GENOTYPING ERROR RATES ASSOCIATED WITH ALTERNATIVE SOURCES OF DNA FOR THE NORTH AMERICAN RIVER OTTER

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ABSTRACT: The ability to accurately genotype individuals may depend upon interactions among numerous variables including the source of DNA, the sample preservation method, the reliability of the molecular marker being used, and the species under study. The use of feces as an alternative source of DNA is becoming increasingly popular; however DNA quality and quantity often are compromised when using fecal material as an alternative source of DNA. The use of poor quality DNA can introduce numerous problems in downstream applications including amplification failure and genotyping errors. The preservation method used for collecting feces in the field is known to influence DNA quality; however, decisions concerning storage mediums are often made independently of information concerning the likelihood of genotyping error. In this study we use North American river otter (*Lontra canadensis*) scats and anal jelly secretions as alternative sources of DNA for amplification of microsatellite loci and examine the influence of 5 preservation methods on rates of amplification and genotyping error in this species. The occurrence of 2 types of microsatellite genotyping errors (allelic dropout and false alleles) was assessed relative to both DNA source and preservation method. The proportion of genotyping errors also was determined for each microsatellite locus to investigate locus-specific error rates. DNA source, preservation treatment, and microsatellite locus all influenced our ability to obtain accurate genotypes, demonstrating the need for conducting preliminary studies before large-scale noninvasive genetic sampling efforts are attempted. Baseline work must be performed prior to the collection of fecal material for any species to determine both the appropriate collection protocols and loci for minimization of genotyping errors.

KEY WORDS: feces, genotyping errors, *Lontra canadensis*, noninvasive genetic sampling, river otter.

Methodologies that enable biologists to obtain accurate estimates of the density and other demographic characteristics of a population should be important components of wildlife conservation programs. Studies based on direct observation or mark and recapture studies frequently have been used to reliably estimate the size of wildlife populations (Flowerdew et al. 2004, Lopez et al. 2004). However, these traditional approaches for estimating the size of wildlife populations may be of limited use or inappropriate for rare or solitary species. For some species, surveys of field sign (e.g., tracks and feces), rather than more statistically rigorous methods (e.g., mark-recapture), have been commonly used to monitor populations (Mason and Macdonald 1987, Kruuk 1992, Ruiz-Olmo et al. 2001). Unfortunately,

demographic trend data based on such indices of abundance often provide biased or difficult to interpret results because of incomplete or nonrandom sampling of populations, especially when the indices are not accompanied with techniques that facilitate determination of the identity and gender of the individual leaving the field sign. Consequently, these indices may incorrectly portray the status and characteristics of populations, thus, limiting the ability of biologists to make informed decisions for implementing conservation strategies (Kruuk and Conroy 1987, Ruiz-Olmo et al. 2001).

In attempts to increase the accuracy of population estimates, many researchers have begun combining noninvasive population counts with molecular genetic techniques. This approach not only enables the unique identification of individuals within populations, but also the elucidation of population attributes such as sex ratio, parentage, social structure and home range or territory size (Kohn et al. 1995, Kohn and Wayne 1997, Oka and Takenaka 2001). Historically, tissue samples, collected directly from a carcass or captured animal, were needed for application in molecular genetic studies. However, recent advances in molecular biology permit isolation of an individual's DNA from a variety of materials including feathers, hair, feces, urine, sloughed skin, and eggshells without capturing or disturbing the animal (Taberlet et al. 1999, Murphy et al. 2002, Segelbacher 2002, Roon et al. 2003).

Fecal samples in particular, which can be easily located and collected for most wildlife species, have become increasingly popular as sources of both mitochondrial and nuclear DNA. In recent years, fecal DNA samples have been successfully utilized for various applications, including investigations to identify species (carnivores: Foran et al. 1997, seals: Reed et al. 1997, canids: Paxinos et al. 1997, mustelids: Hansen and Jacobsen 1999 and Riddle et al. 2003), identity of individuals (orangutan: Immel et al. 1999, mountain lions: Ernest et al. 2000, wolves: Lucchini et al. 2002, Pyrenean brown bears: Taberlet et al. 1997), identify sex (Eurasian otter: Dallas et al. 2000, red deer: Huber et al. 2002), determine population size (seals: Reed et al. 1997, coyotes: Kohn et al. 1999, mountain lions: Ernest et al. 2000), determine social structure (Asian elephants: Fernando and Lande 2000), investigate phylogeography (Asian elephants: Fernando et al. 2000), and assign parentage (bonobos: Gerloff et al. 1999, gibbons: Oka and Takenaka 2001).

Despite the recent popularity and success in applying noninvasive DNA-based techniques, there remain disadvantages associated with isolation of nuclear DNA from fecal material. Some limitations include the presence of polymerase chain reaction (PCR) inhibitors (co-purified excremental substances like bile salts, microorganisms, digestive enzymes, plant polysaccharides, mucus, and bilirubin; Deuter et al. 1995, Kohn and Wayne 1997) and high levels of bacterial- or enzymatic-mediated DNA degradation (Frantzen et al. 1998, Murphy et al. 2000). Each of these factors may increase the probability of failed PCR amplifications and/or genotyping errors at microsatellite loci (Kohn and Wayne 1997, Taberlet et al. 1999, Creel et al. 2003). These complications can escalate the cost and limit the application of genetic methodologies for extracting and analyzing fecal DNA. In fact, to obtain reliable genotypic data for individuals a single sample often must undergo multiple DNA extractions and associated PCR amplifications for each locus because of frequent genotyping errors associated with the analyses of nuclear DNA derived from fecal material (Taberlet et al. 1996, Kohn and Wayne 1997). Unfortunately, redundant application of extraction and amplification protocols can become prohibitively expensive for widespread application of these techniques in the field.

The most common genotyping errors encountered when using fecal DNA can be attributed to the amplification of false alleles and allelic dropout. False alleles (i.e., the detection of alleles that do not occur in the individual from which the DNA sample is derived) may be attributable to contaminating DNA (from other species or other otters) or slippage artifacts generated during the initial cycles of PCR (Taberlet et al. 1996). Allelic

dropout occurs when one of the two alleles present at a heterozygous locus fails to amplify in an individuals' DNA sample, resulting in a false homozygote genotype for the individual at a particular locus (Talberlet et al. 1999). The occurrence of allelic dropout can result from the use of degraded DNA, which may inhibit the amplification of long DNA fragments (>300bp; Frantzen et al. 1998) or from sampling stochasticity when pipetting template DNA (Taberlet et al. 1999).

Genotyping errors associated with analysis of microsatellites often are common in studies using fecal material as a source of DNA and, consequently, are of particular concern among researchers using this approach to generate data for genetic studies. For example, genotyping errors can severely bias population estimates through their influence on the probability of correctly establishing the identity of an individual. In fact, genotyping errors have been shown to inflate population estimates up to 200% (Waits and Leberg 2000) and also can deflate population estimates if multiple individuals are erroneously shown to have identical genotypes or if too few microsatellite loci are used to identify individuals (Waits et al. 2001). Inflated genotyping error rates associated with analyses of fecal material can negatively influence the utility of these methods for any application requiring individual identification or accurate estimates of allele frequencies within populations (e.g., parentage, genetic structure, population assignment, etc.).

Ultimately, the reliability and feasibility of using fecal samples as sources of DNA for microsatellite genotyping, regardless of the application, depends on a combination of factors, which can influence amplification success and levels of genotyping error. These interacting factors include the age of the fecal sample, the portion of the fecal mass sampled, preservation methods used for fecal storage, DNA extraction protocols and the microsatellite loci selected for genotyping (Lathuilliere et al. 2001, Wehausen et al. 2004). Although much attention has been focused on optimization of fecal preservation methods for successful amplification of DNA (Frantzen et al. 1998, Murphy et al. 2000), only recently have researchers begun to consider the influence of storage media on the likelihood of microsatellite genotyping errors (Murphy et al. 2002, Roon et al. 2003). Also, little consideration has been given to the possibility that variance in genotyping error exists among microsatellite loci and that the magnitude of this variance also may be influenced by preservation methods.

A clear need exists for further exploration into the potential sources of variation in amplification rates and genotyping error associated with fecal DNA samples. The high degree of variability across species in the performance of preservation methods and in levels of genotyping error suggest that genetic investigations involving fecal DNA may need to be optimized on a species- and locus-specific basis (Lathuilliere et al. 2001). Our goals in this research were to explore the feasibility of using freshly collected North American river otter (*Lontra canadensis*) fecal material and anal jelly secretions (a mucous-like substance often associated with river otter fecal deposition) as sources of DNA for genetic studies and, in doing so, determine a collection and analysis procedure for obtaining reliable genotypes. Specifically, we were interested in determining if the source of DNA (hereafter referred to as "DNA source") and preservation methods influenced overall amplification potential and microsatellite genotyping error rates. Additionally, we examined variation in genotyping error rates for individual microsatellite loci both within and among preservation methods and DNA sources.

The river otter was chosen as a model species for this research for several reasons, including: 1) recent work by Dallas et al (2003) and Jansman et al (2001) suggests that the use of fecal material as a source of DNA for microsatellite genotyping may be feasible for the Eurasian otter (*Lutra lutra*); 2) a suite of polymorphic DNA-based markers has now been developed specifically for the North American river otter (Beheler et al. 2004); 3) adequate samples (both tissue controls and fecal samples) for North American river otters were

available from captive individuals; and 4) there is a great potential to apply fecal DNA analyses for the conservation of this important and widespread North American species.

METHODS

Sample Collection

Fecal samples were collected from three river otters, trapped in the Adirondack Mountains of New York during 2000-2001 and subsequently held in captivity at Frostburg State University before release into Pennsylvania as part of the Pennsylvania River Otter Reintroduction Project (PRORP; Serfass et al. 1993). Otters were fed a diet of trout (*Oncorhynchus* spp.) and smelt (*Sillago* spp.), and fecal masses were collected from each otter within 4 hrs of deposition.

During the study, 27 fecal masses were collected from the 3 otters (13 from the first otter, 9 from the second, and 5 from the third otter). Each fecal mass was divided into 3 equal sections (left end, middle section, right end). The left and right end sections of each individual fecal mass were grouped together and referred to as "tips" because of the ambiguity in standardizing the front and back of each fecal sample. Three 0.2 g subsamples were taken with a sterile razor blade from each of the 3 sections.

Subsamples from 17 of the fecal masses (from 2 otters) were used to investigate the utility of 3 preservation methods. The subsamples from each of the 3 sections were then placed into a 2 ml tube representing one of 3 preservation methods: 1) Silica desiccant and freezing at -80 ^{o}C (n = 51), 2) DMSO/EDTA/Tris/Salt solution (DET) and freezing at -80 ^{o}C (n = 50), or 3) freeze only: freezing at -80 ^{o}C (n = 51). To simulate fecal collection in the field, subsamples collected in silica desiccant and DET were kept at room temperature for 48-72 hours in their respective preservation matrix before being placed in an ultra-cold freezer (-80 ^{o}C). Subsamples assigned to the freeze only trial were placed in the ultra-cold freezer immediately upon subsampling. During the study an additional 15 anal jelly secretions were collected from the captive otters. Three subsamples were taken from each anal jelly secretion and placed in one of the 3 preservation treatments described above.

The remaining 10 fecal masses were divided into the 3 sections and subsampled as described above and these subsamples were distributed into 2 further preservation treatments (5 fecal masses per treatment): 1) 100% ethanol at room temperature (n = 15 subsamples: 10 tips and 5 middles) or 2) 100% ethanol frozen at -80 ^{o}C (n = 15 subsamples: 10 tips and 5 middles). Subsamples placed in the ethanol followed by freezing preservation treatment were held in ethanol at room temperature for 48 hrs before freezing. No anal jelly secretion subsamples were placed in the ethanol at room temperature or the ethanol followed by freezing preservation treatments.

DNA samples from the blood of each of the otters were collected to serve as positive controls for genotyping of fecal and anal jelly secretion derived DNA. Otters were housed separately, thus there was no doubt regarding which individual produced a fecal mass or anal jelly secretion.

DNA Extraction

All DNA extractions were performed in a sterile laminar flow hood to prevent contamination. DNA was isolated from each fecal and anal jelly secretion subsample using a QIAmp stool kit (QIAGEN Inc., 28159 Avenue Stanford, Valencia, CA 91355). The manufacturer's protocols were followed explicitly with one modification: in the final step of the extraction procedure, DNA was resuspended in 50 µl of the Buffer AE provided in the kit, rather than in 200 µl as recommended by the manufacturer. We made this modification to increase the template concentration of the final elution, which was demonstrated through initial tests to increase PCR amplification success rates. To monitor for contamination,

negative controls (no fecal/anal jelly material added) were included each time an extraction was performed. These negative controls were included in the PCR amplification trials using the microsatellite locus LCf9 (see below) where the absence of PCR product verified that no contamination had occurred.

PCR Amplification Trials

To assess amplification success of DNA among preservation methods we used a primer set for the monomorphic North American river otter microsatellite locus LCf9 (F: GCCCTAAGACCCTCCTTCTC, R: TGCCATTGAAATCCAACTTGT; T_A of 55 ^oC; unpublished) to amplify a 309 bp product from the DNA extracted from each fecal and anal jelly secretion subsample. The LCf9 microsatellite locus was chosen for the amplification trials based on its reliability (easily amplifiable in DNA samples of low concentration) and its large allele size (which made it easy to visualize on an agarose gel). PCR amplifications were attempted twice using the DNA from each subsample and the outcomes were scored as successful or unsuccessful. We defined a PCR amplification as successful only if a product of the correct size (based on the size of the positive control from the captive otters) was detected in at least one of the amplifications.

DNA samples were prepared for PCR amplification in a sterile laminar flow hood and additional negative controls (no DNA added) were included in each PCR amplified sample set to monitor for contamination. Positive controls also were amplified for the LCf9 microsatellite locus each time a set of subsamples was processed. PCR amplifications were performed in 10 uL reactions using a PTC-225 Gradient Cycler (MJ Research) and followed the protocols outlined in Beheler et al (2004). PCR conditions were as follows: 20 ng of template DNA**,** 0.2 mM of each dNTP, 10X PCR buffer (Eppendorf), 0.2 uM of each primer, 1.5 mM $MgCl_2$ and1 unit of Hotmaster hot start Taq DNA polymerase (Eppendorf). The PCR profile for LCF9 was 95^o C for 2 min, then 35 cycles of 94^{o}C for 30 s, 55 ^{o}C for 30 s, 70^{o}C for 30 s for, then 70^{o}C for 10 min. PCR products were visualized on a 1% agarose gel containing ethidium bromide for size comparisons with positive controls and verification of amplification success.

Genotyping and Genotype Verification

Allelic dropout is impossible to detect in homozygotes, therefore, our analysis of genotyping error in DNA from fecal and anal jelly subsamples was restricted to microsatellite loci that were determined to be heterozygous in the captive otters from which the samples originated. Thus, genotypes were obtained for the 3 otters at nine microsatellite loci using template DNA isolated from their blood. Using these data, we identified 6 microsatellite loci for which at least one of the three otters was heterozygous. These six polymorphic microsatellite loci (RIO 02, RIO 04, RIO 05, RIO 06, RIO 07 and RIO 08) were developed specifically for North American river otters (Beheler et al. 2004). Subsamples selected for genotyping error trials at each locus were restricted to the otter or otters that demonstrated heterozygosity at that locus. Thus, fecal and anal jelly secretion subsamples from the 3 captive otters were not distributed uniformly across loci for the genotyping error trials.

To assess the influence of preservation method (silica desiccant, DET, freezing, ethanol at room temperature and ethanol followed by freezing), fecal type (fecal tip, fecal middle, or anal jelly secretion), and microsatellite locus (RIO 02, RIO 04, RIO 05, RIO 06, RIO 07 and RIO 08) on occurrence of genotyping errors, a DNA sample set was identified from those DNA extractions that successfully amplified a product in the amplification success trials (described above using LCf9). We excluded all samples from this subset that did not amplify products of the correct size during the initial amplification trials. From the subset of DNA samples exhibiting successful amplifications, 2 subsamples of DNA from both fecal material and anal jelly secretions were selected from each preservation treatment

(except ethanol - see below) and were genotyped at each of the 6 microsatellite loci selected for genotyping error trials. Each DNA subsample selected for analysis was genotyped repeatedly until 5 unambiguous genotypes (clean, easily identifiable peaks) were obtained at each locus (or until the DNA sample was exhausted).

For fecal samples preserved in ethanol, 4 subsamples of DNA were selected and genotyped at 4 microsatellite loci. Each DNA subsample selected for analysis was genotyped repeatedly until 5 unambiguous genotypes (clean, easily identifiable peaks) were obtained at each locus (or until the DNA sample was exhausted). Fecal subsamples used for the 2 ethanol preservation treatments represented only 2 of the 3 captive otters (fecal masses exclusively from only one otter were used for the ethanol at room temperature treatment and fecal masses exclusively from a second otter were used for the ethanol followed by freezing preservation treatment). Thus, genotyping error trials involving the ethanol preservation treatments could only be conducted at those loci for which the 2 otters were heterozygous (RIO 05, 06 and 07 for ethanol at room temperature and RIO 06 and 08 for the ethanol followed by freezing).

PCR amplifications for each microsatellite locus were performed in 10 uL reactions using a PTC-225 Gradient Cycler (MJ Research) and followed the protocols outlined in Beheler et al (2004). PCR conditions were as follows: 20 ng of template DNA**,** 0.2 mM of each dNTP, 10X PCR buffer (Eppendorf), 0.2 uM of each fluorescently labeled primer (except RIO08 where we used 0.4 uM), and either 1.5 mM $MgCl_2$ (RIO02, 04, 05, 06, 07; Eppendorf) or 3 mM $MgCl_2$ (RIO07, 09), and 1 unit of Hotmaster hot start Taq DNA polymerase (Eppendorf). The PCR profile for RIO02, 04, 06, 08 was 95^{o}C for 2 min, then 35 cycles of 94^{o}C for 30 s, annealing temperature (see Beheler et al. 2004) for 30 s, 70^{o}C for 30 s, then 70^{o}C for 10 min. The profile for RIO05 and RIO07 differed slightly with 40 cycles and an added 45 min extension at 60^{o}C. PCR products were visualized on a 1% agarose gel containing ethidium bromide to confirm successful amplification.

The PCR-amplified microsatellites then were combined into groups of two or three, added to ROX400HD internal lane standard (Applied Biosystems) and electrophoresed through a 5% polyacrylamide gel (Long Ranger Singel Packs; Cambrex) on an ABI 377-XL DNA sequencer. For quality control purposes, combination sets of representative alleles of known size for each locus were constructed and loaded in every 12th lane on each gel. Allele sizes were determined using GeneScan 3.1 (Applied Biosystems) and Genotyper 2.5 (Applied Biosystems).

Analyses

Amplification Success - Amplifications were considered successful if a PCR product of the correct size amplified in at least one of the two trials. For each treatment combination we calculated the percentage of successful amplifications. These percentages were obtained by dividing the number of successful amplifications by the number of attempted amplifications. We calculated the overall percent of successful amplifications for the entire dataset, for each DNA source (pooled across preservation methods) and for each preservation method (pooled across fecal mass section and DNA source).

Genotyping Errors - For each otter we compared genotypes derived from fecal mass and anal jelly secretion DNA to the corresponding reference genotype derived from blood samples as a means of assessing the presence of false alleles or allelic dropout. False alleles occurred when an allele observed in the fecal mass/anal jelly secretion genotype of a given individual was undetected in the reference genotype of that individual. Conversely, allelic dropout occurred when an allele observed in the reference genotype of an individual was undetected in the fecal mass/anal jelly secretion genotypes of that individual.

The proportions of genotypes exhibiting either false alleles or allelic dropout at each locus were calculated for each combination of DNA source and preservation method.

Average proportions of genotypes exhibiting either false alleles or allelic dropout were calculated for each combination of DNA source and preservation method pooled across loci. Locus-specific averages for the proportions of genotypes exhibiting false alleles or allelic dropout also were calculated for each DNA source (pooling across preservation methods).

The overall proportions of genotypes exhibiting errors (combining both false alleles and allelic dropout) at each locus were calculated for each combination of DNA source and preservation method examined. Average proportions of genotyping errors were calculated for each DNA source and preservation method combination, pooled across loci. Locus-specific averages for the overall proportions of genotyping errors also were calculated for each DNA source (pooling across preservation methods).

Because the overall utility of each microsatellite locus can be judged both as a function of the effort expended to acquire each genotype and the incidence of genotyping error at the locus, we also examined locus-specific amplification success rates for the genotyping error experiment. To accomplish this, we recorded the number of amplification attempts required to obtain five unambiguous genotypes at each locus for each subsample used in the genotyping error experiment. For each combination of DNA source and preservation method examined, a locus-specific index of amplification success was calculated by dividing the number of amplifications attempted by the number of genotypes obtained for each subsample (e.g., if 15 amplifications were required to obtain 10 genotypes for evaluation of genotyping error at the locus RIO 02 when using fecal material stored in DET, then the amplification index value for that combination of variables would be 1.5). Average values for the index of amplification success, pooled across loci, also were calculated for each combination of DNA source and preservation method.

Using the estimates for overall genotyping error and the index of amplification success, we calculated an index of utility for each DNA source, preservation method, and locus combination evaluated. The index of utility was created by adding the proportion of overall genotyping errors to the amplification index value for each DNA source, preservation method, and locus combination evaluated. A perfect score of the index for a particular combination of DNA source material, preservation matrix, and locus would be 1.0, indicating that there were no genotyping errors and that every amplification attempt resulted in a scorable genotype. Average values for the utility index also were calculated for each DNA source and preservation method combination (pooled across loci), for each locus, and each DNA source (pooling across preservation methods).

RESULTS

Amplification Success

Overall, successful amplification occurred in 74% (336/454) of all PCR attempts. For all treatment combinations the majority of samples (85%) either amplified a product of the correct size during both trials (rather than only once) or did not amplify at all. All positive controls amplified, whereas none of the negative controls amplified. Differential amplification success rates were observed between DNA sources (Table 1). DNA extractions from anal jelly secretions exhibited a greater overall amplification success (0.91) than fecal masses (0.39), regardless of whether the fecal mass was sampled at the tip (0.44) or the middle (0.27). Overall differences in amplification success also were observed between preservation treatments, regardless of DNA source (Table 1). Subsamples preserved in DET amplified more consistently than other preservation treatment (0.75). Subsamples stored in ethanol at room temperature had the lowest overall amplification success rates (0.33). PCR amplification success rates for fecal mass subsamples stored in DET were higher than those for other preservation methods for each section (0.77 tips, 0.50 middles) and combined (0.68; Table 1). Fecal mass samples frozen at -80° C had the lowest amplification success rates of

all the preservation techniques investigated, both in terms of fecal mass section (0.24 tips and 0.12 middles) and combined (0.22; Table 1). Percent amplification success values for fecal mass tips (range: 0.24 to 0.77) were consistently higher than those of fecal mass middles (range: 0.12 to 0.50) for all preservation treatments with the exception of samples stored in ethanol at room temperature (0.30 for tips verses 0.40 for middles).

DET was the most efficient preservation method for anal jelly secretions and all anal jelly secretions preserved in this buffer amplified successfully (100%; Table 1). Anal jelly samples preserved in silica desiccant or frozen at -80° C each had percent amplification values of 0.87 (Table 1).

Genotyping Errors

When averaged across loci, fecal mass samples collected and frozen in ethanol had the lowest proportion of false alleles (0.05), whereas those frozen at -80° C had the highest (0.36; Table 2). Also, when averaging across loci, allelic dropout occurred least often in fecal masses stored in frozen ethanol (0.00) and most often in fecal masses stored in silica desiccant (0.25). When the data for anal jelly secretions were averaged across loci, proportions of genotypes with false alleles ranged from 0.13 for samples stored in silica desiccant to 0.25 for those stored in DET (Table 2), and proportions of genotypes with allelic dropout ranged from 0.14 for samples frozen at -80° C to 0.40 in samples stored in DET (Table 2).

The overall genotyping error (false alleles and allelic dropout combined) averaged across loci was highest for those that had been frozen at -80° C (0.60) and lowest for samples collected in ethanol with subsequent freezing (0.05; Table 2). Overall, genotyping error rates for anal jelly samples, when averaged across loci, ranged from 0.28 for samples frozen at -80° C to 0.65 for samples stored in DET (Table 2). For the dataset combined (all samples pooled for DNA source, preservation method and loci) 0.40 of all genotypes contained false alleles or experienced allelic dropout.

Locus-specific averages for the proportions of genotyping errors attributed to false alleles and allelic dropout were highly variable among loci for each DNA source (Table 2). For fecal samples, the loci RIO 05 and RIO 08 exhibited the lowest proportion of genotypes with false alleles (0.00 and 0.07, respectively) and RIO 07 had the highest proportion of genotypes with false alleles (0.58; Table 2). Allelic dropout in fecal mass samples occurred least often in genotypes for the loci RIO 05 (0.07) and RIO 08 (0.09), and most often in genotypes from the locus RIO 04 (0.73; Table 2). Patterns of false alleles observed in anal jelly secretion samples followed those observed for fecal masses, with the loci RIO 05 and RIO 08 exhibiting no genotypes containing false alleles and RIO 07 exhibiting the highest proportion of genotypes with false alleles (0.40; Table 2). Allelic dropout in anal jelly secretion samples occurred least often in genotypes for the loci RIO 02 (0.03) and RIO 05 (0.07) and most often in genotypes from the locus RIO 04 (0.50; Table 2).

Fecal samples exhibited the lowest occurrences of overall locus-specific genotyping errors (false alleles and allelic dropout combined) for microsatellite loci RIO 05 and RIO 08 (0.09 and 0.15, respectively), and the highest levels of overall genotyping error for the locus RIO 04 (0.90; Table 2). The fewest locus-specific genotyping errors were observed for the anal jelly secretion samples at the loci RIO 05 (0.07) and RIO 02 (0.23), and the highest at the locus RIO 04 (0.87; Table 2).

Values for the index of PCR amplification success for each combination of DNA source, preservation method and locus examined are in Table 3. Many samples required more than 5 PCR amplifications to obtain 5 scorable genotypes (Table 3). Index values ranged from 1.0 to 5.0 over all combinations of DNA source, preservation method and loci, indicating a wide variance in amplification success rates among the various combinations of variables. When the data were pooled across loci, average index values ranged from 1.0 to

1.5 among the various combinations of DNA source and preservation methods, with fecal masses stored in silica desiccant performing most poorly (1.5) and anal jelly secretions frozen at -80 °C performing the best (1.0; Table 3).

Table 1. Polymerase chain reaction (PCR) amplification success rates for North American river otter fecal mass (tip or middle section) and anal jelly secretion subsamples stored using 5 different preservation techniques; 1) silica desiccant and freezing at –80°C, 2) DMSO/EDTA/Tris/Salt solution (DET) and freezing at -80 °C, 3) freeze only: freezing at -80 °C, 4) ethanol and stored at room temperature (RT), or 5) ethanol and freezing at –80°C. Two amplifications were attempted per subsample (*N* = number of subsamples).

Preservation	*N*	Classification[a]			% Amplified Successfully
		0	1	2	
Fecal Mass					
Silica Desiccant					
Tip	34	24	3	7	0.29
Middle	17	14	1	2	0.17
Combined	51	38	4	9	0.26
DET					
Tip	34	8	3	23	0.77
Middle	16	8	1	7	0.50
Combined	50	16	4	30	0.68
Frozen at –80 °C					
Tip	34	25	4	5	0.24
Middle	17	15	1	1	0.12
Combined	51	40	5	6	0.22
Ethanol-RT					
Tip	10	7	1	2	0.30
Middle	5	3	1	1	0.40
Combined	15	10	2	3	0.33
Ethanol-Frozen					
Tip	10	4	0	6	0.60
Middle	5	4	0	1	0.20
Combined	15	8	0	7	0.46
Overall					
Tip	122	68	11	43	0.44
Middle	60	44	4	12	0.27
Combined	182	112	15	55	0.39
Anal Jelly					
Silica Desiccant	15	2	3	10	0.87
DET	15	0	0	15	1.00
Frozen at –80°C	15	2	0	13	0.87
Overall	45	4	3	38	0.91
Feces/Anal Jelly					
Silica Desiccant	66	40	7	19	0.40
DET	65	16	4	45	0.75
Frozen at –80°C	66	42	5	19	0.36

[a]Number of samples with 0, 1, or 2 correct amplification products out of the 2 amplification attempts.

Table 2. Genotyping error rates across 6 loci for all fecal mass DNA preservation treatments and all anal jelly secretion DNA preservation treatments assessed for North American river otters. Averages are presented across loci for all DNA source/preservation treatment combinations as well as across DNA source/preservation treatment for each locus. Overall genotyping error rates reflect the proportion of genotypes with any error (false alleles and allelic dropout combined).

	RIO 02	RIO 04	RIO 05	RIO 06	RIO 07	RIO 08	Average
False Alleles							
Fecal Mass							
Silica	0.50	0.30	0.00	0.20	0.30	0.29	0.26 (15/57)
DET	0.10	0.00	0.00	0.20	0.40	0.00	0.12 (7/60)
Frozen	0.80	0.20	0.00	0.10	1.00	0.00	0.36 (16/45)
Ethanol-RT	NA	NA	0.00	0.32	0.60	NA	0.31 (18/59)
Ethanol-Frozen	NA	NA	NA	0.10	NA	0.00	0.05 (2/39)
Average	0.47	0.17	0.00	0.18	0.58	0.07	0.22 (58/260)
Anal Jelly							
Silica	0.10	0.10	0.00	0.00	0.60	0.00	0.13 (8/60)
DET	0.40	0.70	0.00	0.30	0.10	0.00	0.25 (15/60)
Frozen	0.10	0.30	0.00	0.00	0.50	0.00	0.15 (9/60)
Average	0.20	0.37	0.00	0.10	0.40	0.00	0.18 (32/180)
Allelic Dropout							
Fecal Mass							
Silica	0.00	0.70	0.10	0.10	0.30	0.29	0.25(14/57)
DET	0.20	0.70	0.00	0.10	0.00	0.00	0.17(10/60)
Frozen	0.20	0.80	0.20	0.40	0.00	0.00	0.24(11/45)
Ethanol-RT	NA	NA	0.05	0.16	0.20	NA	0.14(8/59)
Ethanol-Frozen	NA	NA	NA	0.00	NA	0.00	0.00(0/39)
Average	0.13	0.73	0.09	0.15	0.13	0.07	0.17 (43/260)
Anal Jelly							
Silica	0.10	0.70	0.10	0.00	0.30	0.10	0.22(13/60)
DET	0.00	0.20	0.00	0.70	0.50	1.00	0.40(24/60)
Frozen	0.00	0.60	0.10	0.00	0.10	0.00	0.14(8/60)
Average	0.03	0.50	0.07	0.23	0.30	0.37	0.25 (45/180)
Overall Genotype Error							
Fecal Mass							
Silica	0.50	1.00	0.10	0.30	0.60	0.58	0.51 (29/57)
DET	0.30	0.70	0.00	0.30	0.40	0.00	0.28 (17/60)
Frozen	1.00	1.00	0.20	0.50	1.00	0.00	0.60 (27/45)
Ethanol-RT	NA	NA	0.05	0.48	0.80	NA	0.44 (26/59)
Ethanol-Frozen	NA	NA	NA	0.10	NA	0.00	0.05 (2/39)
Average	0.60	0.90	0.09	0.33	0.70	0.15	0.39 (101/260)
Anal Jelly							
Silica	0.20	0.80	0.10	0.00	0.90	0.10	0.35 (21/60)
DET	0.40	0.90	0.00	1.00	0.60	1.00	0.65 (39/60)
Frozen	0.10	0.90	0.10	0.00	0.60	0.00	0.28 (17/60)
Average	0.23	0.87	0.07	0.33	0.70	0.37	0.43 (77/180)

Table 3. PCR amplification success for all fecal DNA/preservation treatment combinations and anal jelly DNA/preservation treatment combinations at 6 microsatellite loci assessed for application with North American river otters. AA represents the number of amplifications attempted to obtain five genotypes at a locus for each sample. AAG represents the number of amplifications attempted per genotype obtained.

	RIO 02	RIO 04	RIO 05	RIO 06	RIO 07	RIO 08	Total
Fecal Mass							
Silica Desiccant							
Genotypes[a]	10	10	10	10	10	7	57
AA	15	10	10	13	10	26	84
AAG	1.5	1.0	1.0	1.3	1.0	3.7	1.5
DET							
Genotypes	10	10	10	10	10	10	60
AA	15	10	10	10	10	15	70
AAG	1.5	1.0	1.0	1.0	1.0	1.5	1.2
Frozen at -80°C							
Genotypes	5	5	10	10	10	5	45
AA	9	6	10	10	10	25	69
AAG	1.8	1.2	1.0	1.0	1.0	5.0	1.5
Ethanol-RT							
Genotypes	NA	NA	20	19	20	NA	59
AA	NA	NA	20	21	23	NA	64
AAG	NA	NA	1.0	1.1	1.2	NA	1.1
Ethanol-frozen							
Genotypes	NA	NA	NA	20	NA	19	39
AA	NA	NA	NA	21	NA	31	52
AAG	NA	NA	NA	1.1	NA	1.6	1.3
Anal Jelly							
Silica Desiccant							
Genotypes	10	10	10	10	10	10	60
AA	12	12	10	10	10	17	71
AAG	1.2	1.2	1.0	1.0	1.0	1.7	1.2
DET							
Genotypes	10	10	10	10	10	10	60
AA	11	10	10	10	10	21	72
AAG	1.1	1.0	1.0	1.0	1.0	2.1	1.2
Frozen at -80°C							
Genotypes	10	10	10	10	10	10	60
AA	12	10	10	10	10	10	62
AAG	1.2	1.0	1.0	1.0	1.0	1.0	1.0

[a]Number of genotypes obtained.

When the data from genotyping error and the index of amplification success were added to create the index of utility, fecal mass samples preserved in ethanol and frozen at -80 ^{o}C emerged as the best preservation method with an index of utility value of 1.4, whereas samples frozen at -80 ^{o}C without any preservative performed the worst (2.5). A slightly different pattern emerged for anal jelly samples with frozen samples performing the best (1.3) and DET preserved samples performing the worst (1.9). The most drastic differences in overall utility are seen between loci. The microsatellite locus RIO 05 emerged as a highly reliable locus (regardless of DNA source or preservation method) for both amplification

success and genotyping errors, with a near perfect index of utility value (1.1). In contrast, RIO 08 was the least reliable locus with an index of utility value of 2.5 (Table 4).

Table 4. Assessment of the overall utility of each locus and preservation treatment with respect to genotyping errors and PCR amplification ability for application with North American river otters. Values represent the overall genotyping error rate added to the amplification index (AAG) developed in Table 3. A value of 1.0 is perfect with no false alleles, no allelic dropout, and 1 amplification per genotype attempted.

	RIO 02	RIO 04	RIO 05	RIO 06	RIO 07	RIO 08	Average
Fecal Mass							
Silica	2.0	2.0	1.1	1.6	1.6	2.3	1.8
DET	1.8	1.7	1.0	1.3	1.4	1.5	1.5
Frozen	2.8	2.2	1.2	1.5	2.0	5.0	2.5
Ethanol-RT	NA	NA	1.1	1.6	2.0	NA	1.6
Ethanol-Frozen	NA	NA	NA	1.2	NA	1.6	1.4
Average	2.2	2.0	1.1	1.2	1.8	2.6	
Anal Jelly							
Silica	1.4	2.0	1.1	1.0	1.9	1.8	1.5
DET	1.5	1.9	1.0	2.0	1.6	3.1	1.9
Frozen	1.3	1.9	1.1	1.0	1.6	1.0	1.3
Average	1.4	1.9	1.1	1.3	1.7	2.0	
Overall Average	1.8	2.0	1.1	1.4	1.7	2.3	

DISCUSSION

Our study demonstrates the usefulness of otter fecal masses and anal jelly secretions as alternative sources of DNA for genetic analyses. The use of alternative sources of DNA in population genetic studies holds great promise; however, steps need to be taken to ensure accurate results. Researchers should strive to increase amplification success rates and decrease the occurrences of genotyping errors through the correct choice of a sample collection and analysis protocol. We have shown that DNA source and section (fecal masses (tips vs. middle) and anal jelly secretions), preservation method for storing samples, and the individual loci used for genotyping all greatly influence amplification success and genotyping error rates. A few studies have explored the effect of sample source/section (Goossens et al. 1998, Wehausen et al. 2004) and preservation method (Frantzen et al. 1998, Murphy et al. 2000, Murphy et al. 2002) on amplification success and genotyping error rates, however, locus-specific effects (Lathuilliere et al. 2001) have received little attention.

Studies exploring the effectiveness of various fecal DNA preservation methods have had differing results (Wasser et al. 1997, Frantzen et al. 1998, Murphy et al. 2000, and Murphy et al. 2002). Silica desiccation was the preferred preservation in some studies (Wasser et al. 1997), whereas in others, ethanol (Murphy et al. 2002) or DET (Frantzen et al. 1998) were favored. In this study, samples stored in DET buffer exhibited the highest amplification success rates regardless of DNA source or section. Fecal mass tips stored frozen in ethanol also amplified consistently, whereas fecal masses collected in silica desiccant or frozen had the lowest PCR amplification success rates. The differences in preservation preferences observed between such studies suggest that the ideal fecal preservation method may be species-specific. Consequently, preservation techniques should be tested to ensure high rates of amplification success before sampling is conducted.

The occurrences of false alleles and allelic dropout were influenced by DNA source, fecal mass section, preservation method, individual microsatellite loci, and interactions among these variables. Genotyping errors resulting from false alleles and allelic dropout were not as prevalent in samples frozen in ethanol compared with the other preservation methods. Fecal masses preserved in 100% ethanol at -80 ^{o}C did not exhibit any allelic dropout. However, this may have resulted from the combination of preservation and locus

effects because the two loci examined for these samples had the lowest occurrences of allelic dropout. The low error rates observed using samples preserved in ethanol coincides with a study by Dallas et al. (2003) in which scats from Eurasian otters preserved in ethanol had low proportions of allelic dropout and false alleles (0.021 and 0.012, respectively). Consequently, ethanol may be an appropriate preservation method for studies involving otter species.

Genotyping error rates in this study are higher than those reported in other studies (Lathuilliere et al. 2001: 13-20% false alleles and 0-6% allelic dropout, Murphy et al. 2002: 6% false alleles and 7% allelic dropout). However, we are confident in estimates of error in this study because of the inclusion of an otters' reference (blood derived) genotype with its fecal/anal jelly derived genotypes. Despite the high levels of genotyping error detected in this study, error rates clearly are reduced when certain combinations of DNA source, preservation method, and loci are employed. Thus, the selection of appropriate source material, preservation method and suite of loci should result in reliable genotypes.

When planning a genetic study using alternative sources of DNA, researchers should consider a variety of issues (DNA source, section of sample collected, preservation method, and locus robustness) and conduct preliminary studies to determine their influences on the reliability of analysis. Because the overall utility of fecal derived DNA in genetic studies can be judged both as a function of the effort expended to acquire each genotype and the incidence of genotyping error, amplification success rates and genotyping error rates should be assessed for each treatment combination and locus examined. Cost is a limiting factor in studies involving non-invasive samples and, therefore, the development of a reliable and efficient protocol prior to the implementation of a large-scale study would reduce project costs and any ambiguity associated with interpretation of the data.

This study clearly demonstrates the need for conducting preliminary studies before a large-scale noninvasive genetic sampling effort is attempted. Background work must be done for a species to determine a collection protocol and the appropriate loci to reduce genotyping errors. The ability to extract DNA from alternative sources means very little if the DNA is of poor quality or error-prone loci are used during analyses. Consequently, the tradeoffs between reliable amplification and genotyping errors and the interaction between preservation method and loci analyzed should be considered before a preservation method is chosen. Optimized collection and analysis procedures will reduce cost as well as produce more accurate genotypes thereby reducing the potential for erroneous population estimates.

For future studies with river otters, we recommend that fecal mass tips be collected in either DET buffer or 100% ethanol and kept at -80 ^{o}C until DNA isolation. Anal jelly secretions also should be collected whenever they are available. The use of the loci RIO 05, RIO 06, and RIO 08 also would be advisable for the reduction of genotyping error (however the use of RIO 08 could increase project costs because it is difficult to amplify). The other loci examined also could be useful assuming steps were taken to assess possible errors. These recommendations would greatly increase the efficiency of future large-scale population studies involving the North American river otter.

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