Tools and Technology Article

Low Genotyping Error Rates and Noninvasive Sampling in Bighorn Sheep

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ABSTRACT Noninvasive DNA sampling allows studies of natural populations without disturbing the target animals. Unfortunately, high genotyping error rates often make noninvasive studies difficult. We report low error rates (0.0–7.5%/locus) when genotyping 18 microsatellite loci in only 4 multiplex polymerase chain reaction amplifications using fecal DNA from bighorn sheep (*Ovis canadensis*). The average locus-specific error rates varied significantly between the 2 populations (0.13% vs. 1.6%; $P < 0.001$), as did multi-locus genotype error rates (2.3% vs. 14.1%; $P < 0.007$). This illustrates the importance of quantifying error rates in each study population (and for each season and sample preservation method) before initiating a noninvasive study. Our error rates are among the lowest reported for fecal samples collected noninvasively in the field. This and other recent studies suggest that noninvasive fecal samples can be used in species with pellet-form feces for nearly any study (e.g., of population structure, gene flow, dispersal, parentage, and even genome-wide studies to detect local adaptation) that previously required high-quality blood or tissue samples. (JOURNAL OF WILDLIFE MANAGEMENT 72(1):299–304; 2008)

DOI: 10.2193/2006-006

KEY WORDS bighorn sheep, genotyping error, microsatellite DNA, noninvasive genetics, *Ovis canadensis*, remote sampling.

Noninvasive DNA sampling from feces, shed hair, feathers, sloughed skin, or urine allows studies of free-ranging animals without capturing or even disturbing them. Fecal samples are especially useful when studying endangered species because feces is the only material not requiring Convention on International Trade in Endangered Species permits for transportation internationally. Feces can also yield information about physiological hormones (e.g., stress and reproductive condition), diet, and parasite load, unlike other noninvasively sampled material (e.g., shed hair).

A major disadvantage of noninvasive sampling is high microsatellite genotyping error rates that often occur due to low-quantity and low-quality DNA or polymerase chain reaction (PCR) inhibitors in feces (Taberlet et al. 1999). Microsatellites have become the marker of choice for noninvasive studies because of their high polymorphism and relative ease of amplification (Luikart and England 1999). Because microsatellite genotyping error rates can exceed 30% per locus (Gagneux et al. 1997), researchers often must repeat genotypings to determine the correct genotype for each locus in each individual studied (Taberlet et al. 1999, Pompanon et al. 2005). The per-locus genotyping error rate is defined as the proportion of single locus genotypes with one or both alleles scored incorrectly.

Repeat genotyping analyses increase costs in time and consumables. Thus, it would be helpful to 1) minimize error rates and 2) co-PCR amplify (multiplex) several loci in a single reaction tube. Multiplexing saves time and consumes less DNA per locus analyzed, which is important in noninvasive studies that typically yield limited quantities of DNA. Unfortunately, multiplexing likely causes higher error rates, at least at some loci, because it can be difficult to optimize amplification conditions for multiple loci in the same PCR. Finally, error rates must be especially low when genotyping many loci in order to keep the multi-locus error rate reasonably low. The multi-locus error rate is the proportion of multi-locus genotypes that contain $\geq$1 error (e.g., $\geq$1 allelic dropout over all loci genotyped). The multi-locus error rate is important to consider when identifying individuals, analyzing parentage, or conducting assignment tests, which are based on multi-locus genotypes. Few studies have considered multi-locus error rates and even fewer noninvasive studies have used $\geq$10–12 loci. Our objectives were to develop noninvasive genotyping methods for many microsatellite loci in multiplex PCRs while minimizing and

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Table 1. Polymerase chain reaction multiplexes, polymorphism levels, and genotyping error rates per locus for 21 microsatellite loci typed in Glacier National Park (GP; 16 individuals, 8/sampling location North [N.] and South [S.]) and Thompson Falls (TF; 16 individuals, 8/sampling location East [E.] and West [W.]), Montana, USA. Samples were collected in the fall and winter of 2005 from Glacier National Park and Thompson Falls, respectively. We show heterozygosities (exp [H_e] and obs [H_o]) for 2 sampling locations each within GP and TF to avoid Wahlund effects causing a deficit of heterozygotes.

Locus	N_A[a]	Allele length range (base pairs)	N. GP H_e	N. GP H_o	S. GP H_e	S. GP H_o	E. TF H_e	E. TF H_o	W. TF H_e	W. TF H_o
MAF36[c,h]	5	89–105	0.508	0.500	0.742	0.625	0.500	0.750	0.375	0.492
MAF48[c,h]	3	117–129	0.570	0.500	0.375	0.500	0.117	0.125	0.375	0.305
MAF209[c,h]	6	105–117	0.664	0.875	0.742	0.750	0.727	0.875	0.625	0.664
FCB304[c,h]	5	131–139	0.648	0.900	0.758	0.750	0.594	0.375	0.750	0.703
FCB266[c,h]	6	80–94	0.664	0.500	0.695	0.750	0.688	0.750	0.500	0.602
HH62[c,h]	6	117–134	0.740	0.800	0.680	0.875	0.672	0.375	0.875	0.727
MAF33[d,h]	4	122–128	0.703	0.625	0.609	0.500	0.633	0.875	0.875	0.695
ADC[d,h]	4	80–92	0.219	0.250	0.622	0.429	0.219	0.250	0.375	0.430
AE16[d,h]	7	75–93	0.292	0.333	0.766	0.625	0.724	0.429	0.625	0.719
SR8[d,g,i]	5	224–248	0.611	0.667	0.528	0.500	0.734	1.000	0.875	0.781
ILST011[d,j]	6	274–292	0.580	0.800	0.765	0.857	0.750	0.500	0.625	0.648
ILST30[d,g,j]	2	172–182					0.736	1.000	0.667	0.806
INRA185[d,g,h]	6	245–269					0.672	0.875	0.875	0.641
SOMAb[e,j]	4	96–120	0.656	0.875	0.648	0.625	0.477	0.500	0.500	0.531
KRT2[e,j]	2	135–137	0.430	0.625	0.000	0.000	0.539	0.625	1.000	0.711
GLYCAM1[e,j]	5	169–177	0.695	0.750	0.711	0.750	0.117	0.125	0.750	0.602
OLADRBps[e,f,h]	9	273–295	0.500	0.667	0.836	0.875	0.508	0.625	0.625	0.430
TCRBV624[f,j]	4	170–176	0.602	0.750	0.320	0.375	0.477	0.500	0.125	0.430
LIF[f,j]	5	108–122	0.500	0.000	0.531*	0.250	0.594	0.625	0.750	0.539
KER[f,j]	4	173–179	0.617	0.375	0.597	0.500	0.555	0.750	0.750	0.555
MMP9[f,j]	4	185–197	0.555	0.750	0.617	0.375*	0.500	0.750	0.375	0.492
$\bar{x}$	3.8		0.62	0.62	0.65	0.67	0.55	0.60	0.63	0.60

[a] N_A = no. of alleles per locus.
[b] Mean of the % of replicate genotypes with an erroneous genotype (i.e., false allele or allelic dropout).
[c–f] 4 groups (c, d, e, and f) of loci multiplexed in polymerase chain reaction together.
[g] These 3 loci yielded no data (blank cells in table) for many individuals, so were not used in error-rate computations. Locus MHC1 yielded no data and is not listed.
[h] Sheep cloned primer sequences. See Maudet et al. 2004*a* and Luikart et al. 1999 for original references and primer sequences.
[i] Goat cloned primers. See Maudet et al. 2004*a* and Luikart et al. 1999 for original references and primer sequences.
[j] Cattle cloned primers. See Maudet et al. 2004*a* and Luikart et al. 1999 for original references and primer sequences.
* $P < 0.01$.

quantifying genotyping error rates for fecal DNA collected from wild bighorn sheep (*Ovis canadensis*).

STUDY AREA

We collected fecal samples from a native herd of bighorn sheep in Glacier National Park and an introduced herd near Thompson Falls, Montana, USA. In these populations, we will eventually use noninvasive sampling to assess the spatial scale of genetic differentiation and test the influence of landscape features on gene flow among bighorn sheep. For this study, we sampled at 2 locations in Glacier Park only 30–40 km apart but separated by a lake and deep valley. We also sampled at 2 locations at Thompson Falls only 10 km apart and separated by a small river and gravel road soon to be converted to a larger paved road.

METHODS

We collected pellets from an isolated pile of droppings from each individual <1 hour after defecation. We placed 4–6 fecal pellets (using a disposable latex glove, sterile tooth pick, or sticks or stones) into approximately 7 volumes (17 mL) of 95% ethyl alcohol (ETOH) and stored them at room temperature (Glacier Park) or at −10° C (Thompson Falls) in leakproof plastic vials.

We extracted DNA approximately 2 months after collection using the DNeasy™ Blood Kit (Qiagen, Valencia, CA) modified to include an initial wash of one fecal pellet for 15 minutes in 350 μL lysis buffer (0.1 M Tris-HCl, 0.1 M ethylene-diamine-tetra-acetic acid, 0.01 M NaCl, 1% N-lauroyl sarcosine, pH 7.5). We used approximately 200 μL of the lysis buffer directly in the extraction protocol as if the sample were blood (per Maudet et al. 2004*b*).

Initial PCR tests compared the reliability of the Qiagen® multiplex PCR kit versus standard AmpliTaq Gold® and its buffer (Applied Biosystems, Foster City, CA) on a multiplex PCR of a pool of 9 loci (located in genes; see below). The multiplex kit far outperformed AmpliTaq Gold in that all loci successfully amplified with this multiplex kit, whereas several did not when using AmpliTaq Gold (in repeated, independent tests on 4 fecal DNA samples). Thus, the remainder of the analyses discussed below used only the Qiagen multiplex PCR kit.

We amplified 22 microsatellite loci in 4 multiplex PCRs using the Qiagen multiplex PCR kit (Table 1). We performed multiplex PCR reactions in a volume of 12.5 μL containing 1 μL of fecal DNA extract, multiplexed primers at a concentration of 0.2 μM each, and 6.25 μL of Qiagen multiplex PCR buffer (2×). Samples were denatured during 15 minutes at 95° C, followed by 55 cycles of 94° C

Table 1. Extended.

Locus-specific error rate GP (%)[b]	Locus-specific error rate TF (%)[b]	Mean error rate (%)[b]
0.0	0.0	0.0
1.7	0.0	0.9
2.4	0.0	1.2
3.3	0.0	1.7
1.6	0.0	0.8
0.0	0.8	0.4
0.0	0.0	0.0
1.0	0.0	0.5
0.0	0.0	0.0
	0.0	0.0
2.9	0.0	1.5
0.8	0.0	0.4
1.6	0.0	0.8
0.8	0.0	0.4
0.0	0.0	0.0
3.3	0.8	2.1
7.5	0.8	4.2
0.8	0.0	0.4
0.8	0.0	0.4
1.6%	0.13%	0.9%

for 30 seconds, primer-specific hybridization temperature for 90 seconds (Table 1), 72° C for 60 seconds, and a final extension at 72° C for 30 minutes. We conducted PCR amplifications in a PerkinElmer Applied Biosystems 9700 thermal cycler.

After the purification step on Qiagen spin columns, we diluted PCR products 25 times for mixes 1, 3, and 4, and only 10 times for mix 2. We then mixed 1 μL of diluted sample with 10 μL of Hi-Di™ Formamide (Applied Biosystems) and 0.1 μL of GeneScan™-350 Rox size standard (Applied Biosystems). We then loaded products on an ABI Prism 3100 sequencer in a 36-cm-long capillary using POP4 polymer (Applied Biosystems). We conducted fragment analyses using GeneMapper software (Applied Biosystems).

We quantified the quality (usefulness) of each locus for producing genotype data using the quality index from Miquel et al. (2006). We computed the locus quality index as the proportion of all PCRs, including failed PCRs, which yield the correct consensus genotype. This index is different from the genotyping error rate (see next paragraph), which considers only those successful PCRs that amplify DNA. We also computed a sample quality index as the proportion of all PCRs (among all loci) that yield a correct (consensus) genotype for an individual pellet sample.

We computed the genotyping error rate per locus empirically for each locus by repeat genotyping 16 individuals from each of the 2 populations (we eliminated 4 loci that did not amplify in numerous samples). We conducted 8 independent repeat PCR amplifications for each locus. The correct genotype for each locus was inferred from the consensus of the 8 replicate genotyping. This was deemed reliable because ≥5 of the 8 genotypes were always identical. In a few cases ($n = 5$), we obtained fewer than 8 genotypes (e.g., due to no PCR product), and we inferred the correct genotype from a reduced number of identical genotypes (e.g., 4 identical genotypes out of 6).

We computed the multi-locus genotyping error rate as the proportion of all multi-locus genotypes with an error (e.g., allelic dropout) for ≥1 locus. We also computed the proportion of multi-locus genotypes with ≥2 errors. Multi-locus genotypes with only 1 or 2 errors were easily detectable because no 18-locus genotypes (or even 16-locus genotypes) should differ by only 1 or 2 alleles, considering the high variability of our markers (Table 1; Waits et al. 2001, McKelvey and Schwartz 2004).

We inferred allelic dropout when a homozygous genotype was observed for 1–3 genotypings out of 8 that were otherwise heterozygous. We never observed >3 homozygous genotypes among 8 replicates for a consensus heterozygote. We computed the locus-specific allelic dropout rate as the proportion of all genotypes (heterozygous and homozygous) at a locus with a dropout (Pompanon et al. 2005). We inferred a false allele when an extra allele appeared in 1 or 2 of the 8 replicate genotypes. We computed the rate of false alleles as the proportion of all genotypes showing a false allele.

We computed heterozygosity and F_{ST} and conducted tests for Hardy–Weinberg proportions using GenAlEx version 6 (Peakall and Smouse 2006). We tested for gametic (linkage) disequilibrium using GENEPOP version 3.3 (Raymond and Rousset 1995). We conducted tests for Hardy–Weinberg and gametic disequilibrium in Glacier within each of 2 sampling locations (north and south) because F_{ST} between these 2 areas was moderately large ($F_{ST} = 0.13$). We also conducted tests at Thompson Falls on each of 2 sampling locations (east and west), although F_{ST} was lower ($F_{ST} = 0.04$). For each location in each population, we sampled 8 individuals (Table 1).

We conducted likelihood ratio tests for differences in single-locus genotyping error rates between populations (Glacier National Park and Thompson Falls) via logistic regression modeling of the probability of a genotyping error. The binomial response variable was the total number of errors in 8 replications, and the fixed factors were population (location) and locus. Consequently, we used 512 binomial observations for the regression analysis and likelihood ratio tests. We tested for multi-locus genotype error rate differences between populations using a contingency table test for homogeneity of error rates between the 2 populations. We used S-PLUS (Insightful, Seattle, WA) for statistical analyses.

RESULTS

Out of 22 loci, 2 loci (MHC1 and ILST30) did not amplify in numerous samples (Fig. 1). Thus, we eliminated these loci from statistical analyses. We also eliminated SR8 and INRA185 from interpopulation comparisons because many Glacier Park samples did not amplify (Fig. 1a). The lower

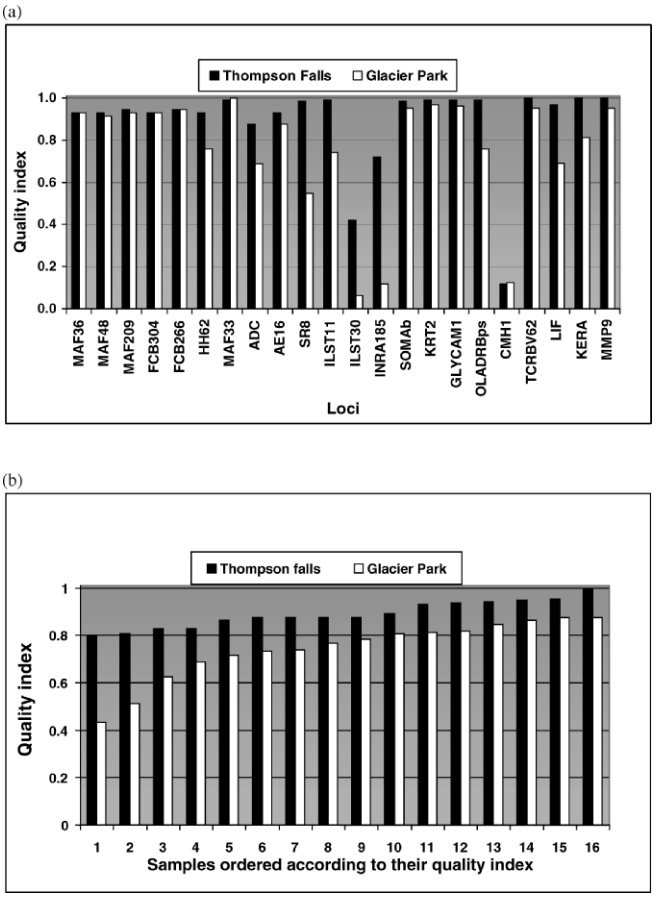

Figure 1. (a) Locus quality index for 21 microsatellite loci genotyped 128 times (16 individuals × 8 replicates) using fecal DNA from Thompson Falls and Glacier National Park, Montana, USA. (b) Sample quality index for 16 samples (21 loci genotyped 8 times) showing generally lower-quality DNA samples from Glacier National Park (mean index = 0.743) compared to Thompson Falls (mean index = 0.890). Samples were collected in the fall and winter of 2005 from Glacier National Park and Thompson Falls, respectively.

quality of individual pellet samples from Glacier Park compared to Thompson Falls is evident from the pellet sample quality index (Fig. 1b).

Heterozygosity levels among loci ranged from 0.12 to 0.91 (Table 1). The total number of alleles ranged from 3 to 9 per locus. We observed significant deviations from Hardy–Weinberg proportions at only 2 loci (LIF and MMP9; $P = 0.007$ for each locus, both in the Glacier population). We detected no strong gametic disequilibrium ($P > 0.01$) for any pairs of loci in either the Glacier or Thompson Falls populations.

Locus-specific genotyping error rates ranged from 0.0% to 0.8% in Thompson Falls and from 0.0% to 7.5% in Glacier Park (Table 1). The loci with highest error rates were LIF and TRCBV, which may result from these primers being designed for cattle DNA sequences (Table 1). Error rates were especially low in Thompson Falls, with only 6 total errors (5 dropouts and 1 false allele) out of 2,560 genotypings (16 loci × 8 replicates × 20 loci). Allelic dropout rates were higher than false allele rates (by approx. 2–5 times; Table 2).

We detected significantly lower locus-specific error rates in Thompson Falls than in Glacier Park ($P < 0.001$; Table 1). We also detected significantly lower multi-locus genotyping error rates (2.3%; 3 of 128 multi-locus genotypings) in Thompson Falls and (14.1%; 18 of 128 genotypings) than in Glacier Park ($\chi^2 = 7.23$, $P < 0.007$). When we dropped the 2 loci with the highest error rates (LIF and TRCBV62), the multi-locus error rates were only 1.6% and 8.6% for Thompson Falls and Glacier National Park, respectively.

DISCUSSION

Our genotyping error rates are among the lowest reported for noninvasive fecal samples. Many studies report single-locus error rates >10–30% for DNA extracted from feces (e.g., Gagneux et al. 1997, Goossens et al. 2000, Murphy et al. 2002). Error rates were low even for some loci with relatively long alleles (>250 base pairs), suggesting relatively limited DNA degradation. For example, OLADRBps had an error rate of 0% in both populations (allele length range: 273–295 base pairs; Table 1). The substantial inter-locus variation in error rates suggests that researchers should often have available additional loci in case some perform poorly during the study.

Our low error rates likely are attributable to several factors. First, we sampled fresh fecal pellets (<1 hr after defecation). Second, DNA degrading enzymes (nucleases) were inhibited quickly by submerging the pellets in a large volume of high percentage (95%) ETOH. Third, washing off only the external mucous layer from pellets (and then discarding them) concentrates DNA (and perhaps many intact cells) while minimizing contamination with PCR inhibitors in the fecal material. Finally, we sampled in fall (Glacier) or winter (Thompson Falls) when the vegetation is less moist and more abrasive, and when fecal matter moves more slowly through the gut. This may promote sloughing of epithelial cells from the intestinal lining. In Glacier Park, pellets

Table 2. Mean per-locus and multi-locus genotyping error rates for 18 microsatellites genotyped in bighorn sheep from 2 populations (Glacier National Park and Thompson Falls, Montana, USA). Samples were collected in the fall and winter of 2005 from Glacier National Park and Thompson Falls, respectively. We computed error rates as the percentage of replicate genotypings resulting in an error.

Population	Mean rate of allelic dropouts	Mean rate of false alleles	Total error rate[a]	Mean rate of non-amplification	Multi-locus error rate
Glacier Park	1.3	0.3	1.6	6.8	14.1
Thompson Falls	0.09	0.04	0.13	2.7	2.3
Overall mean	0.11%	0.15%	0.9%	4.8%	8.2

[a] Single-locus error rate.

collected in October were noticeably less moist than those collected in September.

Lower error rates at Thompson Falls were attributed to sampling later in the year (late Dec vs. Sep–Oct in Glacier) when 1) vegetation was more abrasive or passed through the gut more slowly and 2) ambient temperature was colder, slowing DNA degradation. Also, we put vials collected at Thompson Falls in a freezer (–10° C) within approximately 12 hours after sampling, and they remained there until we shipped them to the laboratory for analysis, whereas we stored vials from Glacier at room temperature. It is uncertain which factor(s) led to lower genotyping error rates for the Thompson Falls samples (vegetation, ambient temperature, or freezing of sample vials). Nonetheless, our study identifies important variables to consider when planning noninvasive studies. Further study of these factors is warranted.

Multi-locus genotyping error rates are important because multi-locus genotypes are used for certain applications such as individual identification, assignment tests, and paternity analysis. In each population, we genotyped 16 individuals 8 times (Table 2), making 128 multi-locus genotypes per population. Although the multi-locus error rates were significantly different between Glacier and Thompson Falls, no individual multi-locus genotypes had >1 error in Thompson Falls, and only 3 multi-locus genotypes had >1 error in Glacier Park. This result is important and encouraging because 2 multi-locus genotypes from 2 different individuals should never differ at only one locus if loci are highly variable and many loci are typed, as in our study. Thus, a 1-locus error is readily detectable and can be removed or corrected.

Relatively low microsatellite genotyping error rates have been reported in only a few other studies using fecal DNA sampled noninvasively. Wehausen et al. (2004) reported average locus-specific error rates of approximately 1.0% from bighorn sheep feces. These authors scraped dried fecal pellets to recover DNA, which apparently tends to be concentrated on the outer surface of pellets. Epps et al. (2005) used the same surface-scrape extraction protocol and reported an average error rate of approximately 2.2%. Fernando et al. (2003) reported an average error rate of approximately 2.0% using DNA recovered from elephant (*Loxodonta africana*) dung (see also Okello et al. 2005). They also scraped the outer surface of the dung pile. Flagstad et al. (1999) reported error rates of 2% and 5% in reindeer (*Rangifer tarandus*) and domestic sheep feces, respectively. These authors used a surface pellet wash similar to the one used here, followed by a magnetic bead DNA extraction protocol (with no proteinase K or phenol–chloroform). All of these studies genotyped only 4–6 loci, except Epps et al. (2005), who genotyped 14 loci. None reported multi-locus genotyping error rates. None used multiplex PCR, except Wehausen et al. (2004), who multiplex amplified 3 pairs of loci.

Interestingly, studies reporting the lowest genotyping error rates tend to be from herbivores (e.g., elephants) and ruminant species with pellet-form feces (deer, sheep, and goats) that can be easily surface-washed or surface-scraped to recover DNA while minimizing the presence of inhibitors of PCR. This suggests that herbivores and surface-wash techniques provide relatively high-quality DNA for PCR amplification. Future studies are needed in other taxa (e.g., carnivores) and in species with different forms of feces (especially round or pellet form) to further assess if surface-wash or -scrape techniques can minimize error rates and improve noninvasive sampling approaches in wildlife studies.

MANAGEMENT IMPLICATIONS

The genotyping error rates reported here are low enough to allow noninvasive fecal samples to be used for nearly any application that previously required blood or tissue samples (Taberlet et al. 1999).

These results suggest we now can genotype dozens of loci and achieve low enough error rates to allow powerful studies or paternity, relatedness (Blouin et al. 1996), dispersal, and forensics (using assignment tests to determine the population of origin of migrants or illegally killed animals; e.g., Cornuet et al. 1999, Manel et al. 2002), and to identify adaptively differentiated populations or loci under selection (e.g., Luikart et al. 2003). We recommend that future studies use PCR multiplexing of multiple (4–6) loci, which saves time and money and consumes less DNA, thereby making noninvasive wildlife genetics studies more feasible.

ACKNOWLEDGMENTS

We thank P. Lundberg and Glacier National Park for help with collection of feces. We thank A. Da Silva, D. Coltman, and G. Obexer-Ruff for providing the names of several microsatellite loci in genes, 2 anonymous reviewers for helpful comments improving the manuscript, and the United States Geological Survey, The Glacier Fund, and the Western Federal Lands Highway Division of the United States Department of Transportation for funding support. G. Luikart was supported by the Portuguese American Foundation for Development and Centro de Investigacao em Biodiversidade e Recursos Geneticos, Universidade do Porto.

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Associate Editor: Waits.