2005)

[d] Holder et al. (1994)

[e] Wilson et al. (1997)

[f] Arevalo et al. (1994)

[g] Bishop et al. (1994)

amplification success was similar among all individuals in the same environment (Dry: $\chi^2 = 3.133$, $P = 0.536$; Wet: $\chi^2 = 2.818$, $P = 0.589$).

Effects of time

Grouping samples by treatment, loci amplification success declined with time for exposed samples ($\chi^2 = 10.672$, $P = 0.031$), but remained the same ($\chi^2 = 3.133$, $P = 0.536$) for samples in the protected environment through 28 days. Amplification success of samples in exposed and protected treatments were similar after 2 days (Mann–Whitney $U = 7.5$, $P = 0.134$), but were different at each following time period (Mann–Whitney $U = 3.5$ [7-days], 0.0 [14, 21, 28 days]; $P = 0.044$ [7 days], 0.007 [14 days], 0.033 [21 days], 0.016 [28 days]). If we ignore loci that failed to amplify, dropout and false allele errors were similar over time when treatments were grouped (Dry: $\chi^2 = 2.369$, $P = 0.499$, Wet: $\chi^2 = 1.369$, $P = 0.713$). Errors also were similar over time when we compared treatments (Mann–Whitney $U = 7.5$ [2 days], 4.5 [7 days], 5.0 [14 days], 4.0 [21 days], 2.0 [28 days], $P = 0.136$ [2 days], 0.065 [7 days], 0.091 [14 days], 0.629 [21 days], 0.219 [28 days]).

Eighty percent of samples in the protected environment and 22% of samples in the exposed environment were successfully genotyped during the experiment. In the exposed treatment, no samples were genotyped without either amplification failure or errors after 7 days. While the time variable alone did not have a strong influence on DNA quality, time + exposure degraded nearly all fecal-pellet DNA after 28 days (Fig. 1). After 14 days, amplification failures and errors occurred in >50% of loci for samples in the exposed treatment (Fig. 1a, b). Excluding loci that failed to amplify and samples that disintegrated, influence of time on error rates per locus were less evident in both protected and exposed treatments.

Discussion

Similar to other studies investigating seasonal precipitation and moisture (Murphy et al. 2007; Piggot 2004), our study showed that rainfall significantly increases degradation rates of DNA from ungulate pellets. In a dry environment, only one error was produced in a sample collected between 0 and 14 days and only 6 errors were produced between 0 and 28 days. There was a high rate of success of the PCR reactions during the entire 28-day period in the dry environment. The results were very different for an unprotected wet environment. Here not only did errors increase after a 7 day time horizon (Fig. 1), but the number of successful PCRs declined markedly.

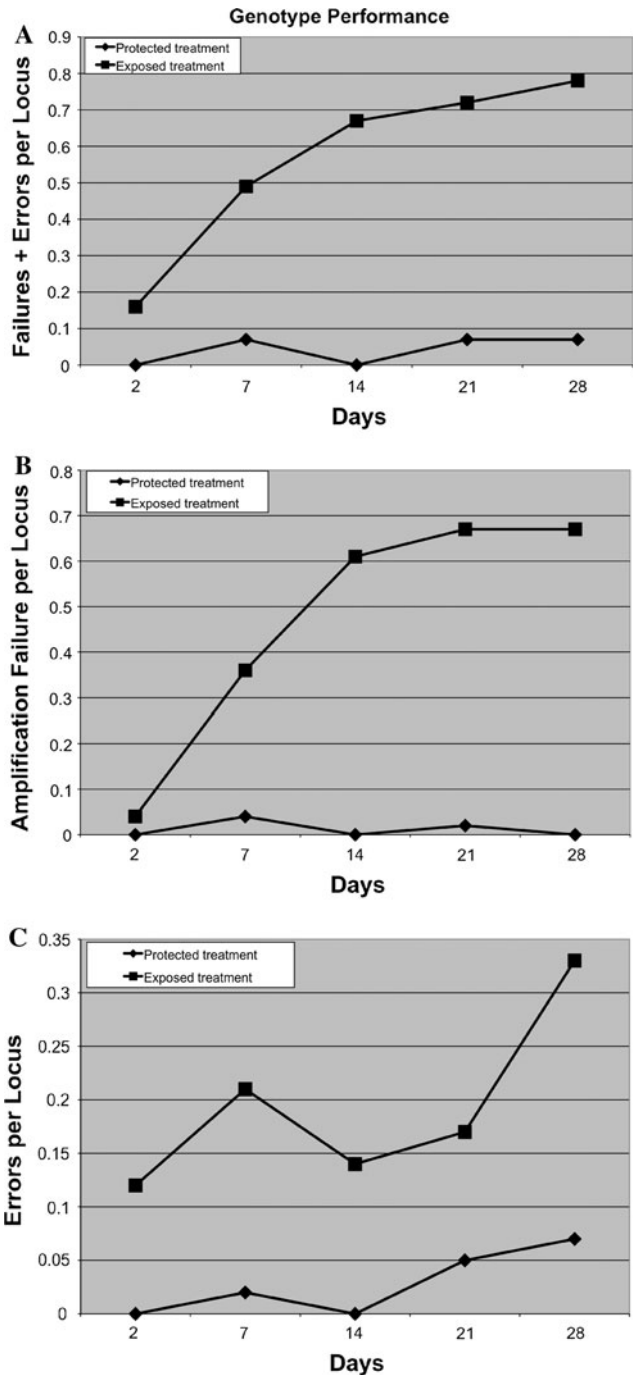

Fig 1 Comparison between genotype performance of DNA extracted from deer fecal pellets stored in protected and exposed environments over 28 days: **a** errors (allelic dropout and false alleles) and amplification failures combined, **b** amplification failures per locus, **c** errors per locus

The high error rate for genotypes from fecal pellets after a short time frame in an exposed environment may represent a hardship for sampling in wet environments; conversely, it removes concerns regarding collecting samples that were deposited prior to the sampling interval of interest. For

instance, clearing feces from plots in a wet environment may not be needed because samples >14–21 days old will fail to provide DNA. However, to reduce testing of samples that fail during PCR, we recommend clearing plots of pellets before initial sampling and not allowing time intervals for pellet deposition to extend beyond 14 days, particularly in wet environments. Addition testing is needed during the winter season to determine if rates of DNA degradation change. Previous studies on pellet persistence (Fisch 1979; Harestad and Bunnell 1987) and seasonal effects (Lucchini et al. 2002) suggest differences will be evident.

We recommend that some form of error checking protocol should be instituted if samples are obtained from a wet environment. In general, error checking protocols take one of three forms: (1) the "multi-tube" approach, where DNA samples are analyzed multiple times to ensure accuracy (Bellemain et al. 2005; Taberlet et al. 1996); (2) quantify the amount of extracted DNA and only analyze samples with adequate yield (Morin et al. 2001); (3) use computer algorithms to detect samples containing genotyping errors and re-run samples that might contain errors (McKelvey and Schwartz 2005; Miller et al. 2002; Valiere et al. 2002). All of these approaches are valid, but it must be recognized that genetic errors must not only be caught, but there must be some ability to demonstrate their removal from a dataset.

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