Noninvasive genetic sampling: look before you leap

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Noninvasive sampling allows genetic studies of free-ranging animals without the need to capture or even observe them, and thus allows questions to be addressed that cannot be answered using conventional methods. Initially, this sampling strategy promised to exploit fully the existing DNA-based technology for studies in ethology, conservation biology and population genetics. However, recent work now indicates the need for a more cautious approach, which includes quantifying the genotyping error rate. Despite this, many of the difficulties of noninvasive sampling will probably be overcome with improved methodology.

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The use of molecular genetic techniques to answer elusive questions in conservation biology and behavioral ecology continues to escalate. This explosive growth can be traced to the development of the polymerase chain reaction (PCR) to amplify specific DNA target sequences[1,2]. Among the different approaches used to obtain DNA from wild animals (Box 1), noninvasive sampling is very attractive to field biologists because the method allows genetic studies of free-ranging animals without having to catch, handle or even observe them[3–5]. The source of DNA can be shed hairs, feces, urine, shed feathers, buccal cells from food wodges, snake skins, sloughed whale skin, eggshells and even skulls in owl pellets[6–9].

In spite of the great promise of these early studies, six or seven years have passed and there are still only a very few comprehensive studies using noninvasive genetic sampling[10–13] (Box 2). This is surprising in view of the high interest of field biologists in this sampling method and the large number of preliminary reports, technical notes and reviews that have already been published on the topic[14–18].

Two opposing viewpoints

Noninvasive sampling can exploit the full potential of DNA analysis

The dominant opinion three or four years ago was that noninvasive genetic sampling could exploit the full potential of DNA analysis and would, hopefully, provide the same information as DNA extracted from blood or tissue samples. This proved to be true for species identification[16] and for intraspecific phylogeographic studies[10,19] requiring PCR amplification of mitochondrial DNA (mtDNA). However, achieving the full potential of DNA markers also means that, by studying appropriate nuclear markers (usually microsatellites), the analysis of noninvasive genetic samples collected in the field can provide individual identification, relatedness estimates, pedigree reconstruction, sex identification, estimates of census and effective population size, and the level of genetic polymorphism within or between populations[17,20–22]. In response to the excitement surrounding noninvasive genetic sampling methods, many ecologists decided to stop taking blood or tissue samples, and, instead, to collect only hairs, feathers or feces; consequently, many studies based on noninvasive sampling were initiated.

Noninvasive sampling has serious limitations

All the limitations of noninvasive sampling methods result from either low DNA quantity, low DNA quality (i.e. degraded DNA), or poor extract quality (i.e. the presence of PCR inhibitors). The more obvious drawbacks are the risk of DNA contamination during the extraction and amplification process, and difficulties of amplifying long sequences because most DNA will be degraded into short fragments[23]. These two problems can be avoided by conforming to stringent guidelines to avoid contamination[24,25], and by choosing PCR primers that amplify short DNA markers (<200–300 base pairs). Unfortunately, some additional and unexpected difficulties have been revealed since 1995. When using hairs, feathers or feces from free-ranging animals, the total amount of DNA available for genetic typing can be very low, and is often in the picogram range. Under these circumstances, three results are possible when genotyping nuclear DNA microsatellite loci: (1) no PCR product is obtained, (2) a PCR product and incorrect genotype are obtained, or (3) a PCR product and correct genotype are obtained. Without repeating experiments or comparing results with DNA samples obtained from blood or tissue, it is difficult or impossible to differentiate between results (2) and (3). When an incorrect genotype is obtained, it is possible that only one allele of a heterozygous individual is detected[11,26–30]. This type of error, called 'allelic dropout', produces false homozygotes (Fig. 1) and can be explained by sampling stochasticity; namely, when pipetting template DNA in a very dilute DNA extract, sometimes only one of the two alleles is pipetted, amplified and detected[30].

Another kind of genotyping error has been detected when amplifying dinucleotide microsatellites: the production of amplification artefacts that can be misinterpreted as true alleles[30]. If such a

Box 1. The three different sampling methods

Destructive sampling: the animal is killed to obtain the tissues necessary for genetic analysis. This sampling strategy was used extensively for isozyme studies, and for mitochondrial DNA (mtDNA) analysis before the polymerase chain reaction (PCR) was discovered. Many researchers have now abandoned it.

Nondestructive sampling: the animal is often captured, and a biopsy or blood sample is taken invasively. Some invasive sampling strategies do not require catching the animal; for example, tissues can be obtained from whales and some other large mammals by using biopsy dart guns[34,35].

Noninvasive sampling: this term should be restricted to situations in which the source of the DNA is left behind by the animal and can be collected without having to catch or disturb the animal. In the literature, nondestructive sampling is often improperly considered as noninvasive. Catching a mammal (or a bird) and plucking a few hairs (or feathers) should not be considered as noninvasive, but rather as nondestructive.

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Box 2. Comprehensive studies using nuclear DNA

Kin selection, social structure and gene flow in chimpanzees (*Pan troglodytes*)[10]
To test hypotheses about social behavior and gene flow, Morin *et al.* collected hair samples from the sleeping nests of free-ranging chimpanzees. Using eight microsatellite loci on 36 individuals, males were shown to be significantly more related than females and to have a significant excess of homozygosity (relative to Hardy–Weinberg expectations), supporting the kin selection hypothesis for evolution of cooperation among males. Furthermore, this pioneer study analysed mitochondrial DNA (mtDNA) polymorphism from 66 chimpanzees to document long-distance dispersal and phylogeographic structure among three subspecies distributed across Africa.

Number, sex and home ranges of Pyrenean brown bears (*Ursus arctos*)[11]
By combining field data on track sizes with microsatellite and Y-chromosome data, we have shown that the population of Pyrenean brown bears consists of at least one yearling and three adult males and one adult female. Only 36 of 247 hair samples and 21 of 105 feces samples provided enough DNA for complete genetic typing at all polymorphic loci using the multiple-tube approach (Box 3). Because of the very low polymorphism (only six out of 24 loci were polymorphic with only two alleles), two individuals could not be resolved using the genetic data alone, but fortunately they exhibited different track sizes. This study demonstrates that a noninvasive genetic approach alone will sometimes not be sufficient to identify individuals in populations with reduced genetic variation.

Mark–recapture experiments using genetic tags[12]
A study of Canadian brown bears demonstrated that it is potentially feasible to estimate population census sizes from genetic tags, without capturing or even seeing the animals. Clumps of hairs were repeatedly collected on barbed wire around baits, and analysed using six highly polymorphic microsatellites. The multiple-tube approach (Box 3) was not necessary, because several hairs from the same clump were used in the DNA extractions to increase the amount of DNA and thus reduce genotyping errors. Nevertheless, two genotyping errors were detected by reanalysing each multilocus genotype that was identified from only a single sample and that differed by only one allele from other genotypes detected many times.

Estimating population size in coyotes (*Canis latrans*)[13]
During a two-week period, 651 carnivore-like feces were collected along six transects in the Santa Monica Mountains of California. After species and sex identification using mitochondrial DNA (mtDNA) and Y-chromosome typing, three highly variable microsatellites identified 30 unique genotypes out of a subsample of 115 feces. The population size was then estimated to be 38 (95% CI: 36–40) by a rarefaction analysis, and 41 (95% CI: 38–45) using a mark–recapture model. This study clearly outlines a general noninvasive method to census large mammals.

'false allele' is generated at a homozygous locus, then the individual would be incorrectly recorded as a heterozygote; if it occurs at a heterozygous locus, then the presence of three 'alleles' might allow the detection of the error[29]. The problem of false alleles was only detected three or four years after the introduction of noninvasive methods, perhaps because most studies dealt with primates and used PCR primers that could amplify human DNA. In these circumstances, false alleles might have been confused with sporadic contaminations by human DNA, and thus were not detected. These types of error occur in less than 5% of PCRs (Refs 29,30), but should not be disregarded as they can lead to erroneous genotyping.

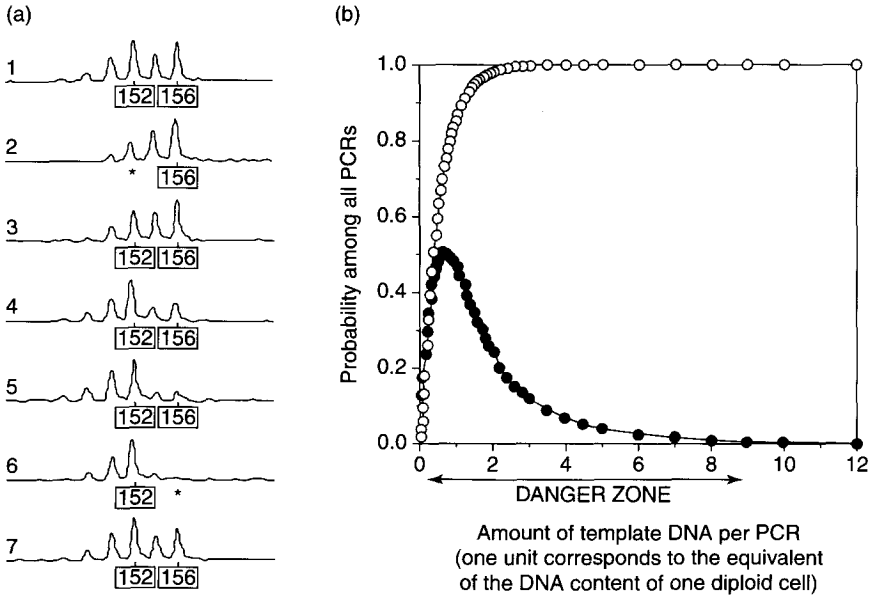


Fig. 1. Allelic dropout. (a) Genotyping experiments on a free-ranging brown bear (*Ursus arctos*) (from Ref. 11). The results of seven independent polymerase chain reaction (PCR) experiments (multiple-tube approach) at a dinucleotide microsatellite locus are shown. Aliquots of a single DNA extract of a shed hair collected in the field were used as template. This bear is a heterozygote with alleles 152 and 156. Allelic dropout occurs in PCRs 2 and 6 (indicated by the asterisk). (b) Probability among all PCRs of obtaining a PCR product (open circles) and allelic dropout (filled circles) according to the amount of template DNA when the PCR conditions allow the amplification of a single target molecule (recalculated from Ref. 30). The probability of obtaining a PCR product increases rapidly with the amount of template DNA, but the probability of allelic dropout decreases less rapidly. Consequently, there is a danger zone when the amount of template DNA is low. This danger zone is particularly insidious when the probability of obtaining a PCR product is close to 100% and the probability of obtaining an allelic dropout is still not negligible. It is very difficult to avoid the danger zone, because the small amount of template DNA used per PCR cannot be quantified precisely and, furthermore, DNA degradation can have the same effect as an extreme dilution.

It is clear that the possibility of obtaining genotyping errors can preclude the completion of a study, although the severity of the impact of such errors will depend on the scientific goal (Table 1). After considering the limitations of noninvasive genetic sampling, many questions arise. For example:

- Is it possible to avoid all these genotyping errors?
- Does the noninvasive sampling approach still represent a reliable alternative for studying the genetics of free-ranging animals?

The challenge of limiting the occurrence and impact of genotyping errors

There is more than one strategy for limiting the occurrence of genotyping errors. Indeed, each step of the genotyping process represents a technical challenge (Box 3). One potential strategy is to obtain more DNA by extracting more material. This solution is possible when using feces or hair tufts from the same animal; however, it can be risky to use many hairs in the same DNA extraction if there is a chance that they might originate from more than one individual. Also, using more material can increase the amount of PCR inhibitors in an extract, which can prevent genotyping even if sufficient quantities of DNA are obtained.

Alternatively, the quality of the samples can be greatly improved by collecting

Table 1. Effects of genotyping errors

Statistical methodology	Example of potential effects of errors	Severity of error
Genetic diversity	Allelic dropout can reduce the observed heterozygosity (H_o), potentially resulting in erroneous concerns about extinction risks caused by inbreeding, low N_e or low genetic variation.	Low–medium
Population structure, *F*-statistics and genetic distance	Erroneous estimates of allele frequencies might change estimates of F_{st}, migration rates (Nm), and phylogenies. Reduced H_o can generate a false Wahlund effect suggesting (erroneously) that substructure exists within the sample(s).	Low
Individual identification for population size estimation (*N*-minimum, *N*-census, N_e)	Genotyping errors might change the number of genotypes detected (*N*-minimum), or the estimate of population census size obtained via mark-recapture studies[a]. Artificial changes in allele frequencies might change estimates of N_e (Footnote b).	Low–medium
Individual identification for assessing relatedness and kinship (paternity/parentage)	Mis- or unassigned paternity could cause erroneous estimation of male reproductive success or mating-system biology. Allelic dropout could increase estimates of inbreeding (via reducing H_o).	High
Population assignment/admixture	Assignment of individuals to the wrong population of origin can cause erroneous estimates of migration rates, sex-biased dispersal, or even the erroneous prosecution of innocent hunters or collectors accused of poaching wild individuals from national parks.	Low–high

[a]From Ref. 41.
[b]From Ref. 42.

hairs, feathers or feces just after the animal leaves them behind. If the samples remain in the field for several weeks before collection, then the DNA could become more degraded and more difficult to amplify. In addition, the DNA will be degraded and give poor results if the method of preservation is not appropriate[23,31]. Thus, preservation methods must be carefully considered and tested. Another option is to multiplex (i.e. co-amplify) several loci during PCR, allowing more efficient use of limited amounts of DNA. However, this approach will require time-consuming adjustments that could be technically difficult or impossible when the quantity and/or quality of template DNA is very low.

The most conservative method of obtaining reliable genotypes from small quantities of DNA is to repeat each DNA amplification independently for each locus several times (multiple-tube approach[30,32]; Box 3). However, this solution is less desirable because it is time-consuming and more expensive than single-tube amplification.

Another potential improvement that is often overlooked is to choose the DNA markers more carefully[33]. First, by using trinucleotide or tetranucleotide microsatellites instead of dinucleotide microsatellites, the risk of obtaining false alleles is reduced. Furthermore, by using more informative markers (i.e. markers with a higher heterozygosity), the same information can be obtained with fewer loci (L. Waits *et al.*, unpublished). As a consequence, more template DNA will be available per PCR, and the risk of allelic dropout will be lower.

When is noninvasive genetic sampling appropriate?

Based on the limitations outlined here, it is clear that some questions cannot be assessed exclusively using noninvasive methodology, simply because the amount and/or quality of DNA available is too low to obtain reliable results. Consequently, researchers should not switch to noninvasive sampling without evaluating whether the question of interest can be solved using this approach. Unfortunately, the disciplines in which noninvasive methods are most needed, ethology and conservation biology, are also the disciplines in which the consequences of genotyping errors can be most serious (Table 1), or in which the application might be most difficult: that is, endangered species with low levels of heterozygosity.

Conducting an appropriate pilot study is the best way to assess whether the noninvasive approach is feasible (Box 4). However, even if the pilot study demonstrates that the noninvasive approach is appropriate, it is still not certain that this method represents the best choice. Indeed, if the multiple-tube approach[30,32] must be used, the total cost of the laboratory work can be five to ten times higher than when using blood or tissue samples. Thus, the main factors that must be weighed are the field constraints involved in capturing the animals and the additional laboratory costs of using noninvasive sampling. Clearly, there are some

Box 3. Major technical challenges and solutions

Sample preservation
It is important to limit the degradation of DNA before extraction. Usually, hairs or feathers are preserved dry, possibly with silica gel. Preservation methods for feces have been thoroughly investigated[23,31], and it appears that the use of desiccating silica gel beads, or a DMSO–EDTA–Tris–salt solution[36] (DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid) gives the best results.

DNA extraction
The classical phenol–chloroform DNA extraction usually gives poor results. Initially, therefore, DNA extraction was a limiting factor to the use of noninvasive sampling, but this technical difficulty has been largely overcome. The Chelex method[37] has proved to be efficient for hairs and feathers. The silica method, using either published protocols[4,38,39] or commercial kits (e.g. QIAamp Kit, Qiagen Inc.), is very effective for feces. There is substantial potential for further improvement in DNA extraction methods.

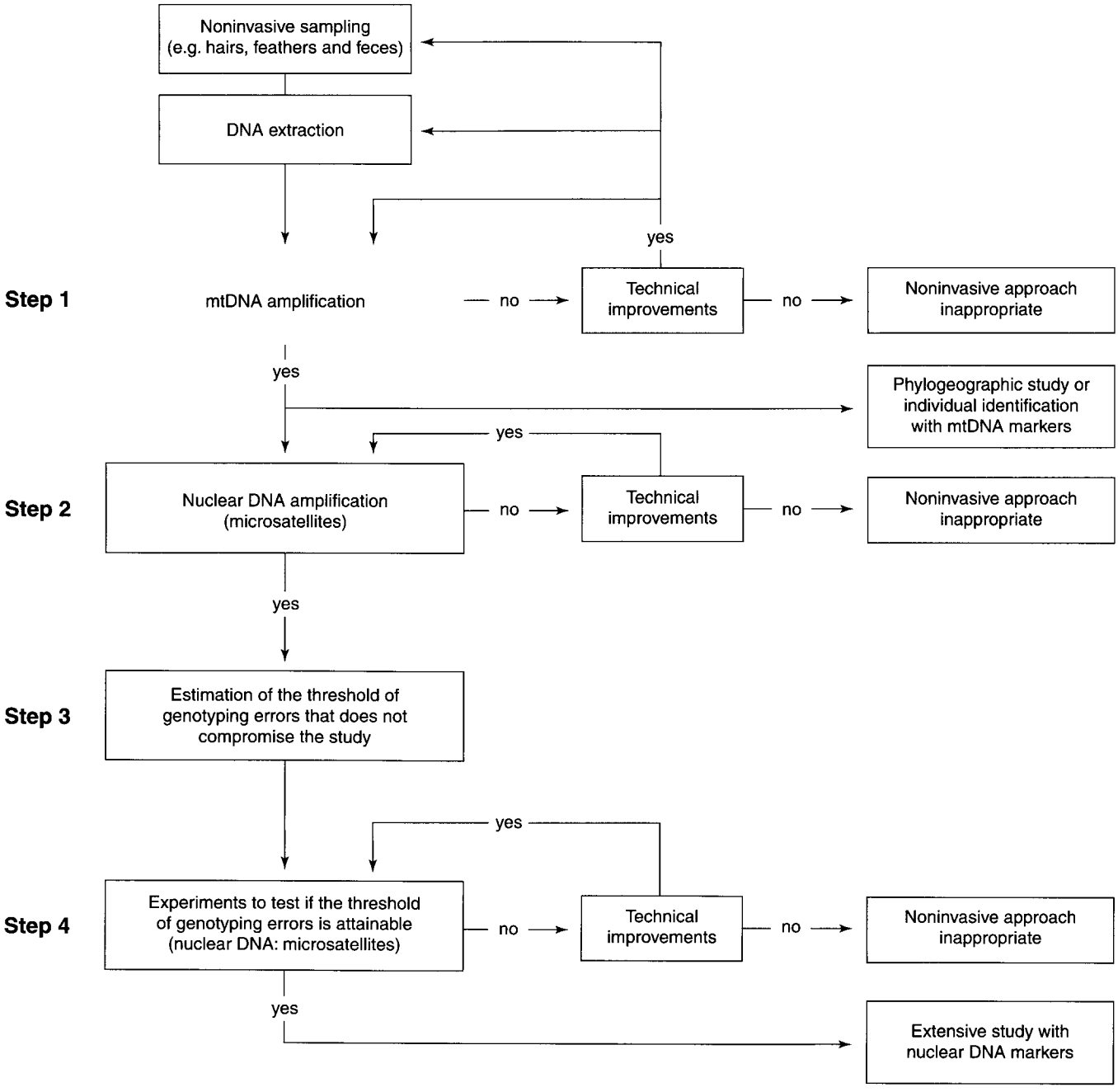
DNA amplification
Recent improvements in *Taq* polymerases that are active only after a 10-min incubation at 95°C (e.g. AmpliTaq Gold™, Perkin-Elmer) reduce non-target amplifications via a hot start polymerase chain reaction (PCR)[40] and thereby allow more PCR cycles without problems. This means that a single target molecule can be detected if the PCR conditions are optimized.

The multiple-tube approach
The purpose of this procedure is to provide reliable genotyping when using very dilute DNA samples. It consists of repeating PCR experiments using aliquots of the same DNA extract[30,32]. The genotype is then deduced by analysing the whole set of experiments (Fig. 1). The multiple-tube approach is the best method to detect and monitor for the three possible errors: allelic dropout, false alleles and sporadic contamination.

Contamination
Methods to avoid contamination are the same as those used in ancient DNA studies. These include:

- Physical separation of the laboratories where pre- and post-PCR experiments are carried out.
- Avoiding handling concentrated DNA extracts in the pre-PCR room.
- Using pipettes dedicated to noninvasive studies and aerosol-resistant pipette tips.
- Continuously monitoring all reagents for DNA contamination (via negative PCR controls).

Box 4. A pilot study to assess feasibility



(Online: Fig I)

Flow chart illustrating the four steps of the pilot study that ought to be carried out before any extensive study based on noninvasive genetic sampling is undertaken. [Note that, in steps 2 and 4, the boxes labeled 'technical improvements' could lead back to boxes titled 'noninvasive sampling' or 'DNA extraction', as in step 1.]

Step 1 consists of amplifying mitochondrial DNA (mtDNA) using genomic DNA that has been extracted using the noninvasive approach. Because mtDNA is present in far higher copy numbers per cell than nuclear DNA, failure to amplify mtDNA indicates that attempts to amplify nuclear loci will be futile.

Step 2 is a repeat of the same type of experiment as in step 1, but using at least one nuclear DNA locus. If nuclear DNA can be amplified without difficulty, then it is possible to proceed to steps 3 and 4. If step 2 fails, then the noninvasive approach is not appropriate, unless technical improvements are achieved.

The purpose of step 3 is to estimate the maximum genotyping error rate that is compatible with the level of confidence required for the question(s) being addressed. This threshold of genotyping error can be estimated either by using analytical equations or by computer simulations[33].

Step 4 is required to determine if it is technically possible to achieve the threshold of genotyping errors estimated in step 3. As the error rate that must be quantified can be very low, the number of amplification experiments to be conducted can accordingly be very large.

situations in which capturing the individuals of interest is not conceivable: for example, in the case of a small endangered population, or in behavioral studies where capturing animals would disturb the system. In these conditions, the noninvasive sampling approach is the only solution.

Another factor to consider is that noninvasive sampling can increase the number of animals that can be sampled in secretive species, and thus make feasible the estimation of important population parameters. Also, the use of feces can provide additional information on

diet, pathogens and reproductive status[17]. When it is possible but expensive to capture the animals, a more thorough cost analysis should be done. Cost–benefit analyses should also weigh the benefits of gaining additional information (e.g. age structure and body condition) that can be obtained only by capturing individuals. One final consideration is that the quantities of DNA obtained using noninvasive sampling are generally not large enough for more than one genetic study. Thus, very little, if any, DNA will remain for analysis in future studies, or future studies might require genetic data that cannot be obtained from the low quantity and quality of the DNA obtained by noninvasive genetic sampling.

The future of noninvasive genetic sampling

After a very enthusiastic beginning, it appeared that technical problems, particularly genotyping errors, would limit the usefulness of noninvasive approaches. However, there is still room for optimism. First, the causes of these genotyping errors are now understood and, accordingly, some viable solutions have been proposed. Second, a more rigorous approach, including an extensive pilot study, can assess the confidence level of the final results by quantifying the genotyping error rate. However, one of the remaining difficulties is that each study is unique, and thus the results from a pilot study on one species cannot be transferred to another species or even to another population with different heterozygosity or sample quality. Fortunately, many of the difficulties of noninvasive sampling are being overcome with improved and rigorous methodology, albeit an approach that currently requires substantially more time and expense in the laboratory.

Acknowledgements

We thank J. Bouvet, L. Gielly, C. Dubois-Paganon, V. Curri, S. Luche, H. Mattock, C. Beckhelling, S. Griffin, E. Uhrès, S. Questiau, N. Escaravage, V. Manceau, B. Goossens, M-P. Biju-Duval, O. Disson and J-J. Camarra for helpful discussions on noninvasive genetic sampling techniques. G.L. was supported by a grant from NSF/NATO.

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