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NONINVASIVE GENETIC SAMPLING TOOLS FOR WILDLIFE BIOLOGISTS: A REVIEW OF APPLICATIONS AND RECOMMENDATIONS FOR ACCURATE DATA COLLECTION

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Abstract: Noninvasive genetic sampling provides great potential for research and management applications in wildlife biology. Researchers can obtain DNA from a variety of sources including hair, feces, urine, feathers, shed skin, saliva, and egg shells without handling or observing animals. These samples can then be used to identify the presence of rare or elusive species, count and identify individuals, determine gender, and identify diet items, or samples can be used to evaluate genetic diversity, population structure, and mating system. We review the recent advancements and techniques used for identifying species, individuals, and gender. We also address the potential pitfalls of noninvasive genetic sampling and provide recommendations for laboratory- and field-based methods to improve the reliability and accuracy of data collected from noninvasive genetic samples.

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Key words: DNA, individual identification, noninvasive genetic sampling, polymerase chain reaction, species identification.

Noninvasive genetic sampling (NGS) is a relatively new data-collection approach with great potential for wildlife biologists. By extracting genetic material from hair, feces, or other DNA sources, biologists are able to collect critical data about wildlife populations without handling, capturing, or even observing individual animals. In 1992, NGS was introduced as a method to obtain genetic samples from rare and elusive brown bears (*Ursus arctos*) in Europe (Höss et al. 1992, Taberlet and Bouvet 1992) and to study social structure in chimpanzees (*Pan troglodytes*; Morin and Woodruff 1992). In the last 12 years, researchers have demonstrated a variety of important applications for NGS ranging from detection of rare species to forensic applications (Table 1).

While NGS holds great promise for wildlife biologists, researchers have highlighted potential weaknesses such as low success rates, contamination concerns, and high microsatellite genotyping error rates (Taberlet et al. 1996, 1997, 1999; Goossens et al. 1998; Waits and Leberg 2000; Creel et al. 2003). As the field has evolved, numerous studies have suggested methods for addressing these weaknesses and producing accurate data using NGS (Taberlet et al. 1999, Mills et al. 2000a, Miller et al. 2002, Paetkau 2003, Broquet and Petit 2004, Piggott et al. 2004). Other authors have provided recent reviews of the use of NGS in con-

servation management of rare or cryptic species (Piggott and Taylor 2003a), detecting and counting rare species (Waits 2004), population estimation (Boulanger et al. 2004, McKelvey and Schwartz 2004), accurate data collection (Paetkau 2003), and addressing genotyping errors (Bonin et al. 2004, Broquet and Petit 2004). Our goals are to provide an overview of the potential of NGS for wildlife biologists and to provide technical advice for accurate and efficient collection of NGS data in the laboratory. We focus on 3 of the most important applications of NGS for wildlife biologists: species identification, gender identification, and individual identification.

EXTRACTING, STORING AND AMPLIFYING DNA

Noninvasive genetic sampling studies have obtained genetic samples from a variety of sources (Table 2). These samples contain mitochondrial DNA (mtDNA) and nuclear DNA (nDNA). Mitochondrial DNA is found in hundreds to thousands of copies per cell, while most cells have 2 copies of the nuclear DNA genome (Birky et al. 1989). Thus, DNA extractions from noninvasive genetic samples have more mtDNA than nDNA, and success rates are higher for mtDNA analyses (Frantzen et al. 1998, Kohn et al. 1999, Poole et al. 2001, Lucchini et al. 2002, Waits 2004). In NGS studies, mtDNA analyses are generally performed for species identification, and nDNA analyses are required for individual and gender identification.

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Analysis of nDNA or mtDNA target regions is achieved using the polymerase chain reaction (PCR; Mullis et al. 1986), which can produce millions of copies of a target sequence starting from as little as a single DNA molecule.

The 2 most commonly used sources of DNA for NGS studies are hair and fecal samples (Table 2). Fecal DNA of the depositor is present in sloughed intestinal epithelial cells, and fecal DNA of prey items is also excreted. Multiple methods have been used to extract fecal DNA including chelex protocols (Paxinos et al. 1997, Palomares et al. 2002), phenol-chloroform (Ernest et al. 2000, Fernando et al. 2000, Oka and Takenaka 2001), diatomaceous earth/guanidine-thiocyanate (Gerloff et al. 1995, Kohn et al. 1995, Lucchini et al. 2002, Idaghdour et al. 2003), magnetic beads (Flagstad et al. 1999), and commercially available silica-binding extraction kits (Farrell et al. 2000, Goossens et al. 2000, Constable et al. 2001, Creel et al. 2003). Fecal DNA extracts often contain high concentrations of PCR inhibitors, and extraction methods are designed to minimize inhibitors while maximizing DNA yield. Multiple studies have evaluated the effectiveness of different extraction methods (Reed et al. 1997, Paxinos et al. 1997, Flagstad et al. 1999, Frantz et al. 2003, Piggott and Taylor 2003b, Wehausen et al. 2004). There is no clear consensus on an optimal method, and the optimal method may vary by species and geographic region. For herbivores, optimal methods of DNA extraction make use of only the outermost material of the fecal pellets, which seems to maximize DNA yield and minimize inhibitors (Flagstad et al. 1999, Fernando et al. 2003, Piggott and Taylor 2003b, Wehausen et al. 2004). Currently, the most commonly used method for extracting DNA from fecal samples is silica-binding extraction kits (Qiagen).

Extraction of DNA from hair samples focuses on cells that are attached to the root of the hair. DNA extraction is generally performed using 1 of 2 methods: chelex protocols (Walsh et al. 1991, Goossens et al. 1998, Woods et al. 1999, Banks et al. 2003b), or commercially available silica-binding extraction kits (Poole et al. 2001, Riddle et al. 2003). Direct comparisons of hair samples extracted by silica-based methods and chelex protocols have demonstrated that silica-based extractions generally have higher DNA yield, amplification success rates, and stability during long-term storage (Poole et al. 2001).

Preservation and storage methods for hair and fecal samples affect DNA amplification success rates (Wasser et al. 1997; Murphy et al. 2000,

2003b; Piggott and Taylor 2003b; Roon et al. 2003). Hair samples are preserved by storing them at room temperature in ethanol (Oka and Takenaka 2001), dry with or without desiccant (Gagneux et al. 1997, Woods et al. 1999), or by freezing (Constable et al. 2001). Roon et al. 2003 compared the effectiveness of silica desiccation and -20°C freezing for brown bear samples stored for up to 1 year before extraction. For mtDNA amplification, there were no declines in success rates over the storage period and no significant differences among preservation methods. However, nDNA success rates decreased significantly ($\sim 20\%$) between 6 months and 1 year of storage regardless of preservation method. Also, nDNA amplification success rates were slightly higher (1–10%) for samples preserved at -20°C . Thus, a combination of silica desiccation and freezing may produce optimal results.

Due to the lower success rates of fecal DNA studies, several researchers have evaluated the impact of DNA preservation methods on DNA quality (Wasser et al. 1997; Frantzen et al. 1998; Murphy et al. 2000, 2003b; Frantz et al. 2003; Piggott and Taylor 2003b). Unfortunately, no method has emerged as clearly superior, with different studies concluding that the optimal storage technique is silica desiccation (Wasser et al. 1998), emersion in ethanol (Murphy et al. 2003b, Frantz et al. 2003), or emersion in DMSO, EDTA, Tris and salt (DETs) buffer (Frantz et al. 1998). The variation in results among studies may be due to species-specific effects (Murphy et al. 2003b), environmental effects (Lucchini et al. 2002, Piggott 2004), or interactions between storage methods and extraction methods (Frantz et al. 2003). Thus, pilot studies with the species of interest may be necessary to determine the optimal method when initiating a new study.

SPECIES IDENTIFICATION

Detecting Rare Species

One of the main applications of NGS is the use of genetic tests to detect rare species using animal sign. The earliest species identification (ID) applications used fecal DNA to differentiate grey seals (*Halichoerus grypus*) and harbor seals (*Phoca vitulina*) (Reed et al. 1997) and to differentiate the endangered San Joaquin kit fox (*Vulpes macrotis mutica*) from other sympatric canids (Paxinos et al. 1997). A growing number of research projects around the world are using hair or fecal samples to document the presence of a target species. In Europe, researchers have documented the current

range of the Iberian lynx (*Lynx pardinus*) using fecal DNA analyses (Palomares et al. 2002). In the United States, the U.S. Department of Agriculture (USDA) Forest Service is using a large-scale hair survey as part of an initiative to gather data on the current distribution of Canada lynx (*Lynx canadensis*) (McKelvey et al. 1999, McDaniel et al. 2000). Throughout North America, a mtDNA fragment analysis method has been used to differentiate brown bears and black bears (*Ursus americanus*) from hair and fecal samples (Woods et al. 1999, Mowat and Strobeck 2000, Murphy et al. 2000, Poole et al. 2001). Restriction enzyme-based techniques were developed to differentiate mtDNA of otter (*Lutra lutra*), American mink (*Mustel vison*), and polecat (*Mustela putoris*) in Europe (Hansen and Jacoben 1999) and in most carnivore species in North America (Mills et al. 2000b, Riddle et al. 2003). An efficient diagnostic test has been developed to differentiate arctic fox (*Alopex lagopus*), red fox (*Vulpes vulpes*), and wolverine (*Gulo gulo*) using 1 conserved primer and 3 different, specific-specific mtDNA primers (Dalén et al. 2004). This diagnostic method is inexpensive and fast because it separates the PCR products by size and does not require a second enzymatic step such as sequencing or digestion with restriction enzymes.

Dietary and Disease Applications Using Feces

Species identification of fecal samples can also be coupled with traditional dietary analysis of scats to evaluate the diets of species whose feces are not clearly distinguishable morphologically. This approach was used by Farrell et al. 2000 to evaluate the dietary niches of sympatric carnivore species in Venezuela and revealed minimal prey partitioning between puma (*Puma concolor*) and jaguar (*Panthera onca*). Reed et al. 1997 developed species ID methods for seal feces and proposed combining DNA analyses with traditional dietary analysis to evaluate the relative impacts of grey (*Halichoerus grypus*) and harbor (*Phoca vitulina*) seals on fisheries. Individual differences in diet of coyotes (*Canis latrans*) have been evaluated by combining genetic individual identification of fecal samples with traditional diet analysis (Fedriani and Kohn 2001).

Another interesting application of species ID analyses using NGS samples is the detection of diet items and pathogens in fecal samples (Kohn and Wayne 1997, Symondson 2002). Currently, there are only a small number of published examples of this type of analysis, but the approach has great potential for wildlife science. Researchers have demonstrated that multiple pathogens can be de-

tected in fecal material using PCR to amplify DNA or RNA of the target organism (Bretagne et al. 1993, Schnuck et al. 1995, da Silva et al. 2003, Whittier et al. 2003). Thus, there is the potential to use fecal DNA analysis to evaluate pathogen exposure or disease prevalence at the individual and population level.

Species identification of diet items is particularly useful for identifying diet items that are not visually distinguishable in feces and has great potential for studies of resource partitioning. When analyzing bear feces, Höss et al. (1992) demonstrated that specific species of plants could be amplified. The DNA of genetically modified corn has also been detected in the fecal samples of domestic cows (Phipps et al. 2003) and pigs (Chowdhury et al. 2003). Genetic analysis of fecal samples from blue tits (*Parus caeruleus*) and great tits (*P. major*) has been used to evaluate Lepidoptera species and proportions in a comparison of dietary niches (Sutherland 2000, Casement 2001). Genetic analyses of feces have even been used to evaluate the diets of extinct sloths (Hofreiter et al. 2000).

Species ID of fecal samples can also be used to identify the predator that killed domestic animals or an endangered species (Farrell et al. 2000, Ernest et al. 2002, Banks et al. 2003a). With this technique, the researcher has the potential to determine the species and individual ID of the predator as well as the species and individual ID of the prey item (Banks et al. 2003a). Regurgitates also provide a potential noninvasive source of genetic samples for wildlife research. Taberlet and Fumagalli (1996) demonstrated the ability to extract DNA from small mammal bones in owl regurgitates and recommend using these samples as a source of DNA for genetic studies of small mammals.

Methodological Considerations

When developing a new species ID protocol, it is necessary to test known samples from across the geographic area that will be studied to account for intraspecific variability. This is primarily an issue if the study requires the differentiation of closely related species whose genetic results could realistically be confused. Ideally, protocols for discriminating between taxa should not rely on a single genetic change and should focus on PCR amplification of short fragments (<300 bp). Short fragments will amplify better from low quantities of degraded DNA (Frantzen et al. 1998, Murphy et al. 2000, Roon et al. 2003), and protocols will be more robust to intraspecific variation when genetic tests rely on variation at multiple nucleotide

positions. As when using any other approach to species identification, the molecular detection of a species in an unusual or previously undocumented area should be treated with caution, and every effort should be made to confirm the result. Material permitting, this could include re-extraction and reanalysis in the lab, but it could also include analysis with nongenetic methods (e.g., morphological analysis of hair) and increased sampling efforts in the field. Leftover materials should also be archived. For example, many of the hair shafts from which we removed follicles for genetic analysis have been used in radio-isotope studies, often years after the genetic work was completed (Felicetti et al. 2004).

GENDER IDENTIFICATION

In mammals and birds, gender is determined by whether an individual has 2 copies of the same sex chromosome (the homogametic sex; females in mammals and males in birds) or 1 copy of each of 2 different sex chromosomes (the heterogametic sex). In principle, a genetic gender test need only establish the presence or absence of the chromosome that is specific to the heterogametic sex. In practice the absence of a chromosome can appear the same as failure to amplify that chromosome, a circumstance that is likely to arise when using minute quantities of DNA, as in many NGS studies (Taberlet et al. 1996, 1999). Specifically, failure to amplify the Y-chromosome in a male mammal or the W-chromosome in a female bird will give rise to an erroneous conclusion of gender ID.

There are several features in a gender ID test that can minimize the chance of incorrectly identifying the heterogametic sex. First, every effort should be made to find a single pair of PCR primers to amplify a marker that occurs on both chromosomes but that has different length alleles on those 2 chromosomes. An excellent example of this approach involves the analysis of chromosome-specific length variation in the chromo-helicase-DNA-binding (CHD) gene. This test appears to work in all nonratite birds (Griffiths et al. 1998, Fridolfsson and Ellegren 1999).

No mammalian gender test possesses all of the qualities of the CHD-based tests in birds. For example, a length variation in the amelogenin gene has many qualities of the CHD tests, and it works in species as different as cows and bears (Ennis and Gallagher 1994, Poole et al. 2001), but most of the species on which we have tested this marker appear to have equal-length alleles on the 2 sex chromosomes. A test based on zinc-finger genes

works on a wider range of species (Shaw et al. 2003), but it requires PCR amplification of a relatively long fragment (>800 bp), which will compromise success rates when working with sparse DNA samples. Another common test amplifies an autosomal or X-linked fragment alongside an *SRY* fragment that identifies the presence or absence of the Y-chromosomes. This test was first applied in brown bears (Taberlet et al. 1993) and has also been used in seals (Reed et al. 1997), otters (Dallas et al. 2000), cougars (Ernest et al. 2000), elephants (Eggert et al. 2003), and wolves (Lucchini et al. 2002). This 2-gene test is vulnerable to changes in PCR conditions that could differentially affect the relative amplification strength of the 2 markers, leading to a mistaken identification for a sample of the heterogametic sex.

The use of conserved-mammalian, gender-ID primers also leads to 2 other potential problems that need to be avoided. First, it is better to use primers that have been shown not to work on humans, otherwise researchers need to wear gloves and take other precautions to avoid contaminating the samples with their own DNA in the field and in the laboratory. Second, in fecal DNA extracts, it is possible to amplify the predator and the prey (Ernest et al. 2000, Murphy et al. 2003a). Thus, for fecal DNA work on carnivores, it is optimal to have species-specific gender ID primers or take additional precautions to verify the accuracy of gender ID results (Waits 2004).

If the absence of a single-marker test makes it necessary to use 2 different markers for the 2 chromosomes, then the primers should be situated so that the PCR product for the gender-specific marker amplifies at least as strongly as the other marker. This helps to ensure that the gender-specific marker will not be out-competed during the amplification reaction in samples containing low quantities of DNA. The easiest way to make 1 PCR product amplify more strongly than another is to move the primers closer together, since short target sequences generally amplify more robustly. We designed primers that produce excellent ZFX/ZFY + *SRY* gender results in mustelids, canids, and felids, but we have been unable to find a single set of primers that works across the order carnivora (D. Paetkau, Wildlife Genetics International, personal communication). In summary, there is considerable room for improvement on the currently available gender tests for mammals.

A unique aspect of a gender analysis, at least relative to markers like microsatellites, is that it carries inherent biological meaning. For example, if

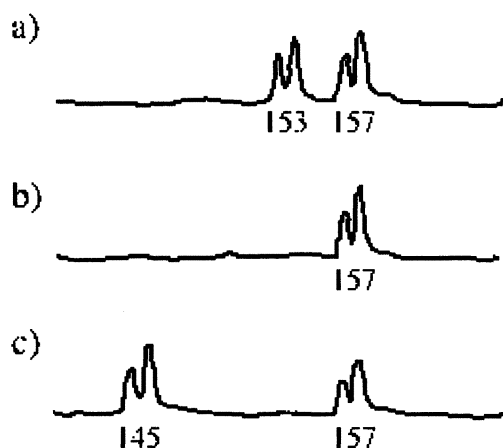


Fig. 1. Electropherogram of microsatellite genotyping errors for a heterozygous genotype. Alleles are labeled and described by size in base pairs: (a) true genotype, (b) allelic dropout or false homozygote error, and (c) false allele error (allele 145) and allelic dropout (allele 153).

we collect a single sample from an individual and make an error in its microsatellite genotype, this will have no impact on the accuracy of the data interpretation in the context of a capture–mark–recapture (CMR) study. By contrast, if we make an error in recording the gender of this sample, we alter the biological understanding of the animal in question. For this reason, researchers should routinely evaluate gender ID error rates and establish protocols to ensure accurate results. For example, it may be necessary to duplicate analyses of all female results in mammals and all male results in birds. At a minimum, very stringent thresholds for signal strength should be established as a means to control errors.

INDIVIDUAL IDENTIFICATION

Individual ID of noninvasive genetic samples, which is generally achieved through analysis with a number of microsatellite markers, is most often used to obtain a minimum or mark-recapture population estimate. Currently, noninvasive genetic sampling has been used to obtain minimum or mark–recapture population estimates in at least 13 different species (Table 1). After identifying individuals, the microsatellite data may also be used to address research questions related to genetic diversity, gene flow, phylogenetic relationships, paternity, social structure, or forensics (Table 1).

There are 2 main challenges to collecting accurate data on individual identity using noninvasive genetic samples. Researchers must: (1) analyze enough loci to have adequate resolution to dis-

tinguish individuals and (2) minimize genotyping errors. If data are not collected for an adequate number of loci, then the number of individuals in the dataset will be underestimated (Mills et al. 2000a, Waits and Leberg 2000, Waits et al. 2001). In contrast, genotyping errors (Fig. 1, allelic dropout, false alleles) will inflate the number of individuals in a dataset (Taberlet et al. 1999, Waits and Leberg 2000, Creel et al. 2003, Roon et al. 2005b). Increasing the number of loci will improve the resolution of any genetic study, but in NGS studies this will also increase the probability of observing a genotyping error in a multilocus genotype. Determining the optimal number of loci for identifying individuals in NGS studies will require balancing the 2 sources of error while considering the research questions and the nature of the samples.

Determining the Minimum Number of Markers

Before initiating a study of individual identity in a new population, researchers should evaluate levels of genetic variation. We recommend genotyping approximately 30 individuals from the study population using 10 to 15 microsatellite loci. The loci can then be ranked in order of decreasing variability (excluding markers that are difficult to amplify or score), and the most variable set of 5, 6, or more markers can be chosen based on suggested guidelines (Waits et al. 2001, Paetkau 2003). Genetic assignment of individual identity may not be feasible in small, insular populations, where genetic variability is likely to be low.

A critical variable in considering the power of a selected marker system is the number of individuals that are likely to be sampled. For example, a study that collects samples from 10 individuals would have 45 pairs of individuals $[(10 * 9) \div 2]$, each of which would have a probability of having identical genotypes. If the marker system has a match probability (probability of 2 individuals matching for the markers analyzed) of 1 in 1,000, then the chance of incorrectly grouping samples from 2 different individuals in this study is $\sim 45 \div 1,000$, which most researchers would consider to be an acceptable risk of error. By contrast, if the same marker system was used to assign individual identity to samples drawn from 250 individuals in this population, the true number of individuals would be underestimated by 30 or more because there would be 31,125 pairwise comparisons between individuals $[(250 * 249) \div 2]$, each of which has a 1/1,000 chance of having matching geno-

Table 1. Applications of noninvasive genetic sampling.

Application	Species	Citation
Detecting rare species	Andean mountain cat, brown bear, Grey wolf, Iberian lynx, Kit fox, New England cottontails	Perovic et al. 2003, Romain-Bondi et al. 2004, Valiere et al. 2003, Palomeres et al. 2002, Paxinos et al. 1997, Kovach et al. 2003.
Estimating population size	Brown bear, black bear	Taberlet et al. 1997, Bellemain et al. 2005, Woods et al. 1999, Mowat and Strobeck 2000, Poole et al. 2001, Paetkau 2003.
	Cougar, coyote, Eurasian badger, Forest elephant, grey wolf, Humpback whale, otter, pine marten, Seals, wolverine, wombat	Ernest et al.2000, 2002 Kohn et al.1999, Frantz et al.2003, Eggart et al.2003, Lucchini et al. 2002, Creel et al. 2003, Palsbøll et al. 1997, Dallas et al. 2003, Mowat and Paetkau 2002, Reed et al. 1997, Flagstad et al. 2004, Banks et al. 2002, 2003.
Evaluating social structure	Chimpanzee, rhinoceros, Marmot, orangutans	Morin et al. 1994b, Garnier et al. 2001, Goossens et al. 1998, Utami et al. 2002.
Genetic diversity and gene flow	Humpback whales, orangutan, bonobos, Grey wolf, brown bears, elephants	Palsbøll et al. 1997, Goossens et al. 2005, Gerloff et al. 1999, Lucchini et al. 2002, Kohn et al. 1995, Fernando et al. 2000.
Detecting hybridization	Red wolf	Adams et al. 2003.
Detecting disease	Gorilla, fox, lemur	Whittier et al. 2003, Bretegne et al. 1993, Da Silva et al. 2003.
Detecting diet items	Brown bear, barn owl, invertebrates	Hoss et al. 1992, Taberlet and Fumagalli 1996, Symondson 2002.
Predator identification of kills	Cougar, coyote,dingo, felids/canids	Ernest et al. 2002, Williams et al. 2003, Banks et al. 2003a, Farrell et al. 2000.
Ivory tusk region of origin	Elephant	Wasser et al. 2004.

types. Clearly, the threshold for acceptable match probabilities needs to be scaled to the number of pairs of individuals that are likely to be sampled.

The next variable that merits consideration is the degree of consanguinity in the study population. Close relatives have much higher match probabilities than unrelated individuals (Woods et al. 1999, Waits et al. 2001), but the proportion of close relatives in a given population will vary depending on the size and isolation of the population. For example, Belant et al. (2005) studied an insular black bear population that was estimated to consist of 26 individuals. They found that the proportion of pairs of genotypes that matched at 3 of 5 markers was about 10 times higher (1 in 100 pairs) than that observed with marker systems of similar variability in continental black bear populations (Paetkau 2003). Without having a way to quantify the different levels of consanguinity in these populations, it is impossible to calculate a match probability that accurately reflects the chance of finding identical genotypes in pairs of individuals drawn at random from these populations. This limits the value of calculated match probabilities for predicting the power of marker systems.

A more direct empirical alternative to calculated match probabilities is to draw inference about match probabilities by extrapolating from the ob-

served frequencies of pairs of genotypes that match at all-but-1 or all-but-2 markers (1MM- or 2MM-pairs). Testing with samples from known individuals has shown that 1MM-pairs are approximately 10-fold more common than 0MM-pairs (errors) and that 2MM-pairs are approximately 100 times as common as 0MM-pairs, at least for typical 6-locus bear studies (Paetkau 2003). No matter what the study species or marker system, we always expect fewer matches at all markers than at all-but-1 markers. Thus, the most direct method to assess marker power at the end of a project is to graph the distribution of genotype similarity (Fig. 2). The ideal distribution will have no more than 1 or 2 1MM-pairs, and it will have <10 2MM-pairs. In such a situation, there is little chance that the true number of individuals was underestimated, and the researcher can proceed with downstream analyses without adjusting for this source of error.

The challenge with deciding how many markers to run is that one doesn't actually know how well the markers will perform or how many individuals will be sampled until the end of the study. Since every data point that is analyzed uses DNA and has a chance of error, the costs of analyzing too many markers extend beyond scheduling and budgetary considerations. Therefore, the most prudent approach is to use 1 more marker than appears to be necessary until enough data have been collected

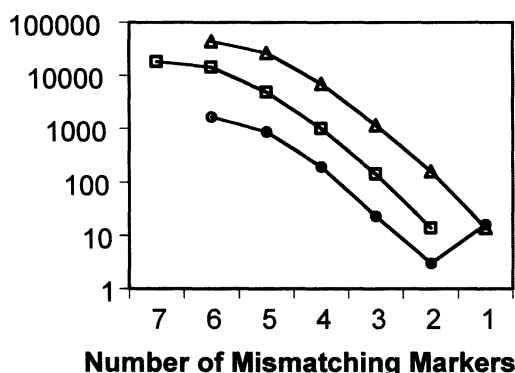


Fig. 2. Examples of mismatch (MM) distributions illustrating the 2 ways, both problematic, in which an excess of 1MM-pairs can be obtained. (1) A 6-locus project that used an insufficiently powerful marker system and is thus likely to have sampled 1 or more pairs of individuals with identical multilocus genotypes (triangles). (2) A 6-locus project prior to re-analysis of 1MM-pairs, all of which were subsequently found to have been caused by genotyping errors (circles; Kluane grizzly bear data taken from Paetkau 2004). (3) An ideal, 7-locus project in which the distribution decays smoothly and rapidly, and where there are no 1MM-pairs and all 2MM-pairs have been confirmed through replication (squares).

to show that 1MM-pairs would still be rare if the number of markers was reduced.

Controlling Genotyping Errors

One source of 1MM- and 2MM-pairs is the use of an insufficient marker system (above), but a second source of these similar pairs of genotypes is when inaccurate genotypes (Fig. 1) are recorded for different samples taken from the same individual. Most of these errors are expected to create 1MM-pairs, since the rate of genotyping errors is generally lower than a few percent per locus in studies using multiple hair samples and a few fecal DNA studies (Goossens et al. 1998, Sloane et al. 2000, Dallas et al. 2003, Paetkau 2003). Indeed, re-analysis of samples involved in 1MM- and 2MM-pairs indicates that errors affect a single marker far more often than they affect 2 markers in the same sample (Paetkau 2003).

Since NGS samples are prone to genotyping errors (Taberlet et al. 1996, Gagneux et al. 1997, Goossens et al. 1998), accurate results can only be obtained if similar pairs of genotypes are confirmed through data replication. There are 3 forms of data replication that are used for this purpose. One form of data replication is detecting multiple samples with the same genotype. Since the most common types of errors (i.e., allelic dropout, false amplification) are not expected to be reproducible, the very observation of a partic-

ular multilocus genotype in multiple samples provides strong support for the accuracy of that genotype. This replication of multilocus genotypes in independent samples often confirms the results for many samples. For example, 96% of the 1,716 samples that were assigned to individuals in 1 black bear study (Ocala Population; Paetkau 2003) had genotypes that were observed in at least 1 other sample.

A second form of data replication is to specifically re-analyze the mismatching markers/loci for similar pairs of genotypes. This approach was first applied to 1MM-pairs by Palsboll et al. (1997), and it has since been expanded and refined by others (Woods et al. 1999, Banks et al. 2003b, Paetkau 2003, Roon et al. 2005b). This targeted replication involves a small portion of the overall dataset, so it is efficient in terms of time and cost (Roon et al. 2005b). However, simulations have suggested that this method will not be sufficient to remove genotyping errors and associated bias in population estimation when per locus error rates are >5% (Roon et al. 2005b). Ultimately, researchers should seek to lower per locus error rates to 1 percent when using this error-checking protocol. Recently developed PCR preamplification protocols provide great promise for decreasing error rates and increasing success rates from NGS sources (Bellemain and Taberlet 2004, Piggott et al. 2004).

The last form of data replication is repeated re-analysis of every sample at every locus, a method originally defined as the multiple tubes approach (Taberlet et al. 1996). In considering the relative merits of targeted, marker-specific re-analysis and wholesale re-analysis, thought must be given to the total amount of DNA that is available and per sample error rates. Theory predicts that even slight decreases in the amount of DNA that goes into a PCR reaction can lead to dramatic decreases in success rates and data accuracy, particularly with those samples that contain little enough DNA to be at significant risk of error (Taberlet et al. 1996). Empirical evidence supports this prediction, with rates of allelic dropout in single-locus, heterozygous genotypes being approximately 70 times higher for samples that fail at >50% of markers during the first round of analysis than in samples with higher success rates (Paetkau 2003).

If one proposes to analyze each sample 10 times, while saving DNA for downstream analyses of gender, relatedness, or population genetics, one must use <10% of the extracted DNA per round of analysis. By contrast, a study that plans to use selected re-analysis can comfortably use 30% of the

Table 2. Noninvasive sources of DNA for wildlife research.

DNA source	Guild	Species	Citation
Hair	Carnivores	American martin, brown bear, felids, ocelot	Foran et al. 1997 <i>b</i> , Taberlet and Bouvet 1992
	Primates	Chimpanzee, gibbon, gorilla, orangutan	Mills et al. 2000 <i>b</i> , Weaver et al. in press.
	Rodents	Marmots	Morin and Woodruff 1992, Oka and Takenaka 2001, Field et al. 1998.
	Marsupials	Wombat	Goossens et al. 1998.
Feces	Primates	Baboon, bonobo, langur monkey, orangutan	Taylor et al. 1998.
	Carnivores	Badger, brown bear, canids, cougar, lynx, mustelids, otter, seals	Constable et al. 1995, Gerloff et al. 1995, Launhardt et al. 1998, Goossens et al. 2000.
			Frantz et al. 2003, Höss et al. 1992, Paxinos et al. 1997, Ernest et al. 2000, Palomeres et al. 2002, Hansen and Jacobsen 1999, Foran et al. 1997 <i>a</i> , Dallas et al. 2000, Reed et al. 1997.
			Vege and McCracken 2001.
			Wehausen et al. 2004, Fernando et al. 2000,
	Herbivores	Bats	Huber et al. 2002, Kovach et al. 2003, Flagstad et al. 1999, Garnier et al. 2001.
		Bighorn sheep, elephant, red deer	Feinstein 2004.
		Rabbit, reindeer, rhinoceros	
	Insects	Butterfly	
	Sirenian/Cetacean	Dugong, dolphin	Tikel et al. 1996, Parsons et al. 1999.
	Birds	Bustards	Idaghdour et al. 2003.
	Marsupials	Quokka, wombat	Alacs et al. 2003, Banks et al. 2002 <i>a</i> .
Urine	Birds	Chicken	Nota and Takenaka 1999.
	Carnivores	Grey wolf	Valiere and Taberlet 2000.
Sloughed skin	Snakes	Timber rattlesnake, corn snake	Bricker et al. 1996.
	Cetaceans	Humpback whale	Valsecchi et al. 1998.
	Insects	Butterfly (exuviae)	Feinstein 2004.
Feathers	Birds	Blue tit, Capercaillie, hornbill, spectacled eiders	Taberlet and Bouvet 1991, Segelbacher 2002, Morin et al. 1994 <i>a</i> , Pierce et al. 1997.
Egg shells	Birds	Spectacled eiders, cowbirds	Pierce et al. 1997, Strausberger and Ashley 2001.
Regurgitates	Carnivores	Grey wolf	Valiere et al. 2003.
	Birds	Barn owl	Taberlet and Fumagalli 1996.
	Primates	Chimpanzee (food wadges)	Sugiyama et al. 1993.
Saliva	Predators	Coyote	Williams et al. 2003.

available DNA during the first round of analysis and still have plenty in reserve for selected data replication, confirmation of weak data, and downstream analysis of additional markers, particularly since the majority of samples will not be candidates for reanalysis or data cleanup. This means that the net result of wholesale re-analysis—beyond increasing costs and slowing schedules—may not be a significant gain in the overall quality of results, at least when working with finite samples like hairs.

In contrast to hair samples, some variant of the multiple tubes approach will generally be the best approach for scat samples because error rates are substantially higher (Goossens et al. 2000, Smith et al. 2000, Frantz et al. 2003, Waits 2004). In fecal DNA samples, the quality of individual results is currently limited by the inhibitory compounds that co-purify with the DNA, thus limiting the amount of DNA extract that can be used in a given reaction. Furthermore, the amount of material that is available for re-extraction is also relatively large with most fecal samples, which means that

analyses can be repeated many times without running out of material. Thus, the strategy of minimizing error and maximizing success rates by using more DNA is not available with scat samples, but the potential for repeated rounds of re-analysis is much greater. Two recent studies developed new multiple-tube approaches that appear to be more time and cost efficient (Miller et al. 2002, Frantz et al. 2003) than the original method (Taberlet et al. 1996). Quantitative PCR has also been suggested as a method to accurately estimate the DNA quantity of noninvasive samples so that researchers can select the highest quality samples and match the error-checking protocol to the quality of the samples (Morin et al. 2001).

As with the risk of underestimation (i.e., matches between individuals at all markers); mismatch distributions can provide the final confirmation that a dataset is not unduly affected by genotyping error. Undetected errors will create a spike of 1MM-pairs (Fig. 2), and a dataset should not be considered complete until each genotype that is involved in a 1MM- or 2MM-pair is con-

firmed through carefully documented data replication at the locus or loci in question.

While data replication should be capable of ensuring the accuracy of individual identifications, analysis of additional markers can provide an additional confirmation at the end of the project. This additional analysis would normally be applied to 1 representative sample from each genotype (individual) that was identified during the main analysis, and it is normally undertaken with population genetic or relatedness analyses in mind. The ability to choose samples that produced strong data during the first round of analysis means that the extra analysis will be less prone to failed results and genotyping error than the original analysis. Once data are available for an excess number of markers, it becomes possible to use statistical methods to ask whether the data contain an excess of similar genotypes, as expected in the presence of undetected error (McKelvey and Schwartz 2004). Undetected errors will not be found if the data replication was carried out thoroughly, but the extra confirmation of data quality will be reassuring.

OVERARCHING TECHNICAL CONSIDERATIONS

Avoiding Contamination

Due to the small quantities of DNA involved, contamination risk is greatly elevated with NGS samples compared to samples taken from blood or tissue (Taberlet et al. 1999). Thus, special precautions must be taken to minimize contamination during field collection and in the laboratory. Field researchers should collect samples that are as fresh as possible since DNA degrades over time (Piggott 2004), and researchers should not touch samples with bare hands unless the markers that they are using have been shown not to amplify human DNA. Ideally, the items used to collect samples would be disposable and changed between each sample or washed and sterilized by flame between each sample. Researchers should avoid collecting samples that might contain a mixture of DNA from 2 individuals through sampling design (Sloane et al. 2000, Wilson et al. 2003), and laboratory protocols should be designed to detect and remove mixed samples from the dataset (Alpers et al. 2003, Roon et al. 2005a).

In the laboratory, DNA extraction and PCR set up should be spatially separated (e.g., separate rooms, or preferably separate floors of same building or different buildings) from DNA that has been amplified. To avoiding transferring amplified DNA into the pre-amplification working

space, the flow of supplies, equipment, and people should always move from pre-amplification to post-amplification laboratory space. Any movement of materials from the post-amplification to pre-amplification space is likely to transfer amplified DNA and greatly increase the probability of contamination. Contamination can also be minimized by focusing on laboratory cleanliness and good lab technique. Benches, pipettes, and laboratory equipment should be wiped down daily or after each batch of samples are processed. Some samples, such as fecal samples, are particularly messy, and researchers may need to wipe down the extraction area and change gloves between samples to avoid cross-contamination. Careful pipetting and the use of electronic pipets or multi-channel pipets can minimize the potential for contamination. Filter tips can also be used to avoid the transfer of extracted or amplified DNA to the inside of pipette barrels, although pipets that have been used for amplified DNA should never be used for pre-amplification steps without thorough decontamination.

Even more important than the specific steps that are taken to control contamination, it is critical that researchers demonstrate the efficacy of their chosen techniques by including a minimum of 1 negative control in every DNA extraction and PCR. These negative controls should be processed and analyzed in the same method as all other samples. Records for the negative controls should be maintained for future inspection. The potential for contamination creates a special challenge with techniques that involve >1 enzymatic reaction such as sequencing or preamplification techniques (Piggott et al. 2004, Bellemain and Taberlet 2004). The practical challenges of keeping negative controls clean will be more complex than with single-step processes, such as PCR-based analysis of length variation (including gender analysis, some species tests, and microsatellite analysis [see below]).

Practicing Good Lab Technique

The accuracy of NGS data can also be improved by practicing the following procedures that we classify as good lab techniques. Positive controls should be included in all PCR reactions and gels. These samples indicate whether reagents and PCR conditions are performing well. They can also be used to detect other problems such as sample mix up, sizing inconsistencies, and microsatellite genotyping errors. With sufficient use, positive controls can form the basis of estimates of error rates. To minimize human er-

ror, each DNA extraction, PCR set up, sequencing reaction, electrophoresis run, and other repetitive procedures should be performed with an established, written protocol. At each step of the process the researcher can check off the action after it is completed. Not only does this provide documentation for future reference, but it reduces the chance that irreplaceable samples will be wasted through something as trivial as forgetting to add a reagent.

Many NGS studies require the researcher to process hundreds to thousands of samples, which creates challenges for data handling and record keeping. Procedures should be designed to minimize or eliminate hand labeling or manual data entry, and records should be kept for each laboratory procedure indicating who performed the work, which batches of reagents were used, which protocols were followed, and which samples were analyzed. Records must also be kept on where samples are ultimately stored, and electronic data should be backed up on an established schedule, including backups to an offsite location.

The laboratory work required for accurate NGS data collection is technically challenging and tedious; thus, thorough training is necessary for students or technicians. We recommend that each new researcher in the laboratory should complete a training period using known samples or should work under the direct, full-time observation of an experienced worker until they have gained the skills needed to work independently. In some cases, NGS work may be a new direction for an established genetics laboratory, and we suggest that students/technicians visit another NGS laboratory to obtain training. Additional quality control can be achieved by cross-examination and duplicate analysis of gels by 2 different individuals in the laboratory. The laboratory should keep a database on human errors to quantify the possible range of error, to raise the awareness of human error, and to help researchers learn about and avoid potential sources of error.

Validation and Quality Assurance

As with any analytical procedure, DNA-based methods need to be validated before they are applied, and they need to be continuously monitored for accuracy. While these issues are not specific to DNA-based methods, tests of genetic methods do need to consider the specific sources of error to which those methods are vulnerable. The major issue of specific relevance to NGS methods is that the low quantities of DNA in noninvasively collected samples can lead to inaccurate PCR results, either through increased sensitivity to contamination

or through failure to amplify 1 of 2 alleles in a heterozygous genotype (Fig. 1, allelic dropout; Taberlet et al. 1996). Depending on the particular sample type used in a given study, the rate of these errors could be as low as a fraction of a percent of single-locus genotypes (Goossens et al. 1998, Sloane et al. 2000, Paetkau 2003). This means that testing would need to be quite extensive to produce precise estimates of error rates (Bonin et al. 2004, Hoffman and Amos 2005).

While validation is an important aspect of any protocol, there are several reasons why ongoing tests are of at least equal importance. As already noted, sample type and quality will vary between studies, and it will vary even over time and space within studies (Lucchini et al. 2002). For example, a rainstorm could reduce the quality of hair or fecal samples during 1 session of a multisession study, or in 1 region of a geographically large study. For this reason the most relevant estimates of error for a given study come from analyses that were repeated within the study, either as positive controls (above) or through wholesale replicate analysis (ie. multiple tubes approach; Taberlet et al. 1996). Any laboratory that is focused on NGS analyses will run hundreds or thousands of replicate analyses per year, making this a very rich source of information on data reproducibility.

A form of testing that has been a source of controversy is blind testing by nonlaboratory personnel. Because the sample sizes for these blind tests tend to be limited, it is rare that such tests would form a richer source of information than the routine positive control samples. Nonetheless, blind tests have value insofar as they remove the need for field workers to have blind trust in a methodology with which they may lack familiarity. Facing the prospect of blind testing is also healthy for laboratory workers, since it reduces the chance of complacency. Blind testing loses value if the testers are not absolutely certain of the correct result, or if the testing is handled in a way that erodes relationships between laboratory and field personnel or erodes public confidence in the research results such as occurred in the National Lynx Survey (Dalton 2002, Mills 2002, Thomas and Pletscher 2002).

MANAGEMENT IMPLICATIONS

The use of NGS has become an important tool in wildlife biology and has great potential for future applications. Recent controversies have questioned the accuracy of data generated in NGS studies highlighting the political sensitivity (Dalton 2002, Mills 2002, Thomas and Pletcher 2002) and

the importance of rigorous standards and guidelines. Future NGS projects should give the utmost attention to developing and documenting scientifically rigorous standards of data collection. Wildlife managers should familiarize themselves with the potential pitfalls of laboratory data collection and take an active role in ensuring that these problems are adequately addressed in the protocols of the collaborating laboratory. We also emphasize that laboratory analysis is only 1 component of NGS research and our review does not address many of the challenges faced in field collection. It is important that field biologists utilizing NGS methods work together to develop a rigorous set of guidelines and considerations for accurate data collection. We believe that NGS is becoming one of the most efficient and accurate methods for estimating population size of animals and detecting the presence of rare species. However, additional evaluations of the strengths and weaknesses of alternate sampling strategies (Boulanger et al. 2004) and estimation approaches (Miller et al. 2005) are greatly needed. Our review has also highlighted the potential and current under utilization of DNA analysis of fecal samples to evaluate disease prevalence, diet and resource partitioning among species, individuals and populations.

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