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ADVANCES AND DIFFICULTIES OF MOLECULAR TOOLS FOR CARNIVORE CONSERVATION IN THE TROPICS

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ABSTRACT. — Genetic data are widely used to test ecological and evolutionary hypotheses that can be applicable to the conservation of wild carnivore populations. We present the most typical information derived from genetic data such as species identification, sex determination, and individual identification, and we address the practicalities of collection and preservation of the most common non-invasive genetic samples. A review of the most widely used molecular markers (mitochondrial DNA, microsatellites, SNPs) and the latest technological developments (whole genome amplification, next generation sequencing) for genetic analysis of wild populations is also included, as well as a few tools for DNA analysis. Finally, we recommend a series of measures to increase the potential success of a genetic study in a wild carnivore species.

KEY WORDS. — carnivore, conservation genetics, molecular markers, non-invasive sampling, population genetics

INTRODUCTION

Many species of carnivores are endangered, elusive and rare, occupy inaccessible areas, and occur at low densities. Direct individual count and behavioural observations can be prohibitive and/or intricate. Therefore, specific tools have been used to study carnivores, such as satellite tracking and radio telemetry, remote sensing, camera trapping, modeling, and molecular genetics (Gottelli et al., 1994; Soisalo & Cavalcanti, 2006; Zhan et al., 2006; Claridge et al., 2009; Wilting et al., 2010). In particular, genetic information has become an integral part of species' management and conservation. However, the most challenging task is the acquisition of adequate samples from wild carnivores that will provide good quality DNA to collect such genetic information. Realistically, obtaining suitable samples such as hair and faeces from wild carnivores in a tropical forest is challenging. We outline the challenges that a carnivore geneticist will face along with the types of genetic samples that can be used in carnivore non-invasive genetics. We also discuss the best ways of sampling and preserving samples, as well as the genetic markers and the software available for analyses. Finally, we provide a list of recommendations for conducting non-invasive genetic studies.

WHAT CAN WE DETECT IN A CARNIVORE GENETIC SAMPLING?

Genetic sampling and laboratory techniques, as well as statistical methods have improved in the recent years and continue to do so. Genetic markers contribute to the conservation of species by aiding forensics and legal actions (Manel et al., 2002) and by resolving many aspects of species biology and ecology (Table 1). In particular, species and individual identifications, as well as gender determination, can be detected in carnivore genetic sampling and can help wildlife managers conserve carnivore species in protected and unprotected areas:

1. **Species identification**: The best way to ascertain a carnivore species is to sequence a region of the mitochondrial DNA, especially the 16S rRNA region (see Hoelzel & Green, 1992; Mills et al., 2000), and compare

Table 1. Applications of different genetic markers and references.

Applications	References
Identification of sympatric species	Davison et al., 2002; Dalén et al., 2004; Reed et al., 2004; Lopez-Giraldez et al., 2005; Nagata et al., 2005
Assessment of the distribution of a species through population census (capture/recapture)	Kohn et al., 1999; Schwartz et al., 1999; Mills et al., 2000 for a review; Prugh et al., 2005
Mapping of distribution and abundance of species in a specific landscape	Cushman et al., 2006; Cushman et al., 2009
Estimation of population size abundance	Kohn et al., 1999; Ernest et al., 2000; Mills et al., 2000; Wilson et al., 2003; Bellemain et al., 2005
Estimation of effective population size	Creel et al., 2003; Bellemain et al., 2005
Exclusion and assignment of parentage, relatedness and kinship patterns	Blundell et al., 2004; Wagner et al., 2007
Identification of the individuals' sex and estimation of the sex ratio in a population	Reed et al., 1997; Taberlet et al., 1997; Dallas et al., 2000; Hedmark et al., 2004; Pilgrim et al., 2005; Seddon 2005; Sugimoto et al., 2006
Inference of phylogeographical relationships	Taberlet & Bouvet, 2004
Measure of dispersal patterns and individual movements	Taberlet et al., 1997
Inference of population structure through population assignment	Randi & Lucchini, 2002
Estimation of the degree of isolation of subpopulations	Stow et al., 2001; Cushman et al., 2006
Monitoring the changes in genetic variability	Lachish et al., 2010
Document disease status and perform evolutionary studies of viral genomes from faecal samples	Steinel et al., 2000
Monitoring of hybridisation and hybridisation effects	Adams et al., 2003; Schwartz et al., 2004
Identification of dietary items	Farrell et al., 2000; Fedriani & Kohn, 2001

the sequences to those reported in a genetic database such as GenBank. DNA barcoding, which uses short and standardised DNA sequences (again typically from a mitochondrial gene), can also be used to identify known species and to discover new species (Herbert et al., 2004; Savolainen et al., 2005). A restriction enzymes technique such as Restriction Fragment Length Polymorphism (RFLP) can also be used (see Paxinos et al., 1997; Mills et al., 2000; Dalén et al., 2004). Restriction maps can be generated showing potential restriction sites with a corresponding enzyme. These restriction sites can then be tested using known tissue samples from different carnivore species, and then used to identify species from non-invasive samples (faeces or hair) collected in the field.

2. **Sex determination**: For every survey carried out for management of a particular carnivore species, it is important to determine the sex ratio in the population of that species to know whether there is a potential breeding population within the area of inference. A typical gene used to identify sex in carnivores is the SRY gene (the testes determining factor), which is present only on the male Y chromosome (Kurose et al., 2005). When a sample from a male is analysed with SRY-specific primers, only one specific product ("band") should be amplified and detected on an electrophoresis gel; if the sample belongs to a female, no bands should be present. Unfortunately, a negative result (i.e., no band) can mean either that the sample originated from a female, or that it was of low quality and did not contain adequate amounts of DNA. To determine whether a given sample is of low quality, co-amplification of a single-copy nuclear locus of the appropriate size, which amplifies regardless of gender, must be performed along with the amplification of the SRY gene. Failure of the nuclear locus to amplify would mean the DNA was of poor quality and the results must be discarded. It is recommended to carry out multiple repeats (at least three) for accuracy (Kurose et al., 2005; Pagès et al., 2009). A second method for identifying sex is to sequence a gene in the zinc-finger region (ZF) of the X and Y chromosomes. In felids, the ZFY (male) band has a three-base pair deletion compared to the ZFX. Thus, a male carnivore species will show two bands on an electrophoresis gel (i.e., a band for the X chromosome and a band for the Y chromosome, which vary in length because of the deletion on Y), whereas a female will only show one band (i.e., females have two X chromosomes, with no length variants; see Pilgrim et al., 2005). Finally, the amelogenin gene, which codes for proteins found in tooth enamel, has a 20-base pair deletion on the Y chromosome of some species. Therefore, it provides a possibility of determining the gender for felids (Pilgrim et al., 2005) as well as ursids (Poole et al., 2001; Yamamoto et al., 2002).

3. **Individual identification**: The most common markers used to identify individuals in a population are nuclear markers: microsatellites. The number of microsatellites necessary for individual identification depends on the amount and distribution of genetic variation in the species, which is characterised by the probability of identity (Waits

et al., 2001). This in fact depends on the population and its history, whether it is a small and inbred population (with little variability) or a large outbred population. Microsatellites have not been identified and characterised for every species of carnivores. One alternative is to test suitable microsatellite primers that have already been developed for a closely related species. The other alternative is to develop microsatellites specifically for the species or population of interest. Today, a number of commercial companies can quickly develop variable microsatellites for a target species at a relatively reasonable cost (US$10,000–US$15,000). Once sufficient power to discriminate between individuals is achieved, the resulting microsatellite genotypes can be compared to determine the number of unique individuals. When employing microsatellites to identify individuals with non-invasively collected genetic samples, it is important to minimise and quantify the error rate (Taberlet et al., 1996). Repeated amplifications using several independent DNA extractions (see Navidi et al., 1992; Taberlet et al., 1996; Goossens et al., 1998; Taberlet et al., 1999 for a review; Goossens et al., 2000) are a minimum requirement. Software such as GIMLET can assist with identification of false homozygotes and false alleles by comparing the repeated genotypes and the associated consensus genotype for each sample (Valière, 2002).

WHAT KIND OF SAMPLES CAN WE GET IN A TROPICAL FOREST?

DNA samples that have been used to study wild populations of carnivores include hairs (Taberlet et al., 1997; Ruell & Crooks, 2010) and faeces (Kohn et al., 1999; Ernest et al., 2000; Davison et al., 2002; Frantz et al., 2003; Pires & Fernandez, 2003; Flagstad et al., 2004; Hung et al., 2004; Prugh et al., 2005). Other sources of DNA for carnivores can be urine (Valière & Taberlet, 2000; Hedmark et al., 2004), bones (i.e., museum samples), teeth (Wandeler et al., 2003) but these samples are very uncommon in the tropics due to rapid degradation.

Hair. — Plucked hairs with root material are the best source of hair DNA, providing adequate storing conditions are used. Hair samples without follicles can provide positive but unreliable DNA. One advantage of hair over faeces is that hair contains fewer chemical inhibitors that could restrict amplification of DNA (Jeffery et al., 2007). Another advantage is that contamination from other DNA sources (e.g., prey DNA found in scat) are minimised with hair. However, fewer cells and therefore less DNA are generally available in a hair sample than a faecal sample. Prior to launching a survey, it is highly advisable to conduct a pilot study to determine the rate of success of obtaining DNA from the hair of the target species under normal survey conditions. For example, it is strongly recommended to collect more than 10 hairs per individual (see Goossens et al., 1998) because the large variation in DNA amplification success due to factors such as the morphological characteristics of the species' hair, the environmental conditions under which the sample is collected, storage and laboratory methods (Jeffery et al., 2007), and sometimes social characteristics of the species (Karamanlidis et al., 2010).

Different methods of collecting fresh hair samples from wild carnivores have been described. Hair traps based on barbed wire around trees and sticky tape can be extremely useful for carnivores such as bears, canids, and felids (Belant, 2003; Weaver et al., 2005; Bremner-Harrison et al., 2006; Zielinski et al., 2006; Long et al., 2007a; Garcia-Alaniz et al., 2010).

Faeces. — Another source of DNA is epithelial material from the digestive tract, which is found in and around the surface of faecal material. In general, faecal samples are large enough to allow multiple extractions and this presents an important advantage compared to the number of extractions and the amount of DNA that can be extracted from hairs of a single individual. However, the greatest problem with faecal analyses is the presence of chemical inhibitors that could restrict the amplification of DNA. Amounts and quality of faecal DNA are known to vary by species, temperature at time of collection, age, season (dry or wet seasons), preservation method, species diet, storage time, and extraction protocol (Murphy et al., 2002; Maudet et al., 2002; Piggott & Taylor, 2003; Nsubuga et al., 2004). Another problem is co-amplification of DNA from prey species (Chaves et al., 2012). One recent technique based on next-generation sequencing can help identifying the different preys in the faeces of a carnivore species and distinguish the DNA of these preys from the host (Pompanon et al., 2012; Shehzad et al., 2012a, 2012b). Finding faecal samples of carnivores in a tropical forest would probably be the most challenging part of a genetic study using non-invasive sampling. Opportunistic findings (along transects) are likely to be time-consuming and ineffective. Probably the best method would be to use scat detection dogs (see Smith et al., 2003; Wasser et al., 2004; Smith et al., 2005; Long et al., 2007b), if canines are allowed into areas of the species of interest.

Other samples. — There has been relatively little study of the success of obtaining DNA from urine, regurgitates, saliva, or menstrual blood. These materials would be extremely difficult to collect in a tropical forest and would provide lower quality DNA compared to hair and faeces. Finally, a few studies have used carnivore scent marks as sources for DNA. Brown hyena (*Hyaena brunnea*) scent marks, stored in absolute ethanol, were tested for their potential to provide DNA, which was extracted using a modified salt-chloroform method (Malherbe et al., 2009). Scent marks secreted by giant pandas (*Ailuropoda melanoleuca*) provided enough DNA to amplify the mitochondrial D-loop, the cytochrome b gene and the Thr tRNA gene regions (Ding et al., 1998).

SAMPLE COLLECTION AND PRESERVATION

It is important to maximise reliable genetic analyses from non-invasive samples, and therefore to minimise contamination and degradation of the samples during and after collection. It is also important to know that non-invasive samples such as

hair or faeces will begin to degrade immediately after being deposited by the animal, and depending on field conditions (especially tropical climates), degradation can be extremely rapid. For example, faecal samples may not persist for more than a few days under tropical conditions or in places where dung beetles and other scavengers will rapidly decompose the faeces. Bear in mind that several factors can influence the quality of your samples and consequently, the quality of DNA that can be extracted: 1) UV radiation: samples must be protected from the sun as UV radiation can degrade DNA; 2) Moisture: plastic bags hold moisture; consider using silica gel to allow dessiccation of the samples; 3) Age: the fresher samples are, the higher quality DNA; 4) Avoid contamination: use a separate container for each sample, sterilise the tools between each collection and avoid containers that can leak (e.g., use parafilm to seal vials); and 5) Use the appropriate reagent to preserve samples (e.g., ethanol, silica gel, RNA later).

CONTAMINATION

Contamination is probably the most important factor to consider during sample collection and processing. Contamination can occur in the field or laboratory and can be a major concern for non-invasive sampling studies. Considering that the target sample may comprise only a few cells at the end of a hair or at the surface of a faecal sample, it is important to limit contact with material that can contaminate the target sample. It is highly recommended to use latex gloves and sterile mechanical devices (e.g., tweezers, wooden picks) for handling those samples in the field. Gloves should be changed between the handling of different samples, and mechanical devices should be sterilised with ethanol and a lighter or replaced between samples. These precautions can be difficult to follow in a tropical (humid) environment (especially wearing gloves), but it is extremely important to adopt sterile procedures when handling non-invasive samples. The success of any non-invasive genetic study can depend on these basic rules.

In the laboratory, it is recommended to have separate facilities for storing and extracting DNA from non-invasive samples. Bleaching and UV irradiation of benches are also compulsory. Finally, it is very important to run a negative control (e.g., samples comprising distilled water only) to detect laboratory contamination.

LONG-TERM STORING OF GENETIC SAMPLES

It is extremely challenging to preserve samples such as hair and faeces. However, it is very important to properly plan storage and label samples as it will ensure their integrity. If samples are properly stored and you can avoid hydrolysis, oxidation, alkylation, UV radiation, and physical cleavage through freeze-thaw cycles, DNA can persist for many years. Roon et al. (2005) evaluated optimal storage methods and DNA degradation rates for brown bear hair samples. Samples were preserved using silica desiccation and –20°C freezing over a 1-year period. Amplification success rates decreased significantly after six months, regardless of storage method. It is therefore important to minimise delays between hair collection and extraction to maximise amplification success rate. However, hair samples are usually stored in paper envelopes, since plastic bags produce static that make hair manipulation difficult and increase contamination risks. However, paper envelopes can retain moisture, which can promote bacterial growth and DNA degradation.

For faecal samples, different storage methods have been tested for several species. It is vital that DNA degradation by nucleases is minimised as much as possible. Storage methods include dehydrating samples by air-drying (Farrell et al., 2000), silica gel beads drying, freezing at –20°C (Ernest et al., 2000), ethanol treatment, or saturating samples in a buffer containing high concentrations of salts or other chemicals interfering with enzymes (e.g., DETs buffer, see Piggott & Taylor, 2003). Another method that seems to produce reliable results involves soaking samples in ethanol followed by desiccation with silica (Roeder et al., 2004). While samples stored in silica showed the lowest DNA concentration, the two-step method yielded significantly more DNA in high quality samples. Murphy et al. (2002) tested five preservation methods on brown bear faeces (90% ethanol, DETs buffer, silica-dried, oven-dried then stored at room temperature, and oven-drived stored at –20°C) at four time intervals (one week, one month, three months, and six months) for both mtDNA and nDNA. The ethanol-preserved samples had the highest success rates for both mtDNA and nDNA. These authors recommended preservation of faecal samples in 90% ethanol when feasible and the drying method when collecting in remote field conditions. In a previous study, Murphy et al. (2000) evaluated four drying methods for brown bear faces, with the freeze-drying and oven drying producing the best amplification rates. A recent tissue storage reagent, called RNA*later*® (Ambion, Inc.), has been successfully used to store faecal samples in several studies (Johnson et al., 2007; Vlcková et al., 2012). However, it is a very expensive reagent and can be difficult to procure in tropical countries. Discrepancies between studies are likely due to factors relating to the species (e.g., omnivores versus carnivores, species with high-lipid versus low-lipid diets), environmental conditions (e.g., mesic versus xeric, many freeze-thaw cycles versus constant cold), field and laboratory protocols (e.g., duration of storage, speed of sample drying, laboratory extraction technique, dessication protocols), and study objectives (e.g., individual versus species identification). Piggott & Taylor (2003) noted an interaction between storage method and extraction technique in the laboratory (i.e., certain extraction techniques performed better with certain storage methods, and vice versa). These results strongly support conducting a pilot study to explore the performance of various storage and extraction techniques for the species of interest.

DNA EXTRACTION

Hair. — The most popular method for extracting DNA from hairs is the Chelex-100® and proteinase K method developed by Walsh et al. (1991). However, Vigilant (1999) obtained better results using Taq polymerase PCR buffer as the extraction buffer. In our experience, using PCR buffer, water and proteinase K in a small extraction volume works very well for shed hairs (Goossens et al., 2005).

Faeces. — Cells containing DNA are not uniformly spread throughout faeces, and two or three extracts should be made per sample (see Goossens et al., 2000). It is also important to use a method that involves fewer steps and sample transfers, although the removal of substances that may inhibit PCRs usually requires repeated purification processes involving several centrifugation steps. We recommend using the QIAamp Stool mini kit (QIAGEN), which has given reliable results in carnivores (Bonin et al., 2004; Hedmark et al., 2004). Other methods have been described including: silica-based method (Boom et al., 1990), magnetic beads (Flagstad et al., 1999), diatomaceous earth method (Gerloff et al., 1995), Chelex-100® (Walsh et al., 1991), and surface wash followed by spin column purification (Piggott & Taylor, 2003). We strongly recommend a pilot study as one extraction technique may work for some species but may not for others. Optimal extraction methods will depend on field conditions, location, season, size, and age of the samples (see Taberlet et al., 1999; Piggott, 2004).

MOLECULAR MARKERS

The choice of a molecular marker will depend on the question of interest. Each marker has its own appropriate use and the costs and difficulty of genetic typing must be taken into consideration. The two most commonly used markers in non-invasive genetics are mitochondrial DNA and nuclear microsatellites. Mitochondrial DNA (mtDNA) is found in the mitochondrion in many copies per cell. It is inherited clonally through the maternal line only and its DNA sequences evolve approximately an order of magnitude more rapidly than chromosomal DNA in the nucleus of the cell. Although mtDNA evolves rapidly, by accumulating 'point' mutations in the DNA bases, there are numerous regions sufficiently conserved to provide comparisons and enable markers to be developed across closely related species (Kocher et al., 1989). The primary usage of mtDNA is in phylogeny (Thomas et al., 1989) and phylogeography (Avise et al., 1987; Tomasik & Cook, 2005), and identifying genetically distinct units for conservation (Moritz, 1994; Sato et al., 2009), but it can also be used to examine genetic distinctiveness within populations (Waits et al., 2008) although its behaviour (as a maternally inherited marker) is strongly affected by patterns of female philopatry and dispersal. It can also be used to identify species hybridisation (see Pilgrim et al., 1998) and differentiate patterns of male and female gene flow (Schubert et al., 2011).

Microsatellites are found mainly in the nuclear chromosomes and are present in thousands of copies scattered throughout the genome (Li et al., 2002). They are highly variable, with up to 20 alleles per locus being common and heterozygosity at any given locus can commonly be up to 80%. The application of 10–20 of these loci can provide the researcher with both an individual-specific genotype and an estimate of the genetic similarity between individuals. Microsatellites are commonly used in studies of paternity and social structure and are the tools of choice in behavioural studies (Kays et al., 2000). Microsatellite primers can produce polymorphic markers in related species, but cross-species utility does have its evolutionary limits, and you may need to isolate, characterise and develop markers in your study species. Before embarking in expensive and time-consuming cloning, look into GenBank® (http://www.ncbi.nlm.nih.gov/genbank/) and scientific journals such as Molecular Ecology Resources and Conservation Genetics Resources for published markers.

Another type of genetic marker, the single nucleotide polymorphisms (SNPs), may ultimately replace microsatellites and become the marker of choice to study the ecology and conservation of wild populations as they have the advantage of better conforming to well-characterised models of evolution and allow access to variability across the whole genome (Hinds et al., 2005). Although studies using SNPs are uncommon, Seddon et al. (2005) addressed ecological and conservation issues in re-colonised Scandinavian and Finnish wolf populations using 24 SNP loci. These loci were able to differentiate individual wolves and differentiate populations using assignment tests. Furthermore, SNPs are believed to provide data with absolute scores (i.e., sequence data that is not subject to differences among laboratories and sequencing platforms), thus facilitating international collaboration between researchers studying the same species. To date, the expense of developing SNPs, and questions regarding error rates, ascertainment biases, their effectiveness with non-invasive samples, and within-population variability, have limited their use in conservation genetics (Morin et al., 2004; but see Seddon et al., 2005).

Amplified fragment length polymorphisms (AFLPs) are dominant markers that can be used in parentage, population assignment, gene flow, and migration, although they are less adequate for reconstructing past events and historic patterns of variation (Bensch & Akesson, 2005). However, their use in non-invasive analysis is likely to be limited due to the requirement for quite large amounts of template DNA and large fragment sizes.

Recent innovations such as multiplex PCR and whole genome amplification and Next Generation Sequencing (NGS) technologies are providing new tools for genetic analysis of wild populations. Multiplex PCR (Henegariu et al., 1997) systems for comparative genotyping are well developed in human forensics and are now being used in wild carnivores (see Nonaka et al., 2009; Roques et al., 2010). Piggott et al. (2004) developed a multiplex pre-amplification method

to improve microsatellite amplification and error rates when using faecal DNA. QIAGEN has developed a multiplex kit, which we have commonly used for genotyping of DNA extracted from non-invasive samples such as faeces and hair of several mammal species (Goossens et al., 2005).

The recent establishment of whole-genome amplification such as multiple displacement amplification (MDA; Dean et al., 2002) promises to revolutionise non-invasive genetic analysis since in principle it allows the production of large quantities of whole-genomic DNA from minute sources, such as are routinely produced from non-invasive studies. MDA allows the generation of thousands of copies of whole genomes of up to 10 kilobase pairs (kb) in length (Dean et al., 2002). The isothermal MDA reaction utilises the highly processive bacteriophage phi29 DNA polymerase and its DNA strand-displacing activity. In the MDA reaction, random hexamer primers annealed to denatured genomic DNA are extended by the phi29 DNA polymerase to form products up to 100 kb. As the DNA polymerase encounters another newly synthesised DNA strand downstream, it displaces it and thus creates a new single-stranded DNA template for priming. Strand displacement leads to hyperbranched primer extension reactions that may yield milligram amounts of DNA product from just a few nanograms of genomic DNA. Owing to its 3'–5' proofreading activity, the fidelity of the phi29 DNA polymerase is very high with an error rate of $<10^{-6}$ (Esteban et al., 1993), which in turn requires exonuclease-protected primers to achieve a high yield. As the reaction involves no thermal cycling and high molecular weight copies of genomic DNA are produced, the genomic coverage of MDA products is higher than that of the PCR-based whole-genome amplification methods, degenerate oligonucleotide-primed PCR (DOP-PCR) and primer extension preamplification (PEP; Dean et al., 2002). The whole-genome amplification has been used in Felids such as jaguarondi and ocelot (Janecka et al., 2006).

Opposed to the Sanger method, newer sequencing technologies are referred to as next generation sequencing (NGS). NGS represent a variety of strategies that rely on a combination of template preparation, sequencing and imaging, and genome alignment and assembly methods (for full reviews see Shendure & Ji, 2008; Metzker, 2009). Common ground for these technologies is the parallel DNA sequencing platforms whose major advantage is the ability to produce massive volumes of data, even as much as one billion short reads per instrument run (Metzker, 2009). An interesting utility of these new sequencing tools for population genetics is the possibility of discovery, validation, and assessment of genetic markers through a single sequencing step and thus genotyping thousands to hundreds of thousands of markers in tens to hundreds of individuals across almost any genome, even in populations with little or no available genetic information (Davey et al., 2011). A crucial step to the success of these protocols is employment of high-quality genomic DNA, with no RNA contaminants or with DNA from other species (Davey et al., 2011). However, Perry et al. (2010) recently described a modified DNA capture protocol to facilitate efficient and highly accurate re-sequencing of megabases of specified nuclear genomic regions from faecal DNA samples that could open the door to the application of genomic-level analyses of DNA from non-invasive sources (Kohn, 2010).

DNA ANALYSIS TOOLS

Numerous software packages are available for various analyses for genetic studies. We provide sources for several commonly-used software programs used to address important questions in conservation (Table 2).

CONCLUSIONS

Although non-invasive analysis is becoming the only acceptable way to retrieve genetic data from many endangered species, and original problems with reliability are being rapidly resolved and technical innovations such as multiplex PCR kits and whole-genome amplification may soon make this type of analysis the norm, appropriate and thorough experimental designs are critical before embarking on genetic studies of tropical carnivores. We strongly recommend the following steps to increase success in genetic studies of wild carnivores: 1) Define clearly the objectives of your study (what do you want to achieve and is it feasible in the time allocated for your study?); 2) Identify the genetic markers that you will need (i.e., if you use microsatellites, check available markers published), and determine the number of samples that you need to collect in the field; 3) Design a proper sampling methodology that will provide appropriate data for your study's objectives, and as such determine your sample size (i.e., at least 30 unrelated individuals per population for any population genetic study); 4) Collect the right data in the field that will support your objectives (location of the sample, forest type, geographic information); 5) Select the right sample preservation method (check the literature or test in a pilot study, if necessary) and pay particular attention to the labeling of your samples; 6) Carry out a pilot study on the effect of collection season and age of the samples on the reliability of microsatellite genotyping; 7) During collection, try to sample the same faeces at least twice, and always sample the outer layer of the faeces (mucus); 8) Select the right DNA extraction method (check the literature or test it if necessary—pilot study) and extract early (almost all studies that have examined sample quality in relation to time have demonstrated a deterioration of DNA [see Roon et al., 2003]); 9) Select the right loci and the right number of loci for your study; 10) Test the effects of genotyping errors and multi-tubes approach using software such as GEMINI (Valière et al., 2002), you need to minimise genotyping error as much as possible; and finally 11) Select the appropriate analytical methods given your sampling methods and objectives and do not hesitate to consult with experts in genetics and statistics.

To conclude, the study of wild carnivores through non-invasive genetic methods in the tropics is extremely challenging. Not intending to be discouraging, it is our strongest opinion that even following the eleven recommendations described above, a successful study cannot be guaranteed. Thus, utmost

Table 2. Software packages available for various genetic analyses and used to address important questions in conservation genetics. References and/or URL are provided.

SoftwareApplication	Reference and/or URL	
STRUCTURE	population genetics	Pritchard et al., 2000; http://pritch.bsd.uchicago.edu/structure.html
Alleles in Space	spatial and genetic information	Miller et al., 2005; http://www.marksgeneticsoftware.net/AISInfo.htm
GENELAND	population structure	Guillot et al., 2005; http://www2.imm.dtu.dk/~gigu/Geneland/
DnaSP	DNA sequences analysis	Rozas & Rozas, 1999; http://www.ub.edu/dnasp/
GENETIX	population genetics	Belkhir et al., 1996-2004; http://kimura.univ-montp2.fr/genetix/
GENEPOP	population genetics	Raymond & Rousset, 1995; http://genepop.curtin.edu.au/
ARLEQUIN	population genetics	Excoffier et al., 2005; http://cmpg.unibe.ch/software/arlequin35/
LDNe	linkage disequilibrium	Waples & Do, 2008; http://tomato.biol.trinity.edu/programs/index.php/LDNe
DROPOUT	genotyping error	McKelvey & Schwartz, 2005; http://www.rmrs.nau.edu/wildlife/genetics/software.php
MARK	mark-capture-recapture	http://www.phidot.org/software/mark/
PRESENCE	probability of occupation	http://137.227.242.23/software/doc/presence/presence.html
MICROCHECKER	null alleles and scoring errors	van Oosterhout et al., 2004; http://www.microchecker.hull.ac.uk/
GENECAP	capture-recapture estimation	Wilberg & Dreher, 2004; http://wilberglab.cbl.umces.edu/downloads.html
RELATEDNESS	pairwise relatedness	Queller & Goodnight, 1989; http://www.gsoftnet.us/GSoft.html
KINSHIP	pedigree relationships	Goodnight & Queller, 1999; http://www.gsoftnet.us/GSoft.html
DELRIOUS	relatedness	Stone & Björklund, 2001
IDENTIX	relatedness	Belkhir et al., 2002

considerations should be taken when choosing molecular methods as a tool for carnivore conservation.

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