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A METHODOLOGICAL COMPARISON AMONG DNA SOURCE TYPES FOR MOOSE GENOTYPING

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ABSTRACT: Population genetic analyses for moose have been based on DNA extracted from blood and other body tissues. Non-invasive sampling of fecal pellets is another potential source of DNA. We compared DNA extraction from blood, liver tissue, and fecal pellet samples from moose in Minnesota and Yellowstone National Park, USA. Extracted DNA from all source types was sufficient for genotyping using 15 microsatellites. DNA extracted from fecal pellets was of lower quality and quantity than DNA extracted from blood and tissue. We provide comparisons of efficiency and effectiveness of DNA extraction protocols for blood, tissue, and fecal pellets, and demonstrate the suitability of using DNA extracted from non-invasively sampled material in moose.

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Key Words: DNA extraction, fecal pellets, genotyping, microsatellites, moose, polymerase chain reaction, population genetics

An important advance in population and conservation genetics has been the ability to obtain DNA from multiple biological source types (Waits et al. 2005). Blood and muscle are the DNA source types used most often in population genetic research with ungulates. Other tissue samples such as liver that are collected for other purposes can also be used for genetic analysis. Samples obtained from hunter-harvested moose or moose killed in vehicular collisions are likely to be available in unlimited quantities on a relative basis. In contrast, samples may be limited if specimens are collected during moose capture operations, although samples collected during such operations have been used as DNA sources in several population genetic studies of cervids (Finnegan et al. 1999, Coulon et al. 2004, Kangas et al. 2013, Wilson et al. 2015).

More recently, fecal pellets collected non-invasively have been used as an alternative source of DNA. These samples can be collected from multiple individuals over a broad geographic region and are relatively easy to collect, particularly if fieldwork is conducted in winter when fresh fecal pellets are visible on snow and cold temperatures limit degradation of DNA. Fecal pellets would be an ideal DNA source in parks or other areas where non-invasive sampling would be preferred or required.

Non-invasive DNA techniques are useful to study populations for which obtaining tissue samples is not logistically feasible. For example, fecal pellet DNA has been used to estimate population size in roe deer (*Capreolus capreolus*; Ebert et al. 2012) and Sitka black-tailed deer (*Odocoileus hemionus sitkensis*; Brinkman et al. 2011), two species that live in densely vegetated habitats that are difficult to survey. Population size, survival rate, and rate of population

size change have been estimated for a protected subspecies of woodland caribou (*Rangifer tarandus caribou*) in Canada using DNA from non-invasively collected fecal pellets (Hettinga et al. 2012). DNA has also been extracted from fecal pellets of mountain goats (*Oreamnos americanus*; Poole et al. 2011) and red deer (*Cervus elaphus*; Valière et al. 2007).

The quality of DNA extracted from fecal pellets can be problematic. Typically, higher quantity and quality DNA is obtained from body tissues than from fecal samples (Waits and Paetkau 2005, Ball et al. 2007) because DNA from feces is more degraded and more likely to be contaminated. The rate of DNA degradation is also affected by the time since deposition and environmental conditions (Kreader 1996, Piggott 2004, Brinkman et al. 2010b). High temperatures, rainfall, bacteria and fungi, and exposure to UV radiation increase the degradation rate of DNA (Piggott 2004, Brinkman et al. 2010b, Buś and Allen 2014). DNA can be extracted with higher success from moose pellets collected from snow in late spring versus pellets collected after snowmelt and temperatures warm (Rea et al. 2016).

DNA extracted from fecal samples is further affected by the diet. Fecal pellet samples from herbivores contain tannins and other substances of vegetative (diet) origin which increases the number of PCR inhibitors in extracted DNA (Kreader 1996), and they may also contain DNA from plants. Carnivore scat includes DNA from prey species which may inflate DNA concentration measurements, but will not affect results if genetic markers are species-specific (Deagle et al. 2005). In addition to inherent factors involving the sample itself, proper collection and lab techniques, including sample collection, storage methods, and specified extraction protocols, are necessary to ensure quality results from all DNA source types (Waits and Paetkau 2005, Beja-Pereira et al. 2009, Buś and Allen 2014).

DNA quantity and quality are important because they directly affect PCR amplification success, genotyping success, and genotyping error rates (Taberlet et al. 1996, McKelvey and Schwartz 2004, Waits and Paetkau 2005, Ball et al. 2007, Brinkman et al. 2010a). As DNA degrades, nucleic acid residues undergo chemical changes and strands become fragmented (Buś and Allen 2014), and this fragmentation results in lower PCR amplification success and increased genotyping errors (Taberlet et al. 1999). Common PCR amplification problems using degraded DNA include failure of DNA to amplify due to absence of usable DNA and genotyping errors (i.e., false alleles and allelic dropout).

Genetic techniques have been tested using DNA from various source types for several species, but there are few direct comparisons among source types for ungulates (Wehausen et al. 2004, Valière et al. 2007), and none for moose. Moose population genetic research to date has used either DNA from blood or tissue, except for the recent tests with fecal pellets by Rea et al. (2016). It would be useful to know the relative extraction and genotyping success from different DNA sources because source type affects the time and resources required for population genetic studies. We compared DNA extraction from existing samples of moose liver tissue, blood, and fecal pellets. We measured average DNA yield, compared PCR amplification and genotyping success rates, and identified ways to improve extraction efficiency in the protocols.

METHODS

We compared DNA from biological sources collected in northern Minnesota

(MN) and the northern range of Yellowstone National Park (YNP), USA. Samples from MN had been archived, and samples from YNP were collected from free-ranging moose in winter 2013.

Study Areas

Minnesota.— The study area of pellet origin includes northern Minnesota which transitions from mixed conifer-deciduous forests, bog, and swamp in the east to an agricultural matrix in the west. The north-eastern area is characterized by conifer-deciduous forests, conifer bogs and swamps, and numerous small lakes, peatlands, and wet forest throughout. The predominant tree species are white pine (*Pinus strobus*), red pine (*P. resinosa*), quaking aspen (*Populus tremuloides*), paper birch (*Betula papyrifera*), white spruce (*Picea glauca*), balsam fir (*Abies balsamea*), and white cedar (*Thuja occidentalis*). The northwestern area is relatively flat and dominated by aspen parkland and farmland.

Yellowstone National Park.— The study area included the portion of the Northern Yellowstone Elk Winter Range (Houston 1982) located within YNP as well as some creek drainages located outside the park. Vegetation consists primarily of sage steppe and grassland at low elevation (< 2,000 m), and coniferous forests at high elevation (>3,000 m). The most common conifers are lodgepole pine (*Pinus contorta*), Engelmann spruce (*Picea engelmannii*), sub-alpine fir (*Abies lasiocarpa*), Douglas-fir (*Pseudotsuga menziesii*), and whitebark pine (*Pinus albicaulis*). Willow (*Salix* spp.) is present in drainages and other wet areas.

Samples

Minnesota.— Blood samples (hereafter blood) were collected by the Minnesota Department of Natural Resources (MNDNR) and stored on Whatman FTA® Classic Cards (Whatman International Ltd., Maidstone, United Kingdom). They were collected from hunter-harvested (n = 116), GPS-collared (n = 132), and sick (n = 6) moose in 2011–2013 (Fig. 1). Samples for hunter-harvested moose were not available in 2013 after the cancellation of the moose hunt (DelGiudice 2014). Liver tissue (n = 31) samples from sick moose were collected from 2009–2012 throughout northern MN and frozen after collection (Fig. 1). Moose health was determined by MNDNR personnel based on a range of observations such as non-normal behavior associated with neurological impairment, emaciation, and inability to stand upright. Sick moose were either found dead or were euthanized. Sex of moose was determined by direct observation except for certain unidentified/unmarked samples: sample sizes were 148 males, 108 females, and 29 unknown. FTA cards were stored at room temperature and frozen liver tissue was stored at -20 °C until analysis.

Yellowstone National Park.— Fecal pellet samples (n = 489) were collected primarily along drainages in YNP (Fig. 2) during winter months with snow present on

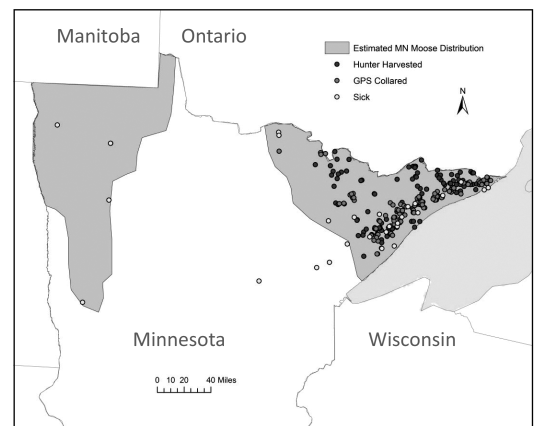

Fig. 1. Location of hunter-harvested (n = 117), GPS-collared (n = 132), and sick (n = 36) moose used in genetic studies in Minnesota, USA.

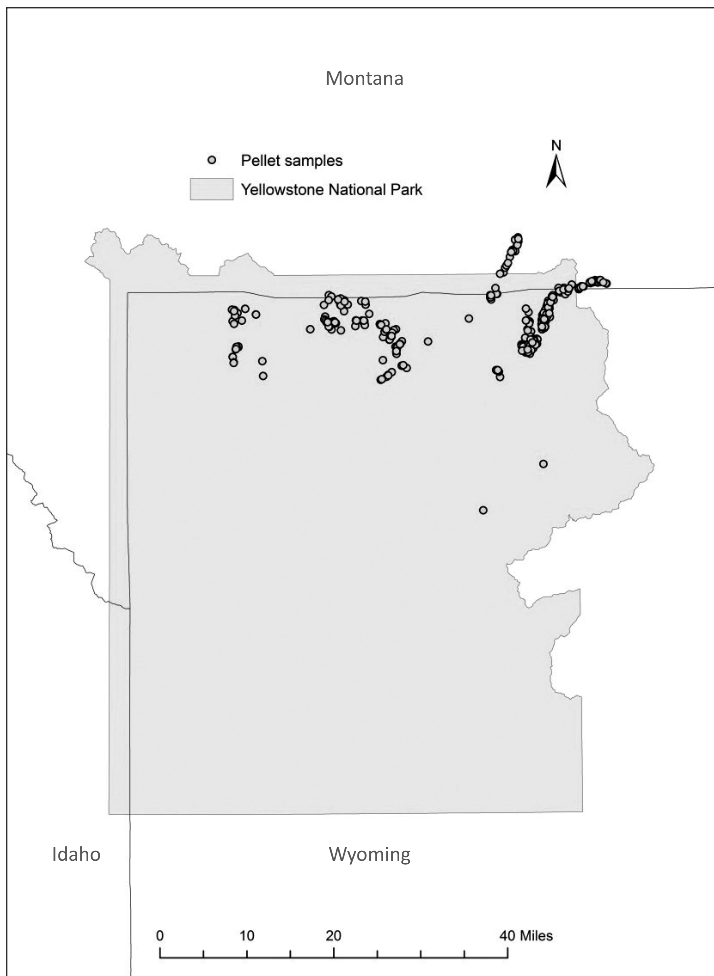

Fig. 2. Location of non-invasively collected moose fecal pellet samples (n = 489) in the northern range of Yellowstone National Park, Wyoming and Montana, USA.

the ground. A minimum of 5 fecal pellets from each deposition pile of each sampled moose was collected and stored in Whirl-Pak® or Ziploc freezer bags. Tissue samples (n = 2) collected opportunistically from dead animals were stored and frozen in Whirl-Pak® bags. Fecal pellets and tissue collected in YNP were kept frozen and sent to the University of Minnesota-Duluth for analysis. Date and time of sample collection, location, and estimated age of the sample was provided for most samples. Sex was determined directly, by collecting fecal pellet samples from an observed animal or inferred from physiological and behavioral clues, such as size of snow bed or presence of a calf. The age of fecal pellet samples at collection was estimated based on direct observation of the moose for certain samples. For fecal pellets found on snow without observing the moose, evidence from the fecal pellets, tracks, snowfall dates, or snow

cover was used to estimate the age of the sample. All fecal pellets were found on snow that had fallen in the current winter, and it is likely that most samples were much less than 4 months old. The maximum age of fecal pellets could not be estimated, but we know that all pellets were deposited in the year collected, and were on or in continuous snow cover until collection. Tissue and fecal pellets were stored at -20 °C until analysis.

Extraction

Blood.—Whole genomic DNA was extracted from one drop of dried blood for each sample (n = 251) using the Fermentas/Thermo Scientific GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Fisher Scientific Inc., Pittsburgh, Pennsylvania) according to the manufacturer's protocol. Each blood drop was taken from individual FTA cards using a 4 mm hand punch. Blood drops were categorized as small, medium, or large with diameters averaging 7.4 mm, 9.8 mm, and 11.8 mm, respectively. Extractions followed kit protocol, with one modification to improve final DNA yield, particularly for small blood drops. Small blood drops were eluted twice with 100 µl of Elution Buffer and then pipetted back into the spin column after being centrifuged, producing a final volume of 100 µl. Medium and large blood drops were also eluted twice, but with new Elution Buffer for the second elution step, resulting in a final volume of 200 µl.

Liver Tissue.—DNA was extracted from 0.02 g of frozen liver tissue (n = 33) using the Thermo Scientific GeneJET Genomic DNA Purification kit (Thermo Fisher Scientific Inc., Pittsburgh, Pensylvania) and the manufacturer's protocol with 4 modifications to improve final DNA yield and purity: 1) overnight incubation with Digestion Solution and Proteinase K instead of the manufacturer's recommended 3–4 h, 2) a minute added to each of the highest speed centrifugation times, 3) a minute added to the elution buffer incubation time, and 4) a second elution step, resulting in a final elution volume of 400 µl.

Fecal pellets.— Three DNA extraction kits were tested during a pilot study using 30 fecal pellet samples: 1) QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, California), 2) Thermo Scientific GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific Inc., Pittsburgh, Pennsylvania), and 3) PowerFecal DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, California). Qiagen's stool extraction kit produced sufficient DNA yield and PCR success with our laboratory techniques (described below), and was chosen for large scale DNA extractions with fecal pellet samples.

DNA was extracted from fecal pellets (n = 489) using two Qiagen DNA extraction kits because the manufacturer discontinued the first kit we used. The first method involved extracting DNA from one whole fecal pellet (n = 301) using the QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, California) and a modified protocol designed to isolate DNA from intestinal cells sloughed off onto the surface of fecal pellets (Estes-Zumpf et al. 2014). Inner fecal pellet material can contain PCR inhibitors that lead to increased variability in PCR amplification and genotyping success rates (Flagstad et al. 1999, Wehausen et al. 2004). To exclude this material from the extraction process, each fecal pellet was submerged in stool lysis buffer (Buffer ASL) from the QIAamp DNA Stool Mini Kit, and agitated to rinse cells off the outer surface instead of vortexing, which can break up the fecal pellet exposing the inner material.

The second method we used to extract DNA from fecal pellets (n = 188) was with the QIAamp Fast DNA Stool Mini Kit (Qiagen Inc., Valencia, California) and the manufacturer's protocol with several modifications to improve final DNA yield and purity, including: 1) centrifuging after step 2 to reduce bubbles caused by vortexing, and 2) reducing centrifuge rates during step 14 to 6,000 x g instead of the manufacturer's recommended 20,000 x g. This protocol required that a portion of a fecal pellet be used, rather than the whole pellet. We used a razor to slice thin layers from the outer fecal pellet material that contained the sloughed off intestinal mucosal cells. For both methods, fecal pellets were kept frozen until processing to prevent thaw and subsequent break up of the pellets. We did not use fecal pellet samples that were frozen together or samples with snow in the collection bag.

Sex Determination

Sex was determined for each sample using the SE47/SE48 primer pair (Brinkman and Hundertmark 2009). This primer pair produces a single band for females and a double band for males by PCR amplifying X- and Y-specific alleles of the amelogenin gene. This method has been used previously with moose and other cervid species (Brinkman and Hundertmark 2009). PCR products for sex identification were visualized using gel electrophoresis on 1.5% agarose gels stained with 10 mg/mL of ethidium bromide.

Genotyping

DNA extracted from blood (n = 248) and fecal pellets (n = 269) was genotyped using one sex-linked and 15 autosomal microsatellites previously used for moose (Table 1). All autosomal forward primers contain an M13 (-21) tail on the 5' end (Schuelke 2000), and PCR products were labeled by incorporating a universal fluorescently labeled M13 (-21) primer (FAM, PET, or VIC) during PCR. For DNA extracted from blood sources, all microsatellites were amplified separately with a total volume of 13 µl containing sterile water, GoTaq DNA Polymerase, and 1x GoTaq buffer (Promega Corporation, Madison, Wisconsin), 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.08 µM forward primer, 0.8 µM reverse primer, 0.8 µM labeled primer, 1% bovine serum albumin (BSA), and 1 µl/reaction DNA. BSA was added to all PCR to bind potential inhibitors and improve amplification specificity (Kreader 1996). The addition of BSA to PCR was initially implemented to increase amplification success using DNA extracted from fecal pellets. It was added to PCR using DNA extracted from blood and tissue for consistency, and to potentially increase PCR amplification success for DNA from each source type.

DNA extracted from fecal pellet sources was amplified similarly using the same set of microsatellite loci; however, an additional step was taken for PCR due to low amplification success rates in a pilot study. For DNA extracted from fecal pellets, microsatellite loci were amplified using either single step or pre-amplification PCR methods (Table 1). Pre-amplification is a two-step PCR method designed to increase the amount of DNA template for amplification and reduce genotyping error (Piggott et al. 2004). Because the success of the pre-amplification method has been questioned (Hedmark and Ellegren 2006, De Barba and Waits 2010), we conducted a pilot study to test this method using DNA extracted from fecal pellets amplified with our microsatellites. We used pre-amplification methods described in Piggott et al. (2004) with PCR mixtures modified for reduced total volume (Tjepkes 2015). The pre-amplification method was used only on loci for which it increased PCR

Table 1. Characteristics of one sex-linked and 15 autosomal microsatellites used in genetic analysis. Autosomal microsatellites were PCR amplified with M13 fluorescently labeled primers, then combined into non-overlapping panels for genotyping. TA is the optimal annealing temperature. The designated PCR method is for DNA extracted from moose fecal pellets; DNA extracted from blood samples was all PCR amplified using the single step method.

Locus	M13	T_A (°C)	Size Range (bp)	PCR Method	Reference
SE47/SE48	-	53	224–260	Single step	[1]
RT30	VIC	54	212–232	Single step	[2]
RT5	FAM	54	168–180	Single step	[2]
RT1	PET	47	247–255	Pre-amp	[2]
RT9	PET	54	138–154	Single step	[2]
BL42	FAM	49	263–285	Pre-amp	[3]
BM848	FAM	54	358–382	Single step	[3]
BM888	VIC	50	191–209	Single step	[3]
BM1225	FAM	50	237–267	Single step	[4]
BM2830	PET	50	127–139	Single step	[4]
CRFA	VIC	53	264–274	Pre-amp	[5]
KCSN	PET	50	206–218	Pre-amp	[5]
IGF-1	FAM	54	123–127	Single step	[5]
Cervid14	FAM	54	227–249	Single step	[6]
NVHRT03	PET	54	124–138	Pre-amp	[7]
NVHRT21	VIC	50	174–190	Pre-amp	[7]

References: [1] Brinkman and Hundertmark 2009; [2] Wilson et al. 1997; [3] Hundertmark 2009; [4] Broders et al. 1999; [5] Cronin et al. 2001; [6] Wilson et al. 2003; [7] Roed and Midthjell 1998.

amplification success and subsequent genotyping success.

Analyses

PCR products were analyzed at the University of Minnesota Biomedical Genomics Center using an ABI 3730xl capillary genetic analyzer. Genotypes were assigned using GeneMarker (v.2.6.0, Softgenetics LLC, State College, Pennsylvania) to score alleles for each locus. Failed or ambiguous allele scores were re-amplified and genotyped again to reduce scoring errors and missing data.

DNA quantification.— DNA concentration for each sample was quantified using a NanoDrop ND-1000 spectrophotometer. Using DNA concentration and total extraction volume, total DNA yield from each extraction was calculated for comparison. The two protocols for DNA extraction from fecal pellets were also compared to determine which method resulted in higher DNA yield. DNA yield was compared between blood, tissue, and fecal pellet samples using ANOVA with Bonferroni post hoc comparisons.

Sex determination.— Sex determination success rates were compared between DNA extracted from blood, tissue, and fecal pellet samples using Fisher's exact test. The accuracy of sex determination using genetic methods was determined by comparing genetically determined sex with recorded sex from direct field observations using DNA from blood, tissue, and fecal pellet

samples. Additionally, genetically determined sex using DNA from fecal pellet samples was compared to recorded sex from indirect field observations.

PCR and genotyping success rates.— Success of PCR amplification of autosomal microsatellites and genotyping success rates were calculated. The autosomal PCR success rate was the proportion of successful PCR amplification attempts used to estimate the amount of effort required in the laboratory for a sample. PCR attempts were classified as successful if they produced viable product that could be used to genotype individuals. Autosomal PCR success rates using DNA extracted from blood and fecal pellets were compared using a χ^2 test for independence.

The genotyping success rate allows for a comparison of overall success using DNA from several DNA source types; however, it does not reflect the amount of effort and resources required to obtain that success. For example, if all 15 loci were successfully PCR amplified on the first attempt, the overall genotyping success rate would be 100% (15 of 15 PCR amplifications were successful) and the autosomal success rate would also be 100%. If fewer than 15 loci were successfully PCR amplified in the first attempt, but then all 15 loci were successfully PRC amplified in a second attempt, the overall genotyping success rate would also be 100%, but the second sample would have required twice as many PCR amplifications. The autosomal PCR success rate would be between 50% (15/30) and 97% (29/30) depending on how many loci were successfully amplified in the second sample.

Genotyping success rate is the proportion of microsatellites for which we were able to obtain genotypes for a given sample, given that a PCR amplification attempt was successful. PCR and allele scoring was attempted a second time for DNA samples if the first attempt failed. Individual genotyping success rates were then averaged for each DNA source type. Genotyping success rates using DNA extracted from blood and fecal pellet samples were compared using two-sample t-tests.

Genotyping error.— A concern when working with lower quality and quantity DNA in fecal samples is the increased risk of genotyping errors such as allelic dropout or false alleles. To estimate genotyping error, PCR and allele scoring was repeated for at least 24 randomly chosen individuals at each microsatellite locus using previously extracted DNA from fecal pellets. However, because many of the original repetitions using DNA extracted from fecal pellet samples did not produce a usable amplicon, 95 additional randomly chosen DNA samples from fecal pellets were repeated at 6 microsatellite loci. Genotyping error was calculated using these duplicated allele scores. We were interested only in estimating genotyping error and did not investigate observed genotyping errors further (i.e., amplifying conflicting loci a third time) as recommended by Taberlet et al. (1996).

Predictors of genotyping success.— To identify ways to improve efficiency and success rates, DNA yield and sex determination success were tested as predictors of genotyping success. Two-sample t-tests were used to determine whether higher DNA yield or PCR amplification success of SE47/SE48 resulted in greater genotyping success. If DNA yield or PCR amplification success leads to greater genotyping success, poor quality samples could be identified and censored, reducing the amount of time and effort spent on those samples.

Effect of fecal pellet age.— Since numerous environmental factors degrade DNA, the time between deposition and collection was investigated to determine an effect on DNA quality and quantity. Time

since deposition was estimated for fecal pellet samples based on observations made in the field during collection, including visual fecal pellet characteristics, moose tracks, and snowfall. Using these data, DNA was separated into 3 fecal pellet age classes: < 24 h (n = 84), 24–48 h (n = 65), and > 48 h (n = 109) since deposition.

DNA yield for each fecal pellet age class was compared using ANOVA to determine if time since deposition affected the amount of DNA obtained from fecal pellets. Sex-linked and autosomal PCR amplification success between age classes was compared using Fisher's exact and χ^2 tests, respectively. Finally, two-sample t-tests were used to determine if age since deposition affected overall genotyping success. Because of potential uncertainty in differentiating fecal pellets deposited < 24 h and 24–48 h since deposition, PCR amplification and genotyping success rates between DNA from fecal pellets collected < 48 and > 48 h after deposition were also compared.

RESULTS

DNA Yield

We extracted DNA from 251 blood samples, 33 liver tissue samples, and 489 fecal pellet samples. Yield of extracted DNA for blood, tissue, and fecal pellet samples was sufficient for genotyping. Blood and liver tissue samples produced the highest average DNA yield, and fecal pellets produced the lowest average DNA yield (ANOVA, $F_{3,769}$ = 113, P < 0.001). DNA extractions using sliced fecal pellets had slightly higher average DNA yield than DNA extractions using whole fecal pellets (Fig. 3).

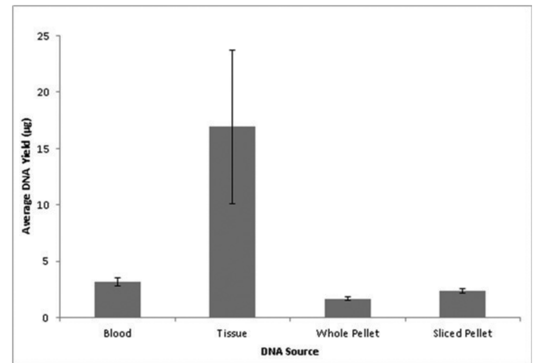

Fig. 3. Average DNA yield extracted from multiple DNA source types. DNA sources were from moose in Minnesota (blood, n = 251; tissue, n = 31) and Yellowstone National Park (tissue, n = 2; fecal pellet, n = 489). Fecal pellets were extracted using either the whole fecal pellet (n = 188) or using outer slices of fecal pellet (n = 301).

Sex Determination

We determined sex with primer pair SE47/SE48 using DNA extracted from all 3 sample sources. Sex determination was successful for 100% of blood samples (n = 251), 91% of tissue samples (n = 33), and 83% of fecal pellet samples (n = 460). Success was significantly greater using DNA extracted from blood than liver tissue or fecal pellets (Fisher's exact test, P < 0.01). The success rates using extracted DNA from tissue and fecal pellets were not significantly different (Fisher's exact test, P > 0.05). We calculated the accuracy of sex determination using these methods by comparing results determined genetically to field records from moose directly observed depositing fecal pellets. Genetic and field sex determination was consistent for 219 of 225 blood samples (0.97), 24 of 25 tissue samples (0.96), and 64 of 67 fecal pellet samples (0.96) when analyzed separately. When sex was determined from indirect evidence in YNP, the genetically-determined and field-determined sex were consistent for 74 of 100 fecal pellet samples (0.74). The 26 fecal pellet samples that resulted in inconsistent sex determination results were from both males (n = 11) and females (n = 15).

PCR and Genotyping Success Rates

We genotyped 517 total samples using DNA from blood (n = 248) and fecal pellets (n = 269) at 15 autosomal microsatellite markers. Average autosomal PCR success rate was higher for blood than fecal pellets (0.81 vs. 0.63, respectively, $\chi^2_1 = 57$, $P < 0.001$). Similarly, average genotyping success rate, the percent of microsatellites that we were able to use if PCR amplification was successful, was higher for DNA extracted from blood than fecal pellets (0.82 vs. 0.76, respectively, $t_{506} = 6.04$, $P < 0.001$).

Genotyping Error

The average genotyping error rate calculated by repeated PCR amplification and allele scoring using DNA extracted from fecal pellet samples was 0.10 for re-analyzed microsatellite loci. PCR did not produce usable amplicons for 3 microsatellite loci (IGF-1, RT5, and CRFA) using DNA extracted from fecal pellets.

Predictors of Genotyping Success

DNA yield and PCR amplification success of the sex-linked primer pair SE47/SE48 were tested in order to determine whether they could be used as predictors of downstream success, particularly genotyping success. DNA yield was not correlated with genotyping success using DNA extracted from blood or fecal pellets (Fig. 4). DNA extracted from fecal pellets that successfully PCR amplified at the sex-linked loci had higher genotyping success at autosomal loci compared to those that failed to amplify at this locus (0.78 [n = 236] vs. 0.59 [n = 31], respectively, $t_{36} = -4.61$, $P < 0.001$). This comparison could not be made for DNA from blood or tissue samples because the PCR success of > 96% produced too few failed samples to test.

Effect of Fecal Pellet Age

Time since deposition was estimated for 447 samples as either < 24 h (n = 178), 24–48 h (n = 114), or > 48 h (n = 155). Average DNA yield was not significantly different between different age classes (ANOVA, $F_{2,255} = 1.73$, $P = 0.18$; Table 5). DNA extracted from fecal pellets collected < 24 h and 24–48 h after deposition had the highest sex determination success rates and were not different from each other (0.85 and 0.92, respectively, Fisher's exact test, $P = 0.10$), whereas DNA from fecal pellets collected > 48 h after deposition had lower success rate than fecal pellets collected < 48 h after deposition (0.74, Fisher's exact test, $P = 0.0003$; Table 2).

In addition, fecal pellet samples were compared to determine whether fecal

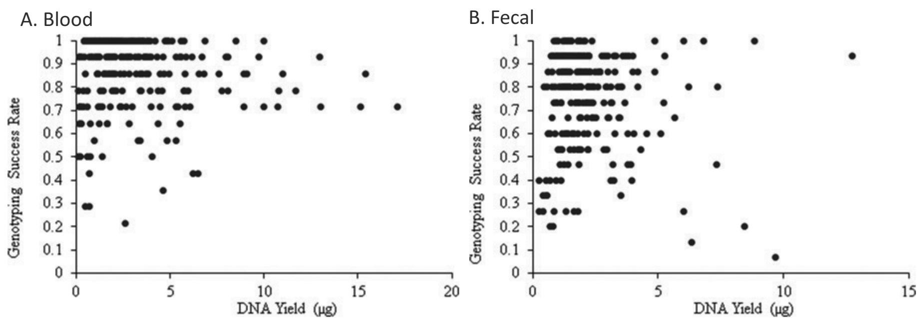

Fig. 4. Genotyping success rate for (A) blood and (B) fecal pellet samples of varying DNA yield (µg) from moose in Minnesota and Yellow National Park, USA.

Table 2. Effect of moose fecal pellet age on DNA yield, sex-linked microsatellite PCR amplification success (sex-linked PCR success), and autosomal microsatellite PCR amplification success (autosomal PCR success) using 15 microsatellite markers using fecal pellets collected >24 (n = 84), 24–48 (n = 65), and >48 h (n = 109) after deposition. Fecal pellets in > 48 h age class ranged from 48 h to 4 months since deposition.

Time Since Deposition (h)	Average DNA Yield (µg)	Sex-linked PCR success	Autosomal PCR success
<24	2.54	0.85	0.67
24–48	2.45	0.92	0.69
>48	2.08	0.74	0.56

pellet age at collection influenced PCR amplification or genotyping success rates. Similar to sex determination success rates, fecal pellets collected < 24 and 24–48 h after deposition had the highest PCR success rates and were not different from each other (0.67 and 0.69, respectively), whereas the success rate for fecal pellets collected > 48 h after deposition was significantly lower (0.56) (Table 2). Autosomal PCR success rate for fecal pellets in the < 24 and 24–48 h age classes was not different (χ^2_2 = 1.31, P = 0.25), but was different from fecal pellets collected > 48 h after deposition (χ^2_2 = 46, P < 0.001). Genotyping success rate was the highest for DNA from fecal pellets collected < 24 and 24–48 h after deposition (0.82 and 0.83, respectively) (Fig. 5). Fecal pellets in the < 24 and 24–48 h age classes were not different (t_{136} = -0.10, P = 0.92), but were different from fecal pellets collected > 48 h after deposition (t_{191} = -5.29, P < 0.001). Fecal pellets collected > 48 h after deposition had the lowest average genotyping success rate (0.69), which was significantly different from the two other age classes. Similar results for PCR amplification and genotyping success rates were obtained when DNA extracted from fecal pellets collected < 48 h after deposition was compared to DNA extracted from fecal pellets collected > 48 h after deposition.

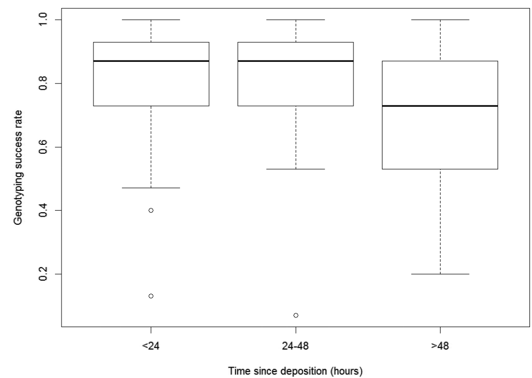

Fig. 5. Effect of fecal pellet age at collection (<24 h, n = 84; 24–48 h, n = 65; >48 h, n = 109) on individual genotyping success rate from moose in Yellow National Park, USA. Age of fecal pellets in the > 48 h age class was unknown, but pellets were collected from on top of snow in the current winter. The age could have been up to 4 months, but was likely less.

DISCUSSION

DNA was successfully extracted from blood, liver tissue, sliced fecal pellets, and whole fecal pellets. Tissue samples produced the greatest amount of DNA per extraction and fecal pellets produced the smallest average DNA yield. Because fecal pellet samples had less available sample and required a greater amount of PCR, these DNA samples were more likely to be exhausted. There is also variability in DNA quantity and quality in fecal pellet samples, even among fecal pellets from the same individual and the

same fecal pellet group (Taberlet et al. 1996). Therefore, it would be best to maximize fecal pellet collection in the field.

DNA yield was not a reliable predictor of genotyping success as expected. DNA yield estimates the amount of DNA in a sample, but it does not provide information on DNA quality, presence of PCR inhibitors, or presence of foreign DNA. Samples with high DNA yield may have DNA degradation, and indeed, we found evidence supporting this because pellets > 48 h old had similar DNA yield as pellets < 48 h old, but lower genotyping success.

DNA extracted from blood had the highest average PCR amplification success for both sex-linked and autosomal microsatellites. This suggests higher quality DNA was extracted from blood than liver tissue, as liver tissue samples had higher average DNA yield. DNA extracted from fecal pellets had the lowest sex determination success, suggesting lower DNA quality and/or increased presence of PCR inhibitors.

Genotyping and PCR Amplification Success

Greater effort was required for genotyping success with fecal pellet samples than blood samples. This was because autosomal PCR amplification success was lower using DNA from fecal pellets compared to blood, a consequence of the lower quality DNA. For regions or populations where non-invasive genetic sampling is the only feasible option, fecal pellet samples are an alternative source of DNA, but only if collected at the optimal time of year. If higher quality samples such as blood or liver tissue are available, these samples are easier to process in the laboratory; however, the effort required to obtain samples is an important consideration. Obtaining samples of blood, muscle, or other tissues can require more effort than obtaining fecal pellet samples, unless collection is in conjunction with other projects.

Reported PCR amplification success using DNA from feces has been variable (Wehausen 2004, Broquet et al. 2007), and is likely due to factors beyond the DNA source. For example, methods of sample collection, storage, and extraction affect DNA quality and downstream success (Roon et al. 2003, Wehausen 2004, Waits and Paetkau 2005). The effectiveness of these methods has been tested, but without clear consensus (Luikart et al. 2006, Beja-Pereira et al. 2009). The lack of consensus is likely influenced by inherent variation among species and environmental variables (Waits and Paetkau 2005); therefore, it is essential to conduct a pilot study before beginning large-scale extractions (Taberlet et al. 1999).

Improvements and Recommendations

We made many attempts to improve PCR success using DNA from fecal pellets, including testing multiple DNA extraction kits and protocols, adding BSA to remove PCR inhibitors, using the pre-amplification method for PCR, and testing a variety of PCR conditions (optimizing PCR master mix ingredients and PCR temperature profiles) for each microsatellite locus.

The average amount of DNA extracted from sliced fecal pellets was greater than that from whole fecal pellets using the surface washing method. A similar comparison using whole fecal pellets and sliced outer fecal pellet material from bighorn sheep found no difference in extracted DNA yield (Wehausen 2004). However, the differences could be due to the substantially larger sample size in our study, and differences in species, environmental conditions, and extraction protocols. We found slicing fecal pellets to be a more time consuming process with increased probability of contamination through exposure to multiple laboratory

surfaces (e.g., cutting surface, razor, and forceps), even though protocols were in place to minimize such. Additionally, accidental inclusion of inner fecal pellet may increase PCR amplification failure and variation among samples (Wehausen 2004). Therefore, although the sliced fecal pellet method resulted in higher average DNA yield, we prefer the whole fecal pellet surface washing method.

Although not quantified, we felt that PCR success using DNA extracted from fecal pellets improved with the inclusion of BSA, although it was not as beneficial when attempting to amplify DNA extracted from blood or tissue. This is because BSA does not have a noticeable effect on PCR amplification success using DNA with low levels of PCR inhibitors (Kreader 1996). Thus, the difference in the effect of including BSA provides evidence for increased levels of PCR inhibitors in DNA extracted from fecal pellets compared to either blood or tissue.

The pre-amplification method for PCR was beneficial for 6 microsatellites. However, this method required twice the number of PCRs, and thus more resources (time and reagents). In some cases the pre-amplification method caused non-specific amplification or amplification patterns that created difficulty for allele scoring, thus, we recommend caution with this method.

Time since deposition affected genotyping success of fecal pellets. DNA is degraded by several environmental conditions (e.g., high temperature, precipitation, UV radiation, and microorganisms), thus the longer it is subjected to adverse conditions, the less likely a fecal pellet will contain usable DNA. Selecting fresh fecal pellets (< 48 h after deposition) will likely result in better quality and quantity of DNA, and improved PCR amplification and genotyping success rates. Success rate was lower when pellets of unknown age were collected, and fecal pellets collected after March had low genotyping success (Rea et al. 2016). However, samples collected in dry or protected areas may have a substantially larger window for collection (Brinkman et al. 2010b). If precise estimates of fecal pellet age were available, it could be possible to determine the age at which fecal pellets should not be collected/used for DNA analysis.

Variability does exist in DNA quality and quantity from fresh fecal pellets. Poor quality samples can be removed from analysis based on PCR amplification success using primer pair SE47/SE48, and other microsatellites could be used for this purpose. However, using a sex-linked microsatellite enables identification of sex, which is often missing from non-invasively collected samples. An additional benefit of using a sex -linked microsatellite is to check for errors in data records, and to evaluate accuracy of identifying the sex of animals not directly observed. In fact, we identified such errors in data from YNP. If the sex-linked microsatellite fails, then it is likely that DNA extraction will not be successful, and the sample could be censored from the data set without investing additional resources of time or materials.

Future work could include testing the effectiveness of sample collection and storage methods. Blood and tissue samples were collected and stored before our study began, and reported methods from other non-invasive genetic studies for collection and storage were used (Carr et al. 2010, Ebert et al. 2012). However, collection and storage methods might have influenced our results, as for example, liver tissue samples from sick moose that were found dead or euthanized. Death and subsequent post-mortem decomposition result in DNA degradation, particularly in liver tissue (Alaeddini et al. 2010). Samples

were stored in -20 °C freezers, but this has not proven entirely effective at eliminating DNA degradation (Dawson et al. 1998), especially for extended periods. Storage beyond 6 months reduces both DNA yield and PCR amplification success for multiple DNA source types (Roon et al. 2003). Extracted DNA quantity and quality from tissues we used may have been improved using samples collected more recently, if DNA had been extracted immediately following collection, or if samples had been stored at colder temperatures. However, it is also significant that despite the potential issues that could have confounded successful DNA extraction, we were able to extract DNA from most of these samples with reasonably high success.

CONCLUSION

Population genetic studies of moose have traditionally used DNA extracted from tissue, blood, or a combination of these sources. The source, methods, and available resources (time and money) which influence the quantity and quality of data typically vary in genetic research with moose. Similar to that found with other ungulate species (Luikart et al. 2006, Brinkman et al. 2010a, Ebert et al. 2012), we have shown that moose fecal pellets are viable sources of DNA. Importantly, fecal pellets can be collected non-invasively which increases the sampling potential in genetic studies of moose. We provide guidelines concerning the optimal collection, storage, and laboratory procedures for using fecal pellets in population genetic studies with moose.

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