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Remote collection of animal DNA and its applications in conservation management and understanding the population biology of rare and cryptic species

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Abstract. Obtaining useful information about elusive or endangered species can be logistically difficult, particularly if relying entirely on field signs such as hair, feathers or faeces. However, recent developments in molecular technology add substantially to the utility of such 'non-invasive' samples, which provide a source of DNA that can be used to identify not only species but also individuals and their gender. This provides great potential to improve the accuracy of abundance estimates and determine behavioural parameters, such as home-range size, individual habitat and dietary preferences, and even some forms of social interaction. Non-invasive samples can also be a useful alternative to blood or tissue samples (the collection of which traditionally has required trapping of animals) as genetic material for applications such as relatedness, population genetic and phylogenetic analyses. Despite the huge potential of non-invasive genetic sampling, the current technology does have limitations. The low quantity and quality of DNA often obtained from such sources results in an increased risk of genotyping errors, which may lead to incorrect inferences, particularly false identification of individuals. Appropriate precautions and pilot studies are required to minimise these risks, and in some cases it may be wise to employ traditional methods when they are adequate.

Introduction

Wildlife field study methods are many and varied, but their utility is limited in applications involving species that are rare, endangered or cryptic. Determining the abundance of such species with any degree of accuracy can be difficult, although such information is vital for developing management strategies. In addition, in-depth studies to examine the population biology of these species via genetic methods may be logistically impossible, particularly if dependent on opportunistic sampling (Kohn and Wayne 1997). For rare and sensitive species, invasive study methods such as trapping (which may also be accompanied by tissue or blood sampling) and fitting of radio-transmitters may be neither feasible nor appropriate (Morin and Woodruff 1996; Taberlet *et al.* 1999). This is particularly so if the species of interest is difficult to trap, or the risk of injury or death is too high for animals already under pressure from predators, disease, shrinking habitat and other factors (Greenwood 1996). In any case, invasive study methods may unavoidably alter the dynamics of a population in ways that are difficult to predict (Harrison *et al.* 1991), and possibly change a behaviour or characteristic being studied (Morin *et al.* 1994*b*; Morell 1995).

Field collection of samples such as hair or faeces can provide some useful information, such as presence/absence and possibly a rough estimation of abundance. However, unequivocal verification that such samples came from the species of interest may not be possible on the basis of morphology alone. By utilising non-invasively collected samples as sources of DNA for molecular genetic marker analysis, many of these shortcomings can be surmounted.

It is useful to distinguish between two types of situation in which collection and use of non-invasive samples for genetic analysis may be desirable. The first is when capture or observation of animals is impossible or inefficient, yet morphological analysis of remotely collected samples such as hair and scats provides only limited information (Kohn and Wayne 1997). Appropriate DNA analyses can greatly extend the utility of such samples by unequivocally determining the species, identity and gender of the individuals from which they came (Kohn and Wayne 1997; Taberlet *et al.* 1999). In the case of faecal samples, identification of plants and animals that make up their diet may be enhanced (Hoss *et al.* 1992; Hofreiter *et al.* 2000; Symondson 2002).

The second major use for non-invasive samples is as an alternative to blood or tissue samples (also referred to as non-destructive sampling, see Woodruff 2003) taken from trapped animals, as a source of DNA for relatedness, population genetic and phylogenetic analyses (Hoss *et al.*

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1992; Kohn *et al.* 1995; Fernando *et al.* 2000). While we draw attention to some studies in which non-invasive samples have been used for these purposes because conventional sampling was not feasible, a summary of the important contributions made by genetic analyses in the study of animal biology is beyond the scope of this review (instead, see Sunnucks 2000).

Although there is intense interest in non-invasive genetic sampling, and numerous publications on the great potential of this new technology, only a relatively small number of comprehensive studies on wild populations have used non-invasive sampling (e.g. Fernando *et al.* 2000; Garnier *et al.* 2001; Vigilant *et al.* 2001; Utami *et al.* 2002). This number is likely to increase, as more researchers become aware of the vast potential for molecular analysis in gleaning useful biological data from non-invasive samples. However, the technology currently has significant drawbacks that mean more traditional methods should perhaps be employed when they are adequate. In this review we aim to present an overview of the tempting range of potential applications for this technology, while warning of their limitations. We explain how these methods work and discuss how they may improve on traditional approaches to the same questions and how they have been put to good use in wildlife-related projects to date. There is no doubt this technology is beginning to revolutionise many areas of the study of elusive and endangered species.

The basis of non-invasive genetic typing

How it works

DNA sequencing of appropriate genes can unequivocally determine the species from which a non-invasive sample was obtained. However, recent developments in molecular technology extend the usefulness of such samples far beyond determination of a species' presence and rough estimation of its abundance (Taberlet *et al.* 1999; Sunnucks 2000). The greatest contribution to this breakthrough was the development of the polymerase chain reaction (PCR) (Saiki *et al.* 1985; Arnheim *et al.* 1990), a method of enzymatically amplifying informative DNA sequences using short pieces of DNA that act as 'primers' for DNA extension. Coupled with DNA sequencing and its surrogates (such as analysis of single-stranded conformation polymorphisms, or SSCP: Sunnucks 2000) this enables the detection of species-, population-, individual-specific and sex-specific DNA signatures from a sample. Even very degraded and low-quantity target DNA, such as that recoverable from many kinds of non-invasively collected samples, can act as PCR template.

The second crucial development, from the point of view of individual identification and other applications requiring high resolution, was the discovery of DNA sequences comprising short repetitive arrays embedded in unique sequences (Tautz 1989). These markers, called microsatellites, exhibit a high degree of variability within populations and can provide individuals with unique DNA profiles when a number of these markers are used in combination. Although development of microsatellite primers is time-consuming they can be used in other closely-related species (usually within the same family) so that the process does not have to be repeated for every species (e.g. Coote and Bruford 1996; Engel *et al.* 1996; Primmer *et al.* 1996; Slate *et al.* 1998; Zhang *et al.* 2001; Zenger *et al.* 2002). Importantly, because any given set of microsatellite PCR primers typically only amplify DNA from closely related species, contaminating DNA (either from bacteria or dietary components in the case of faeces) is unlikely to interfere with interpretation.

The availability of DNA sequence information from the sex chromosomes of mammals and other vertebrates facilitates gender identification of an animal from a non-invasive sample. For example, genetic sex identification in mammalian samples can proceed via PCRs employing primers specific for both Y-chromosomal DNA and an autosomal or X-linked marker (Griffiths and Tiwari 1993; Kohn and Wayne 1997; Sloane *et al.* 2000). Samples yielding products from both markers are deemed to be male and those with only one, female. Alternatively, the presence of a length polymorphism in the amelogenin gene, which has homologues on both the X and Y chromosomes, can be exploited to distinguish between the sexes, as has been demonstrated in great apes (Bradley *et al.* 2001) and black bears (Yamamoto *et al.* 2002). Although sex chromosome DNA has been sequenced in only a small number of animal species, its apparent high level of conservation has enabled the design of 'universal' primers for sex identification in a great diversity of animals (e.g. mammals: Aasen and Medrano 1990; Griffiths and Tiwari 1993; cetaceans: Berube and Palsboll 1996; birds: Griffiths *et al.* 1998; marsupials: Watson *et al.* 1998).

The ability to identify individuals and their sex from non-invasively collected samples offers unprecedented potential to improve the accuracy of abundance estimates and determine behavioural parameters for individuals, such as their home-range size, habitat and dietary preferences, and even some forms of social interaction. Applications for this technology are theoretically restricted only by the ingenuity of the researcher. For example, because both nuclear and mitochondrial DNA (mtDNA) was amplified successfully from rodent and insectivore skulls found in regurgitated barn and tawny owl pellets, Taberlet and Fumagalli (1996) suggested that such samples might be a good source of material for studying population genetics of these small animals. Of course, in many cases a traditional trapping approach may be more efficient for straightforward population genetic analyses, but as Symondson (2002) points out, molecular analysis of owl prey would reveal selective predation on certain genotypes or sexes.

Potential DNA sources

Non-invasive sources of DNA that have proven useful to date include shed or plucked hairs from various primates, marmots, wombats and bears (Morin *et al.* 1994*b*; Taberlet *et al.* 1997; Field *et al.* 1998; Goossens *et al.* 1998*a*; Woods *et al.* 1999; Sloane *et al.* 2000; Constable *et al.* 2001; Banks *et al.* 2002*b*, 2003). DNA analysis of plucked hairs has been successful in providing a variety of data on wombats. Their burrowing nature makes hair collecting relatively easy by the placement of double-sided tape across the burrow entrance, removing hairs from animals as they move in and out of their burrows (Sloane *et al.* 2000; Banks *et al.* 2002*b*, 2003). Hair samples have been collected from Capuchin monkeys (*Cebus olivaceus*) by shooting a tape-covered syringe from an air-powered dart pistol (Valderrama *et al.* 1999), and from free-ranging black bears (*Ursus americanus*) and brown bears (*U. arctos*) by means of hair traps consisting of barbed wire attached to a tree encircling a scent lure (Woods *et al.* 1999). Methods for other species include wrapping bait to force animals to handle it and thus leave a hair sample, and making a corral of tape so animals squeeze their bodies between tape rails to reach bait (Valderrama *et al.* 1999). These novel methods can be adapted to target specific individuals, and will 'pluck' fresh hair, which has higher-quality DNA than shed hair (Valderrama *et al.* 1999; Morin *et al.* 2001).

Another source of DNA is epithelial cells shed from the intestinal lining and deposited in, and on the surface of, faeces (Hoss *et al.* 1992). Such DNA has successfully been analysed from a variety of animals including primates (Constable *et al.* 1995; Gerloff *et al.* 1999; Utami *et al.* 2002), mountain lions (Ernest *et al.* 2000), coyotes (Kohn *et al.* 1999), bears (Taberlet *et al.* 1997), ungulates (Flagstad *et al.* 1999), dolphins (Parsons *et al.* 1999), bats (Vege and McCracken 2001), common wombats (Banks *et al.* 2002*a*) and black rhinos (Garnier *et al.* 2001). More unusual sources of DNA have been wolf urine in snow (Valiere and Taberlet 2000), chimpanzee buccal cells from chewed food remnants (wadges) (Sugiyama *et al.* 1993; Takenaka *et al.* 1993; Hashimoto *et al.* 1996; Morin and Woodruff 1996), sloughed skin from cetaceans (Bricker *et al.* 1996; Valsecchi *et al.* 1998) and for birds, nest materials, feathers, eggshells and urine (Morin *et al.* 1994*a*; Pearce *et al.* 1997; Nota and Takenaka 1999). However, most comprehensive studies have relied on DNA extracted from either hair or faeces, which in many cases is abundant and relatively simple to collect.

Molecular exploitation of 'field signs' for species identification

Scats, hairs or feathers collected in the field are traditionally subjected to a variety of morphological analyses in order to determine which species they are from (Putman 1984). However, there are situations in which such samples may not be reliably identified to species level on the basis of morphology alone (Bulinski and McArthur 2000). Hair morphology, in particular, is often indistinguishable or problematic between closely related taxa (Brunner and Coman 1974; Friend 1978; Valente and Woolley 1982; Taylor 1985). For example, there is no current macroscopic technique that can reliably distinguish black from brown bear hairs (Woods *et al.* 1999). Even highly skilled specialists can misidentify species. In a blind test carried out to determine the accuracy of results from microscopic examination of hairs from 37 mammal species occurring in south-eastern Australia the accuracy and consistency of species identification varied considerably among taxa (Lobert *et al.* 2001): 19 species were reliably identified but the remaining 18 (including *Sminthopsis* spp., *Antechinus* spp., *Petaurus* spp., *Trichosurus* spp., *Gymnobelideus leadbeateri* and *Rattus rattus*) were subject to some degree of identification error (Lobert *et al.* 2001). Most errors were due to intra-taxon variation in hair characteristics.

Misidentification of species from scats is probably common. It has been estimated that faeces are assigned to the correct species in only 50–66% of North American cases (Halfpenny and Biesot 1986). In Australia there are many opportunities to confuse the faeces of sympatric macropod species, such as those of eastern grey kangaroos (*Macropus giganteus*) with red-necked wallabies (*M. rufogriseus*) (Johnson and Jarman 1987) and those of Bennett's wallabies (*M. rufogriseus*) with red-necked pademelons (*Thylogale billardierii*) (Bulinski and McArthur 2000). A recent study to determine the presence of quokkas (*Setonix brachyurus*) at sites in Western Australia has utilised mtDNA analysis to distinguish quokka scats from those of other macropods known to be present (Alacs *et al.* 2003).

Determining the presence and abundance of endangered species and carnivores from morphological analysis of field signs is particularly difficult (Palomares *et al.* 2002). Abundance estimates and feeding ecology of the endangered pine marten (*Martes martes*) is traditionally carried out by morphological identification of faeces in the field by expert surveyors (Davison *et al.* 2002). However, DNA analysis of morphologically identified pine marten faeces showed that the surveyors often failed to distinguish them from those of red foxes (*Vulpes vulpes*) and failed completely when pine martens were at very low densities (Davison *et al.* 2002). A multi-evidence approach incorporating DNA analysis of faeces is recommended for the management of this species, which may be extinct in England and Wales (Davison *et al.* 2002). A similar problem is experienced with the endangered Iberian lynx (*Lynx pardinus*), as faeces may be confused with those of foxes as well as of wild and domestic cats (*Felis silvestris* and *F. catus*) and domestic dogs (*Canis familiaris*) (Palomares *et al.* 2002). The endangered San Joaquin kit fox (*Vulpes macrotis mutica*) is sympatrically distributed with four other canids with similar scat morphology, causing

identification problems, particularly at low population densities (Paxinos *et al.* 1997). By contrast, mtDNA analysis allowed unequivocal species identification from field-collected scats, in surveys for both Iberian lynx and San Joaquin kit foxes (Paxinos *et al.* 1997; Palomares *et al.* 2002).

Identification by molecular means can thus greatly enhance the utility of non-invasive samples for indicating presence or absence of a species. Indeed, as molecular technology becomes cheaper and more routine it may be the method of choice even in situations where traditional morphological analysis is definitive for species identification, particularly as the latter is typically dependent on the availability of experienced practitioners.

Abundance estimates aided by individual identification of 'field signs'

Limitations of traditional methods

Traditional methods of estimating animal abundance are based on direct observational counts of individuals (either free-ranging or following capture), or on indirect signs such as footprints and faeces (e.g. Grigione *et al.* 1999). While direct approaches are effective for many animals, they are inadequate for species that are elusive and/or difficult to trap, and for endangered species for which such methods may be too disruptive. For example, trapping of the highly endangered northern hairy-nosed wombat (*Lasiorhinus krefftii*) affects both its health and behaviour. Analysis of trapping records showed that wombats lost an average of 0.5 kg between first and second captures separated by intervals of up to six months (Hoyle *et al.* 1995). In addition, areas trapped twice in succession had lower population-size estimates for the second trapping period, suggesting that animals may have temporarily left areas disturbed by trapping (Hoyle *et al.* 1995). Other species, such as tammar and parma wallabies, can exhibit strong trap avoidance (Vujcich 1979) and repeated trapping drives in an area may increase trap wariness (Lentle *et al.* 1997). Animals with large home ranges and mobility, such as large carnivores and elephants, are difficult to observe or capture (Grigione *et al.* 1999; Kohn *et al.* 1999; Woods *et al.* 1999; Ernest *et al.* 2000). In any case, the latter may pose unacceptable safety risks to both humans and animals (e.g. mountain lions: McCrown *et al.* 1990; bears: Woods *et al.* 1999; elephants: Eggert *et al.* 2002).

There are also a variety of analytical disadvantages to trapping-based abundance estimation. One is that most trapping techniques are unable to provide 'snap-shot' estimates of population size for many species because they require many months or even years to obtain sufficient sample sizes (Kohn *et al.* 1999). Deceased and migrating individuals may thus be mistakenly included in such population estimates, resulting in overestimates (Kohn *et al.* 1999). On the other hand, poor trapping success may lead to underestimation of population size. For example, non-invasive genotyping of coyote faeces showed that more than two-thirds of the current population may have been missed by long-term ecological surveys, perhaps due in part to a low overall trapping efficiency of only one animal per 58 trap-nights (Kohn *et al.* 1999). Population estimation of red foxes (*V. vulpes*) in Australia is also hampered by low trapping efficiency, and trapped samples may be strongly male-biased, with important ramifications for estimating population parameters (Kay *et al.* 2000). Similarly, trapping data for northern hairy-nosed wombats indicates an excess of males, which in the absence of other evidence, might be interpreted as trapping bias because females are known to be harder to recapture and are more mobile (Alan Horsup, Queensland Parks and Wildlife Service, unpublished). However, a recent census using microsatellite analysis of remotely collected hair samples confirmed the male bias (Banks *et al.* 2003). Finally, misidentification can occur using any tagging system, due to lost ear tags, distorted tattoos, changes in appearance, or lost or malfunctioning radio-transmitters (Woods *et al.* 1999), and any of these may impinge on the accuracy of abundance estimates.

A frequently used *indirect* method of estimating animal abundance is to count faecal pellets. This is of most use if fixed plots are employed to examine trends in abundance (Jarman and Capararo 1997; Bulinski and McArthur 2000). Relying on scat counts alone may lead to important overestimates in population size, as exemplified by the estimate that a small colony of brush-tailed rock-wallabies (*Petrogale penicillata penicillata*) was surviving in the Grampians, Victoria, when, in fact, only a single animal remained (J. Reside, Victorian Brush-tailed Rock-wallaby Recovery Group, unpublished). Examination of footprints left in sand plots is employed for some elusive species (e.g. mountain lions: Smallwood and Fitzhugh 1995). Footprints are only marginally informative, as it is rare to observe a perfectly formed one, and usually very difficult to distinguish between the tracks of closely related species (Triggs 1992). In any case, methods such as these only indicate population trends, as extrapolating absolute abundance from them is problematic (Jarman and Capararo 1997; Bulinski and McArthur 2000). Ultimately, traditional analyses of field signs provide little information on absolute population size and understanding of the demographic, behavioural and life-history strategies of individuals and populations.

What can molecular genetic analysis add?

New molecular methods can help to overcome some of the above limitations by providing accurate identification of remotely collected samples to both species and individual level, allowing direct and relatively unbiased enumeration (Kohn and Wayne 1997; Taberlet *et al.* 1999). At the very

least, this approach can provide an estimate of the minimum number of animals of a particular species in a given area. For example, in one of the first studies of this type, extensive non-destructive (skin biopsies) and non-invasive (sloughed skin) sampling across the North Atlantic Ocean detected 7698 humpback whales and found an unexpected male sex bias in the population (Palsboll *et al.* 1997). Microsatellite profiling and sexing of faeces (in combination with footprint measurements to estimate age) collected from the dwindling Pyrenean brown bear (*Ursus arctos*) population gave an estimated population size of at least one yearling (male), three adult males and one adult female (Taberlet *et al.* 1997). A combination of faecal DNA typing and trapping provided a minimum estimate of 16 mountain lions (seven by capture and nine by faecal DNA analysis) living in, or travelling through, the Yosemite Valley during an 18-month period (Ernest *et al.* 2000).

Collection of non-invasive samples followed by microsatellite genotyping is highly amenable to sampling designs appropriate for mark–recapture estimates of population size, as applied to trap-based estimates. This approach was used to estimate the abundance of coyotes from faeces collected from transects in the Santa Monica Mountains of California (Kohn *et al.* 1999). Abundance of Canadian black bears and grizzly bears over a 64 × 64-km grid was estimated using the hair-trapping method described earlier, followed by microsatellite analysis. More than 1750 hair samples were collected and 1496 of them determined to species level using mtDNA analysis (Woods *et al.* 1999). Of the 54 brown bears that contributed 303 hair samples, only 12 had previously been captured and radio-collared. This was thus a very effective and efficient way of censusing a species that is difficult to observe, exists at low densities and has large home ranges (Mace *et al.* 1994; Mace and Waller 1997).

Applications involving individual identification have also been successful using both hairs and faeces of wombats, animals that are particularly difficult to enumerate by traditional means (McIlroy 1977; Taylor *et al.* 1998). The size of a population of common wombats (*Vombatus ursinus*) in suburban Melbourne parkland was estimated with very narrow confidence limits from faecal DNA (Banks *et al.* 2002*a*). In a similar analysis, but based on DNA profiling of hairs collected on double-sided tape at burrow entrances, the sole remaining population of the highly endangered northern hairy-nosed wombat was estimated to contain 113 individuals (Banks *et al.* 2003). This estimate substantially exceeded earlier (trap-based) ones, which, in combination with other indicators, suggests that the population may recently have increased in size following several years of favourable climatic conditions (Banks *et al.* 2003).

Other applications requiring individual identification

Individual identification by non-invasive genotyping can also be useful for tracking particular individuals in the wild and identifying dispersal events. For example, DNA profiles of captive-raised chimpanzees recorded prior to their release into a native population will facilitate long-term tracking of these individuals (and their offspring), and thus assist in gauging the success of such translocation programmes (Goossens *et al.* 2002). Analysis of 692 'recaptures' of Northern Atlantic humpback whales detected from non-invasive samples confirmed previous assumptions (based on identification and tracking of natural markings) regarding individual and migratory patterns, site-fidelity to summer feeding grounds and mixing in winter breeding grounds (Palsboll *et al.* 1997).

Dietary analysis

The definitive species identification offered by molecular technology, in combination with traditional dietary analysis of scats has made it possible to elucidate the diets of a variety of species whose faeces are not readily distinguished morphologically. For example, this approach allowed Reed *et al.* (1997) to determine the relative impacts of grey (*Halichoerus grypus*) and harbour (*Phoca vitulina*) seals on fisheries, and Hansen and Jacobsen (1999) to better interpret the feeding biology of mink (*Mustela vison*), otters (*Lutra lutra*) and polecats (*Mustela putorius*). In another example, assignment of field-collected scats to each of four sympatric Venezuelan carnivore species using mtDNA analysis, indicated that scat size overlapped considerably among species. This produced a much-altered profile of their dietary niches, which had previously been interpreted on the basis of erroneous assumptions about scat size (Farrell *et al.* 2000).

Another possible application of faecal DNA technology is in the identification of plants and animals consumed by the species of interest, although conventional methods may be better for analysing their actual abundance in the diet (Kohn and Wayne 1997). Nonetheless, in cases where foods are thoroughly digested or difficult to identify, including where hard parts of similar species are difficult to distinguish, molecular identification will be invaluable. Analysis of bear droppings by DNA sequencing of amplification products from the chloroplast *rbc*L gene identified the presence of DNA from the genus *Photinia* (Hoss *et al.* 1992). Although exact identification to species level may have been possible by analysis of a more informative gene, the researchers inferred that the plant was most likely the common *P. villosa*, and that its berries formed a significant component of the bears' diet during late summer when the scats were collected (Hoss *et al.* 1992). Molecular analysis of scats has even been used to infer diet in a long-extinct sloth, whose faeces were discovered in a cave in Arizona (Hofreiter *et al.* 2000), and from an 11 700-year-old rodent midden (Kuch *et al.* 2002).

Molecular identification of prey remains in bird faeces is an alternative to invasive techniques and also a potentially more accurate approach (Sutherland 2000; Casement 2001).

Bird diets are generally determined by labour-intensive direct observation, use of videos or cameras at nest sites, or by more invasive techniques such as stomach pumping (e.g. Walter and O'Neill 1986) or examination of gut contents of dead birds (e.g. Miller and McEwen 1995). MtDNA analysis of faecal samples from nesting blue tits (*Parus caeruleus*) and great tits (*P. major*) identified dietary species of Lepidoptera, and determined that both birds consumed a similar range of species (Sutherland 2000), but in different proportions (Casement 2001).

Non-invasive samples as a novel source of phylogenetic, population genetic and molecular ecological data

Molecular genetic analyses have long provided important information on species biology to complement traditional taxonomic, demographic and behavioural data collection (Sunnucks 2000). However, for many species that might benefit from such analyses, collection of adequate amounts of genetic material via non-destructive sampling from captured animals has been difficult or impossible. In such situations, non-invasive sample collection could provide a valuable new source of genetic information, opening up a myriad of opportunities to study phylogenetic relationships, population genetics, mating systems and dispersal. For example, the burrowing and primarily nocturnal behaviour of wombats makes them difficult animals to study, but assignment tests based on genotypes obtained from common wombat faeces identified immigrants in a population, suggesting that its apparent geographic isolation did not hamper immigration (Banks *et al.* 2002*a*). Similarly, the threatened status and dangers associated with sampling some large mammals precludes the use of invasive genetic sampling. However, analysis of the extent and distribution of genetic diversity in wild Asian elephants (*Elephas maximus*), as well as phylogeographic relationships among populations was possible using faecal DNA analysis (Fernando *et al.* 2000). Also, analysis of paternity and relatedness based on faecal DNA enabled investigation of reproductive success and mating strategies in a wild translocated population of the endangered black rhinoceros (*Dicerous bicornis*). The observation of a substantial male reproductive skew (10 of 19 progeny produced over a 10-year period were fathered by a single male) provided evidence for a possible dominance hierarchy and the first genetic proof of polygyny in the species (Garnier *et al.* 2001). Thus, non-invasive sampling can be invaluable in monitoring reproductive patterns in endangered species, thus assisting in the development of reproductive management strategies.

Two species that are of conservation concern in Europe but difficult to study directly are the brown bear (*U. arctos*) and gray wolf (*Canis lupus*), which have particularly benefited from non-invasive sampling. MtDNA control region sequencing from bear faeces collected from the Brenta mountains of northern Italy (Hoss *et al.* 1992; Kohn *et al.* 1995) indicated the presence of only a single haplotype identifying the bears as being of western, rather than eastern, European origin (Taberlet and Bouvet 1994). This has implications for management and possible augmentation of this highly endangered population (Hoss *et al.* 1992; Kohn *et al.* 1995). Wolf populations have declined substantially in Europe but direct monitoring of packs is difficult owing to the species' elusive nature (Lucchini *et al.* 2002). The current naturally occurring wolf recolonisation of the Alps is of major importance to the conservation of the Italian population in particular, because this may reconnect it to other populations and reverse its long-standing genetic isolation (Lucchini *et al.* 2002). DNA analysis of faeces collected from the Italian Alps identified the number, sex and relatedness of animals in two apparently distinct wolf packs, and suggested that they originated exclusively from the Italian source population (Lucchini *et al.* 2002).

Invasive genetic sample collection is particularly problematic in the primates, whose capture is considered unethical. Instead, DNA obtained from wadges, faeces, plucked hairs and shed hairs collected from night nests has provided a wealth of data on phylogeography, gene flow, social structure and kinship in wild chimpanzees (Constable *et al.* 2001; Vigilant *et al.* 2001), gorillas (Jensen-Seaman and Kidd 2001), bonobos (Gerloff *et al.* 1999), Hanuman langurs (Launhardt *et al.* 2001), orangutans (Utami *et al.* 2002) and lemurs (Nievergelt *et al.* 2002). Microsatellite genotyping was performed on faecal samples from an Indonesian orangutan (*Pongo pygameus abelii*) population that has been the subject of a long-term behavioural study with an emphasis on male reproductive strategies (Utami *et al.* 2002). Paternity analysis using faecal samples showed that males with cheek flanges (a secondary sexual characteristic associated with 'sitting, calling and waiting') enjoyed a similar degree of reproductive success to those without flanges (who employed a 'going, searching and finding' strategy) (Utami *et al.* 2002).

Limitations and considerations in non-invasive genotyping

Despite the advantages of non-invasive sampling in some situations, it is important to note that samples collected this way are usually inferior in reliability to samples such as ear biopsies and blood. Thus, DNA analysis from such sources needs to be rigorously performed and checked (Taberlet and Luikart 1999; Taberlet *et al.* 1999).

Genotyping errors: *detecting and overcoming them*

The main limitations of non-invasive sampling relate to the low quantity and quality of DNA typically recovered from such samples, particularly faeces and shed hairs. This necessitates the use of more laborious and costly DNA extraction procedures than are required for blood and tissue

samples (Taberlet *et al.* 1999; Ernest *et al.* 2000). Furthermore, the ramifications of contamination with high-quality DNA from other sources are sufficiently severe that extra precautions are required during DNA extraction, such as working in a laboratory separate from that in which high quality DNA samples are handled or stored. Perhaps most importantly, analysis of low-quality and low-quantity DNA can be highly error-prone, especially in the case of microsatellites. Two main types of scoring error, unrelated to contamination events, are commonly experienced. Allelic dropout (the stochastic failure of one allele to amplify) leads to heterozygotes being mistakenly identified as homozygotes. The production of amplification artefacts, known as false alleles, can also result in incorrect genotypes (Taberlet *et al.* 1996; Smith *et al.* 2000).

The ramifications of genotyping errors are dependent on the aims of the project. For example, undetected high levels of allelic dropout resulting in erroneously low observed heterozygosity levels will lead to false interpretations with regard to inbreeding and population structure (Taberlet *et al.* 1999). Applications requiring assessment of relatedness and kinship, or individual identification (e.g. for estimating population size), may be particularly severely affected (Taberlet *et al.* 1999). For example, the very different rates of extra-community matings reported for chimpanzee populations reflect genotyping errors due to the use of non-invasive DNA sources, rather than gross mating behaviour differences (Constable *et al.* 2001; Vigilant *et al.* 2001). A comparison across two studies, of genotypes obtained from a Tai forest community in western Africa showed that genotypes for some individuals (9 of 33) had been incorrectly scored for at least one locus in the earlier study using shed hairs, leading to mistaken inferences of extra-community matings (7 of 13 offspring compared with 1 of 14 offspring sired by males outside the mother's social group) (Gagneux *et al.* 2001; Vigilant *et al.* 2001). It is now known that these errors were due to the very low quantities of DNA extracted from shed hair in comparison to faeces (Morin *et al.* 2001; Woodruff 2003).

The rate of microsatellite genotyping error from non-invasive samples may depend on a range of biological and technical factors. For example, Sloane *et al.* (2000) reported very low rates of genotyping error (0.3%) and hence achieved reliable individual identification using DNA isolated from single wombat hairs collected remotely on sticky tape. Follicles of wombat hairs are relatively large, and may contain substantial amounts of DNA, although yield has not been quantified. In addition, the Sloane *et al.* (2000) protocol maximises DNA recovery: hairs are 'plucked' (rather than shed), and DNA is extracted within 24 hours using an efficient protocol in which all the DNA remains in the same tube. Low error rates were also experienced using plucked bear hairs (Woods *et al.* 1999). In contrast, rates of genotyping error were sufficiently high from single hairs plucked from alpine marmots (*Marmota marmota*) that 10 hairs were required to genotype an individual accurately (Goossens *et al.* 1998*b*). It is not clear whether this is due to storage effects, since the DNA from marmot hairs was not extracted immediately, or whether it might suggest a relationship between body size and hair follicle size (and thus DNA quantity). Studies using shed hairs appear to be more error-prone because follicles may contain small amounts of degraded DNA, or alternatively may be absent (Higuchi *et al.* 1988; Vigilant 1999). Morin *et al.* (2001) reported that 79% of chimpanzee hair extracts had no amplifiable DNA and the remainder, very low quantities, leading to substantial rates of genotyping error.

Error rates of genotyping faecal DNA are generally higher than those from hairs, but are highly variable due to many factors. For example, there may be variation between individuals, as reported for orangutans, in which some individuals' faeces never provided amplifiable DNA, while faeces of others always did (Goossens *et al.* 2000). Researchers may also encounter season- or age-related reliability of genotyping. For example, wolf samples collected fresh in winter produced higher-quality DNA extracts than older ones, or those collected during summer (Lucchini *et al.* 2002). Faecal DNA genotyping from alpine ibex (*Capra ibex*) and Corsican mouflon (*Ovis musimon*) suffered only low error rates when carried out on samples collected in winter (99 and 95% of samples respectively) as compared with spring, when only 52% and 59% of samples produced reliable genotypes. A possible explanation for this observation is seasonal dietary and/or climatic characteristics. Both ibex and mouflon eat young buds and shoots with minimal fibre during spring, which may decrease intestinal membrane abrasion and/or increase the passage of material, thus reducing the intestinal cell content in spring faeces (Celia Maudet, University Joseph Fourier, personal communication).

The range of factors capable of affecting rates of genotyping error from non-invasive samples means that experimental protocols developed for one species may not be easily transferable to other study systems or even to other laboratories. Thus it is imperative that pilot studies be carried out for each planned application, to facilitate quantification of error rates associated with various field sampling regimes, as well as storage, extraction and amplification protocols (Taberlet *et al.* 1999). This process will also provide the necessary data for a cost–benefit analysis to determine whether a non-invasive approach is feasible. In this context a useful recent development is the availability of computer programs that allow researchers to simulate and quantify the effects of particular error rates on the outcomes of analyses of genotypic data sets, and their consequent biological interpretation (e.g. Gemini: Valiere *et al.* 2002).

If non-invasive sampling is deemed necessary in a particular study system despite evidence that high error rates

may be experienced, various approaches may help to mitigate this to some extent. Most studies using faecal DNA carry out 3–8 replicate PCRs per sample for each microsatellite to ensure that the correct genotype is obtained (Taberlet *et al.* 1997, 1999; Morin *et al.* 2001), although the original 'multiple tubes approach' suggested by Navidi *et al.* (1992) recommended 10 or more PCR replications. For example, a pilot study on Savannah baboon (*Papio cynocephalus*) faecal DNA showed that accurate determination of relatedness between individuals or social groups required rigorous testing and many replicates (8 to 16 for each locus) (Smith *et al.* 2000). Because such a high degree of replication greatly increases the consumables, costs and time required, it would be very useful if a pre-screening process could identify problematic samples. Two possibilities have been examined to date. The first uses a fluorescent 5′ nuclease PCR assay that measures the amount of amplifiable target DNA rather than total DNA in extracts (Morin *et al.* 2001) (conventional DNA quantification methods are ineffectual as they measure total DNA, which includes degraded target DNA, as well as bacterial and prey DNA in the case of faeces). It appears that template amounts in the range of 26–100 pg, 101–200 pg and more than 200 pg require seven, four and two PCR replicates, respectively, for accurate genotyping (Morin *et al.* 2001), a finding consistent with earlier indications that 56 pg of template DNA was a critical threshold (Taberlet *et al.* 1996). The second possibility for pre-screening applies to hair samples, of which microscopic examination prior to extraction has shown a strong relationship between follicle morphology (indicative of degree of cellular atrophy) and accuracy in genotyping (Kathryn Jeffery and Mike Bruford, University of Cardiff, personal communication).

Contamination of faecal DNA extracts with DNA from dietary items

A source of error peculiar to studies based on faeces is that DNA from dietary items will be co-extracted with that from the species of interest. Depending on the degree of sequence similarity with the PCR primers being used, this contaminating DNA may co-amplify and thus cause errors in interpretation, or simply obscure the intended target. One way to avoid this is to design species- or genus-specific primers. This requires that (a) sequences be available for comparison amongst a wide range of species, and (b) regions of low homology can be identified. While this is generally possible for mtDNA, it is unlikely to be the case for the Y chromosome, which shows a remarkably high degree of sequence conservation (Griffiths and Tiwari 1993). Thus, all currently used mammalian-sexing primers will amplify sequences in a wide variety of mammals. For example, the fact that faeces from known mountain lion females produced a Y-specific PCR product was probably due to the presence of male mammalian prey DNA in the faeces (Ernest *et al.* 2000). To a lesser degree, accurate microsatellite genotyping (and therefore individual identification) may be hampered when the diet of the target species includes individuals of the same or a closely related species. The likelihood that such interference would be detected depends on the degree of diversity in both the predator and prey populations, because detection would require that at least three different alleles be observed at a locus (given that diploid individuals should have no more than two different alleles). Alternatively, microsatellite loci that are known to display non-overlapping allele sizes (such as is seen for gray wolves and coyotes: Roy *et al.* 1994) in focal and prey species will act as species markers in this situation

If the PCR primers themselves cannot be made sufficiently specific to amplify only the DNA of the target species, then the sequence of resulting PCR products will need to be analysed. This will be effective only if the target sequence of the species of interest is known to be distinguishable from those upon which it is likely to prey or scavenge. Such an approach was successfully employed by Lucchini *et al.* (2002) in determining the sex of wolves from faecal samples collected in the field, but this would not be possible in species known to frequently indulge in cannibalism, or predation/scavenging of closely related species.

It is not possible to summarise all possible scenarios that would result in misinterpretation of results due to contaminating DNA in faeces. However, these examples caution that researchers should be aware of the range of species whose DNA will co-amplify with that of the target species, under the planned PCR protocol. If this includes species other than the target, then they should ensure that their methodology will adequately distinguish contaminants.

Methodological considerations

Collection and storage of samples

The most crucial aspect of successful DNA analysis of non-invasive material is ensuring the integrity of the results. Locating non-invasive samples can be difficult and time-consuming, so appropriate well planned field sampling strategies are required. In addition, since the laboratory analysis is somewhat expensive and error-prone, it is essential that the amount and quality of DNA recovered is maximised, requiring optimisation of both storage and extraction techniques.

Design of sample-collection strategies will depend on both the study species and the question being asked. For instance, in abundance estimation, *a priori* information such as home range and territoriality will be useful for estimating the number of transects and size of area to be covered. In species that defecate at communal roost or latrine sites, faecal DNA may not be the non-invasive sampling method of choice, as scats are likely to be contaminated with the DNA

of other individuals. Therefore, the fact that big brown bats (*Eptesicus fuscus*) could be genotyped from faeces collected from the cloth bags in which they were held individually (Vege and McCracken 2001) is unlikely to alleviate the need to capture animals, as bat guano underneath roost sites may effectively constitute a pooled DNA sample. In the light of this, and given that genotyping of faecal DNA is more expensive and error-prone than it is from blood or tissue sources, non-invasive genotyping is currently unlikely to provide new insights into the biology of communally roosting species.

Ideally, all non-invasive samples should be collected from the field as fresh as possible and not touched with bare hands, as human DNA can interfere with some analyses, particularly mammalian sex identification (see above). Hair samples may be best extracted immediately in the field as this is a simple process, but storage in paper envelopes under relatively dry climatic conditions may be sufficient to preserve the DNA (Goossens *et al.* 1998*b*; Woods *et al.* 1999; Sloane *et al.* 2000). In contrast, faeces should ideally be transported to a laboratory for DNA extraction, because appropriate extraction protocols typically require unwieldy equipment and involve many steps, which increases the risk of contamination. If faeces are collected in remote locations, there will be a time lag between collection and extraction, so it is necessary for samples to be adequately stored.

During sample storage it is vital that the opportunity for nucleases to degrade the DNA is minimised. This requires that the molecular environment of the DNA be physiologically inhospitable to enzymatic activity, which can be achieved by either physical or chemical means. For faeces this has involved the sample being dehydrated either by air drying (e.g. pumas and jaguars: Farrell *et al.* 2000; sheep and reindeer: Flagstad *et al.* 1999) or alcohol treatment (e.g. chimpanzees: Constable *et al.* 2001; baboons: Bayes *et al.* 2000; bonobos: Gerloff *et al.* 1999; elephants: Fernando *et al.* 2000), frozen at –20°C (e.g. mountain lions: Ernest *et al.* 2000), or saturated in a buffer containing high concentrations of salts or other chemicals that will interfere with enzymes (e.g. DET buffer: Frantzen *et al.* 1998). The latter was found to be most effective for preserving nuclear DNA from baboon faeces, but the other storage methods mentioned above performed equally well for mtDNA (Frantzen *et al.* 1998).

DNA extraction

Extracting DNA from hair follicles and feathers is simple and quick, allowing large numbers of samples to be processed simultaneously, even in the field with minimal equipment. A commonly used method involves simply boiling hairs or feathers in a suspension of Chelex resin, thus using a single extraction step with consequent minimal opportunity for contamination and loss of DNA (Morin *et al.* 1994*a*; Pearce *et al.* 1997; Taberlet *et al.* 1997; Field *et al.* 1998; Gagneux *et al.* 1999; Vigilant 1999; Sloane *et al.* 2000; Banks *et al.* 2002*b*). Faecal DNA extraction is substantially more time consuming because of the presence of a cocktail of substances that may co-purify with the DNA and inhibit subsequent PCRs. Their removal requires repeated purification steps, usually involving centrifugation, typically performed using filter columns or silica binding beads (Hoss *et al.* 1992; Taberlet *et al.* 1997; Kohn *et al.* 1999; Bayes *et al.* 2000; Bradley *et al.* 2000; Goossens *et al.* 2000; Constable *et al.* 2001; Banks *et al.* 2002*a*).

Preliminary treatment of faecal samples for DNA extraction may depend on the size of the faeces and the way in which they have been stored. Surface-washing to remove intestinal cells from the mucosal layer of the faeces was found to be very effective for air-dried reindeer (*Rangifer tarandus*) and domestic sheep (*Ovis aries*) faeces, resulting in substantially lower genotyping error rates (2%) than whole-sample homogenisation (30%) (Flagstad *et al.* 1999). Some studies involving crushing or homogenising whole or partial faeces have suffered relatively high rates of genotyping errors (e.g. mountain lions, 8%, Ernest *et al.* 2000; wolves, 18%, Lucchini *et al.* 2002). At least for some taxa, surface-washing may be more effective when faeces are small and can be handled easily. However, larger-sized faeces may require different preliminary methods such as scraping the surface crust (as for elephant dung: Fernando *et al.* 2000) or collecting the slurry from the bottom of vials containing ethanol-stored faeces (as for chimpanzees: Constable *et al.* 2001). Given this range of experiences it is clear that a pilot study to assess different preservation and extraction methods is important and the preservation method, in particular, may be dependent on the field conditions and location and size of the faecal sample (Taberlet *et al.* 1999).

Analytical considerations

The success of applications involving the establishment of individual identity, paternity and kinship are highly dependent on levels of genetic diversity in a population, and may require a large number of microsatellite loci in genetically depauperate populations (Kohn and Wayne 1997). Unfortunately, the species for which non-invasive genotyping may be favoured as a method of study – i.e. those that are most endangered – are often those suffering from loss of genetic variation due to small population sizes (Frankham 1995). For example, the critically endangered Pyrenean bear population had such low genetic diversity that a standard genotyping approach could not distinguish two individuals whose presence was therefore indicated only by their different track sizes (Taberlet *et al.* 1997).

Studies to identify individuals by DNA fingerprinting can employ the statistic ‘probability of identity’ (PID), which enables the determination of the number of loci with given levels of heterozygosity required to distinguish confidently

between a pair of individuals (Waits *et al.* 2001). Failure to adequately distinguish individuals could result in an underestimate of population size, particularly if the population is composed of closely related individuals such as siblings, which are much more likely than unrelated pairs to share a genotype (Waits and Leberg 2000). Access to sufficient numbers of polymorphic microsatellite loci may be problematic for several reasons that may typify non-invasive sampling studies. The first is that target species may be lacking in genetic diversity, as indicated above, so that a large number of microsatellite markers will be required to achieve reliable individual identification. Second, the yield of DNA from non-invasive samples may be too low to provide template for amplification of a sufficient number of markers (Taberlet *et al.* 1997). Third, the proportion of markers that reliably amplify from poor-quality DNA, such as that extracted from non-invasive sources, can be very low (e.g. Banks *et al.* 2002*a*). Finally, microsatellite primers have been developed for relatively few wildlife species, and these may not include the more cryptic and rare species for which non-invasive genotyping may be desired. This requires that a suite of markers be developed specifically. Owing to the expense and time involved in this process, the resulting panel of markers may be small.

The need to establish (a) whether the target population exhibits sufficient levels of genetic variation, and (b) whether appropriate markers are available or can realistically be obtained, further illustrates the necessity for carrying out a pilot study prior to launching a large-scale non-invasive sampling program (Taberlet *et al.* 1999).

Conclusion

Non-invasive genetic typing is an exciting and novel technology that has had application around the world and is likely to greatly assist studies on the Australian fauna, which contains many species that are cryptic or endangered. These techniques provide information at the species level, as well as allowing individual identification. The genotypic data obtained may also be used to infer genetic variation, genetically effective population size, recruitment, dispersal, home range, habitat use, kinship and paternity.

For animals whose capture is impractical, non-invasive sampling may be an important or exclusive method for obtaining information. However, techniques are not yet developed to the stage where they can reliably and cost-effectively replace capture and sampling of animals in many situations. Some applications will not justify the expense and effort required. It is important when considering carrying out non-invasive sampling to perform pilot studies in the laboratory and in the field to determine whether the technology is appropriate, and to allow a cost–benefit analysis to be carried out before launching into a large-scale collection project. The constraints and costs of capturing animals need to be weighed against those relating to laboratory analysis of non-invasive sources of DNA. However, the costs of non-invasive genetic sampling are likely to decrease in the future with the availability of higher-throughput machinery and improved technology. Ultimately, the added cost for these techniques will be acceptable for particular species where current methods do not provide optimal results.

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