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Non-invasive genetic analysis in conservation

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INTRODUCTION

A key component of the emergence of conservation genetics as a recognisable subdiscipline of conservation biology over the last ten years has been the development of methods to genetically assess and monitor populations of endangered species non-invasively. The rapid development of methodologies for ensuring the accurate capture of molecular data from elusive, easily stressed or potentially dangerous (!) organisms and concerns over the accuracy of the data produce have prompted a number of excellent reviews on the subject in recent times (e.g. Taberlet *et al.* 1999; Taberlet and Luikart 1999; Piggott and Taylor 2003; Woodruff 2003; Wayne and Morin 2004). Here, we will review the issues and wide-ranging applications of non-invasive genetic analysis without focusing on the molecular technicalities in great detail.

Why non-invasive genetics?

Before the advent of non-invasive genetics and the use of animal by-products such as faeces, shed hair, feathers, bones, fish scales, teeth, etc., obtaining genetic material from wild populations was often ethically (in particular for species listed as endangered and critically endangered under CITES regulations) and logistically extremely difficult. Now such analysis is increasingly possible and the sampling of large populations without visual/physical contact is particularly beneficial for endangered species or if the species studied can transmit or are susceptible to disease (e.g. great ape species whose pathogens are often extremely similar to the researchers studying them). In a century where linking behaviour, social structure, dispersal and population genetic structure has become a new challenge for conservation geneticists, the development of non-invasive sampling and genotyping has provided the opportunity to explore these links and has dramatically opened new areas for research.

Population Genetics for Animal Conservation, eds. G. Bertorelle, M. W. Bruford, H. C. Hauffe, A. Rizzoli and C. Vernesi. Published by Cambridge University Press.

The characterisation of non-invasive material using molecular markers allows biologists to identify and count individuals in wild populations, identify the sex of those individuals and determine their movement patterns, infer parentage, kinship and relatedness, and assess pathogens and diet (see Kohn and Wayne 1997 for a review). The possibility of this approach first came to attention when Higuchi and colleagues demonstrated that single human hair roots could provide enough DNA for molecular genetic analysis (Higuchi *et al.* 1988), thanks to the polymerase chain reaction (PCR). Following this discovery, Morin and colleagues were the first to apply this method to hair collected in nature, studying paternity in a wild population of chimpanzees (*Pan troglodytes*) in Tanzania (Morin and Woodruff 1992; Morin *et al.* 1994c) and gene flow between chimpanzee populations in Africa (Morin *et al.* 1994b). Then, very quickly, during the 1990s, alternative DNA sources began to be exploited in a diversity of organisms: for example faeces in terrestrial (Gerloff *et al.* 1995) and marine mammals (e.g. Reed *et al.* 1997; Valsecchi *et al.* 1998), birds (Pearce *et al.* 1997) and reptiles (Bricker *et al.* 1996).

Subsequently, several studies started to combine and compare the data from different sort of non-invasive samples: e.g. shed/plucked hair and faeces (Taberlet *et al.* 1997; Bayes *et al.* 2000; Smith *et al.* 2000; Constable *et al.* 2001; Vigilant *et al.* 2001). Surprisingly, however, to date relatively few studies have been carried out using exhaustive population sampling using only non-invasive material, possibly due to the financial and logistical constraints involved in collecting and processing very large numbers of samples in this way (but see, for example, Buchan *et al.* 2003; Goossens *et al.* 2005; Zhan *et al.* 2006), but nonetheless, such approaches remain perfectly feasible and will become common in the literature in the near future.

What are the applications?

At the population level and below, the use of genetic markers such as the major histocompatibility complex (MHC) (Knapp 2005), amplified fragment length polymorphism (AFLP), microsatellites, minisatellites and mitochondrial DNA with the potential use of non-invasive samples can be applied in a myriad of contexts (see Table 8.1 for a more exhaustive list of examples), such as individual identification (Taberlet and Luikart 1999 for a review; Waits *et al.* 2001 for a review), species identification (Symondson 2002 for a review; Teletchea *et al.* 2005), exclusion and assignment of parentage (Jones and Ardren 2003 for a review), relatedness and kinship patterns (Ross 2001 for a review), dispersal patterns and individual movements (genotyping in space and time; Gagneux *et al.* 2001), inferring

Table 8.1 *Applications of non-invasive genetics in conservation with examples taken from the literature*

Applications	Examples and references
Individual identification	Hairy-nosed wombat (Sloane *et al.* 2000)
Species identification	Mustelid species (Hansen and Jacobsen 1999; Lopez-Giraldez *et al.* 2005); canid species (Paxinos *et al.* 1997; Davison *et al.* 2002; Dalén *et al.* 2004; Reed *et al.* 2004); seal species (Reed *et al.* 1997); deer species (Galan *et al.* 2005); macropods (Alacs *et al.* 2003); Chinese tiger (Wan *et al.* 2003)
Parentage	Sumatran orang-utan (Utami *et al.* 2002); chimpanzee (Morin *et al.* 1994b, c; Gagneux *et al.* 1997a, 1999; Constable *et al.* 2001; Vigilant *et al.* 2001); black rhinoceros (Garnier *et al.* 2001); Asian elephant (Fernando *et al.* 2000)
Relatedness and kinship	Eastern imperial eagle (Rudnick *et al.* 2005); bonobos (Gerloff *et al.* 1999); chimpanzees (Mitani *et al.* 2000; Vigilant *et al.* 2001)
Dispersal system	Common wombat (Banks *et al.* 2002); bonobos (Gerloff *et al.* 1999); chimpanzees (Morin *et al.* 1994b; Gagneux *et al.* 2001)
Individual movements	Brown bear (Taberlet *et al.* 1997); Hanuman langur (Launhardt *et al.* 2001)
Population structure	*Antechinus agilis* (Kraaijeveld-Smit *et al.* 2002)
Population assignment	Black-footed albatross (Walsh and Edwards 2005); wolves (Randi and Lucchini 2002); Alpine ibex (Maudet *et al.* 2002); African elephant (Wasser *et al.* 2004)
Phylogeography	African elephant (Eggert *et al.* 2002); Asian elephant (Fernando *et al.* 2000); brown bear (Taberlet and Bouvet 1994)
Effective population size	Brown bear (Bellemain *et al.* 2005); Louisiana black bear (Triant *et al.* 2004); gray wolf (Creel *et al.* 2003); chinook salmon (Shrimpton and Heath 2003)
Censusing and capture/recapture	Savannah baboon (Storz *et al.* 2002); badger (Wilson *et al.* 2003); coyote (Kohn *et al.* 1999; Prugh *et al.* 2005); Northern hairy-nosed wombat (Banks *et al.* 2003); black rhinoceros (Cunningham *et al.* 2001)
Hybridization effects and hybridization monitoring	Red wolf and coyote (Adams *et al.* 2003); Canada lynx and bobcat (Schwartz *et al.* 2004)
Identification of ESUs	Bornean elephant (Fernando *et al.* 2003); Larch mountain salamander (Wagner *et al.* 2005)
Reconstruction of phylogenetic relationships	Brown bear (Taberlet and Bouvet 1994)

Table 8.1 (*cont.*)

Applications	Examples and references
Impact of habitat fragmentation and reduced gene flow	Desert bighorn sheep (Epps *et al.* 2005)
Sex determination	Eurasian otter (Dallas *et al.* 2000); red deer (Huber *et al.* 2002); canid species (Ortega *et al.* 2004; Seddon 2005); felid species (Pilgrim *et al.* 2005); wolverine (Hedmark *et al.* 2004); Asian elephant (Vidya *et al.* 2003); birds (Miyaki *et al.* 1998)
Molecular tracking or genetic tagging, genetic monitoring	Brown bear (Taberlet *et al.* 1997; Lorenzini *et al.* 2004; Tallmon *et al.* 2004); mountain lion (Ernest *et al.* 2000); gray wolf (Lucchini *et al.* 2002); chimpanzees (Goossens *et al.* 2002); humpback whales (Palsbøll *et al.* 1997); black and brown bears (Woods *et al.* 1999), wolverine (Flagstad *et al.* 2004)
Disease status	Chimpanzees (Santiago *et al.* 2003); chimpanzees and gorillas (Makuwa *et al.* 2003); carnivores (Steinel *et al.* 2000)
Forensics (DNA barcodes) and legal actions	Birstein *et al.* 1998; Palumbi and Cipriano 1998; Pank *et al.* 2001; Carr *et al.* 2002; Fang and Wan 2002; Manel *et al.* 2002; Shivji *et al.* 2002; Chapman *et al.* 2003; Hebert *et al.* 2003, 2004; Moritz and Cicero 2004; Will and Rubinoff 2004; Barrett and Hebert 2005; Prendini 2005; Schander and Willassen 2005

population structure (Pritchard *et al.* 2000), population assignment (Blanchong *et al.* 2002 for a review) and phylogeography (Avise 2000), determination of effective population size (Kohn *et al.* 1999), population census (capture/recapture) and population size estimation (Schwartz *et al.* 1999; Mills *et al.* 2000 for a review; McKelvey and Schwartz 2004a, b; Paetkau 2004; Lukacs and Burham 2005a, b and Miller *et al.* 2005 for reviews), detection of hybridization events and monitoring of hybridization (Schwartz *et al.* 2004; Willis *et al.* 2004), phylogenetic species designation and the identification of evolutionary significant units (Moritz 1994; Li *et al.* 2004), evaluation of the impact of habitat fragmentation and reduced gene flow among populations (e.g. Stow *et al.* 2001), molecular tracking (e.g. Taberlet *et al.* 1997), sex determination (e.g. Shaw *et al.* 2003), disease status and evolutionary study of viral genomes from faecal samples (e.g. Whittier *et al.* 2004), forensics and legal actions (Birstein *et al.* 1998; Palumbi and Cipriano 1998; Dalebout *et al.* 2002; Manel *et al.* 2002) and dietary

analysis (Farrell *et al.* 2000; Fedriani and Kohn 2001; Deagle *et al.* 2005; Parsons *et al.* 2005).

METHODS AND SAMPLE SOURCES

Sample sources

DNA sample sources that have been used to study wild populations include shed hairs (collected in night nests) from great apes (Morin *et al.* 1994b, c; Constable *et al.* 2001; Goossens *et al.* 2005) and plucked hairs from wombats (Sloane *et al.* 2000; Banks *et al.* 2002b, 2003), Alpine marmots (*Marmota marmota*) (Goossens *et al.* 1998a), capuchin monkeys (*Cebus olivaceus*) (Valderrama *et al.* 1999) and bears (Taberlet *et al.* 1997; Woods *et al.* 1999; Triant *et al.* 2004). Another valuable source of DNA is epithelial material from the digestive tract, which is found in and around the surface of faecal material. Using PCR, DNA was first successfully amplified from a bear faecal sample by Höss *et al.* (1992). Since then, such DNA has been analysed from a variety of mammals including bats (Vege and McCracken 2001); common wombats (*Vombatus ursinus*) (Banks *et al.* 2002a); marine mammals (Tikel *et al.* 1996) including dolphins (Parsons *et al.* 1999; Parsons 2001) and seals (Reed *et al.* 1997); ungulates (Flagstad *et al.* 1999; Huber *et al.* 2002); African (*Loxodonta africana*) (Eggert *et al.* 2002) and Asian elephants (*Elephas maximus*) (Fernando *et al.* 2000); black rhinos (*Diceros bicornis*) (Garnier *et al.* 2001); pine martens (*Martes martes*) (Davison *et al.* 2002), Eurasian badgers (*Meles meles*) (Frantz *et al.* 2003; Wilson *et al.* 2003) and Eurasian otters (*Lutra lutra*) (Dallas *et al.* 2003; Hung *et al.* 2004); kit foxes (Paxinos *et al.* 1997), coyotes (*Canis latrans*) (Kohn *et al.* 1999; Prugh *et al.* 2005), wolves (*Canis lupus*) (Lucchini *et al.* 2002; Creel *et al.* 2003) and wolverines (*Gulo gulo*) (Flagstad *et al.* 2004); mountain lions (*Puma concolor*) (Ernest *et al.* 2000) and Iberian lynx (*Lynx pardinus*) (Palomares *et al.* 2002; Pires and Fernandes 2003); and primates (Constable *et al.* 1995; Launhardt *et al.* 1998; Gerloff *et al.* 1999; Launhardt *et al.* 2001; Oka and Takenaka 2001; Vigilant *et al.* 2001; Utami *et al.* 2002; Lukas *et al.* 2004; Goossens *et al.* 2005). Faeces have been also used in bird species (Segelbacher and Steinbrück 2001), mainly in the great bustard (*Otis tarda*) (Broderick *et al.* 2003; Ydaghdour *et al.* 2003). The most common non-invasive sample used in birds is feathers (Pearce *et al.* 1997; Segelbacher 2002). Taberlet (1991) first showed that a single plucked feather contained enough DNA for genetic analysis, then Morin *et al.* (1994a) amplified DNA from hornbill feathers. In a more recent study, Rudnick *et al.* (2005) used naturally shed feathers to identify Eastern

imperial eagle (*Aquila heliaca*) individuals, generate parentage data and monitor a wild population in Kazakhstan. Other sources of DNA for birds are eggshells (Pearce *et al.* 1997), egg membranes (Nuechterlein and Buitron 2000) and urine (Nota and Takenaka 1999). Urine has also been used for genetic analyses in gray wolf (*Canis lupus*) (Valière and Taberlet 2000) and wolverine (Hedmark *et al.* 2004). Recently, Yasuda *et al.* (2003) described a simple method of DNA extraction and microsatellite typing from urine samples using a DNA/RNA extraction kit that should open avenues for new studies using urine. In fish, old scale samples can be useful as a source of DNA (Nielsen *et al.* 1999). Collections of fish scales can be found in many fisheries in the world and comprehensive genetic studies are consequently being carried out with a temporal perspective on many fish species.

More unusual sources of DNA are chimpanzee wadges (chewed food remnants) containing buccal cells (Sugiyama *et al.* 1993; Takenaka *et al.* 1993), sloughed skin in whales (Valsecchi *et al.* 1998) and snakes (Bricker *et al.* 1996), skin swabbing in dolphins (Harlin *et al.* 1999), eggs in sea turtles (Moore *et al.* 2003), skin mucus in fish (Livia *et al.* 2006) and buccal swabs in amphibians (Pidancier *et al.* 2003). Other biological materials such as teeth and scrimshaw from sperm whales (*Physeter macrocephalus*) (Pichler *et al.* 2001), old teeth in red fox (*Vulpes vulpes*) (Wandeler *et al.* 2003), ivory in elephants (Comstock *et al.* 2003; Wasser *et al.* 2004), meat in whales (Baker *et al.* 1996; Palumbi and Cipriano 1998; Baker *et al.* 2000) and dolphins (Baker *et al.* 1996), Chinese alligators (*Alligator sinensis*) (Yan *et al.* 2005), ostriches (Abdulmawjood and Bulte 2002) and sea turtles (Moore *et al.* 2003), body parts and remains in sharks (Hoelzel 2001; Pank *et al.* 2001; Shivji *et al.* 2002; Chapman *et al.* 2003) and whales (Carr *et al.* 2002; Dalebout *et al.* 2002), sturgeon caviar (Wolf *et al.* 1999), carcasses in deer species (Fang and Wan 2002) can all give reliable results for DNA analysis and are very useful in trade monitoring and wildlife poaching detection of endangered species.

Storage of samples

Hair samples

There are two kinds of hairs that can be used as non-invasive DNA source: plucked and shed. Plucked hairs are by far the best source of hair DNA for both mitochondrial and nuclear DNA analysis while shed hairs can often be problematic for nuclear DNA analysis. Single plucked hairs with root material should provide enough DNA for genetic analysis, providing adequate storing conditions are used (see below). However, we recommend

collecting more than 10 hairs per individual (see Goossens *et al.* 1998b). Valderrama *et al.* (1999) described four methods of collecting fresh hair samples from wild and captive mammals: (1) shooting a rolled strip of duct tape, pressed onto the flat tip of a plastic syringe, from an air-powered dart pistol; (2) making a corral by enclosing a small area with duct tape; (3) wrapping a bait (i.e. to a tree) with duct tape; (4) wrapping inverted tape around the tip of a stick and touch the animal (only for captive animals). Hair traps based on barbed wire around trees (for bears) and sticky tape in rodent tubes can also be useful. Plucked hairs have been used for free-ranging primates (Valderrama *et al.* 1999), wombats (Sloane *et al.* 2000; Banks *et al.* 2003), brown bears (*Ursus arctos*) (Taberlet *et al.* 1994, 1997; Woods *et al.* 1999) and Alpine marmots (Goossens *et al.* 1998a).

For shed hairs, the roots have usually undergone apoptosis before shedding (telogen phase), and much of the nuclear DNA is degraded (Jeffery *et al.* 2007). However, epithelial tissue may be attached to the root of freshly shed hairs and provides a source of undegraded nuclear DNA (Linch *et al.* 1998). Shed hairs are commonly used for great ape studies (see Morin *et al.* 1994b, c; Gagneux *et al.* 1997a; Goossens *et al.* 2005) but can show unreliable results (Gagneux *et al.* 1997b).

Roon *et al.* (2005a) evaluated the optimal storage methods and DNA degradation rates for hair samples. Hair samples from brown bears were preserved using silica desiccation and –20 °C freezing over a 1-year period. Amplification success rates decreased significantly after a 6-month time point, regardless of storage method. It is therefore important to minimize delays between hair collection and extraction if we want to maximize the amplification success rate. However, hair samples are usually stored in clean paper envelopes (Goossens *et al.* 1998a; Woods *et al.* 1999; Sloane *et al.* 2000), since plastic bags produce static that make hair manipulation difficult and increase the contamination.

Faecal samples

For the last 10 years, different storage methods have been tested for faecal samples of different species. It is vital that DNA degradation by nucleases is minimised as much as possible. This can be done by dehydrating the sample by air-drying (Flagstad *et al.* 1999 for sheep and reindeer (*Rangifer tarandus*); Farrell *et al.* 2000 for mountain lions and jaguars (*Panthera onca*)), by silica gel beads drying (Bradley and Vigilant 2002 for gorillas), by −20 °C-freezing (Ernest *et al.* 2000 for mountain lions), by alcohol (ethanol) treatment (Gerloff *et al.* 1999 for bonobos (*Pan paniscus*); Fernando *et al.* 2000 for Asian elephant; Constable *et al.* 2001 for chimpanzees; Goossens *et al.* 2005

for orang-utans (*Pongo pygmaeus*), or by saturating the sample in a buffer (DETs: see below) containing high concentrations of salts or other chemicals interfering with enzymes (Frantzen *et al.* 1998). Frantzen *et al.* (1998) evaluated the effectiveness of these four methods for preserving fresh baboon faeces: drying, –20 °C freezing, 70% ethanol and DMSO/EDTA/Tris/salt solution (DETs). The latter was the most effective for preserving nuclear DNA and the three other methods performed equally well for mitochondrial DNA analysis and for short microsatellite fragments (less than 200 bp) showing that amplification success is dependent on storage method, PCR product size and molecular marker used. In another study, Piggott and Taylor (2003a) evaluated the same preservation methods (together with different extraction methods) but for faecal samples from two Australian marsupial herbivores (*Dasyurus maculatus* and *D. viverrinus*) and one introduced carnivore (*V. vulpes*). Their results showed that the highest amplification and lowest genotyping error rates were obtained with dried faecal sample extracted via a surface wash followed by Qiagen spin column purification. More recently, Roeder *et al.* (2004) compared faecal sample storage in ethanol and silica with a two-step method: soaking of the samples in ethanol followed by desiccation with silica. While the samples stored in silica showed the lowest DNA concentration, the two-step method yielded significantly more DNA in high quality samples. The ethanol and the two-step methods performed equally for lower quality samples. Nsubuga *et al.* (2004) obtained significantly higher amounts of DNA from wild mountain gorillas (*Gorilla beringei beringei*) and chimpanzees faecal samples using the protocol developed by Roeder and colleagues. Moreover, they showed a small negative correlation between temperature at time of collection and the amount of DNA amplified. RNA later (see next paragraph) preservation solution did not produced better results than silica gel beads storage.

In 2002, Murphy *et al.* tested five preservation methods on brown bear faeces (90% ethanol, DETs buffer, silica-dried, oven-dried stored at room temperature, and oven-dried stored at −20 °C) at different time points (1 week, 1 month, 3 months and 6 months) and for two different genetic markers (mtDNA and nDNA). The ethanol-preserved samples had the highest success rates for both mtDNA and nDNA. The 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 faeces, with the freeze-drying and oven drying producing the best amplification success rates. A recent tissue storage reagent, called RNA*later*® (Ambion, Inc.), has been successfully used to store faecal samples in our

laboratory and those of others. It is an aqueous, non-toxic reagent that rapidly permeates most tissues to stabilize and protect RNA in fresh specimens. DNA (and obviously RNA) can be isolated from RNA*later*-stored samples with very good reliability in genotyping results. Only problem: it is an expensive reagent. Faeces in birds can be stored at −20 °C in 90% ethanol (Broderick *et al.* 2003; Idaghdour *et al.* 2003).

Other samples

Urine Urine samples can be used for carnivores and can be easily collected on snow (see Valière and Taberlet 2000; Hedmark *et al.* 2004). It can also be collected in plastic sheets placed under sleeping nests of great apes. Individual chimpanzees or orang-utans often urinate from the side of the nest on awakening and urine can be collected and transferred to storage vials using disposable plastic pipettes. Unfortunately, fermentation and DNA degradation of DNA cells may occur rapidly after urination (Hayakawa and Takenaka 1999), it is therefore recommended to collect as large a volume as possible and transfer it into two volumes of 95% ethanol. Urine can also be used for birds (Nota and Takenaka 1999), and fixed with 70–90% ethanol and stored at –20 °C.

Feathers In general, feathers are stored in envelopes or plastic bags and stored dry until analysis (see Segelbacher 2002). In a study on the Eastern imperial eagle, Rudnick *et al.* (2005) used naturally moulted adult feathers collected from nesting sites. He also plucked developing blood feathers from chicks. Adult feathers were stored dry at room temperature. Developing chick feathers were stored in a lysis buffer (see Rudnick *et al.* 2005 for details).

Wadges Buccal cells from food items (wadges) can be successfully extracted and mtDNA and nDNA can be amplified from the DNA extracted (Hashimoto *et al.* 1996 in chimpanzees). Wadge samples must be transferred to a sterile 50-ml polypropylene tube filled with 90% ethanol and 1 mM Na_3EDTA, to avoid bacterial and enzymatic degradation of the DNA.

Extraction kits and methods

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 (see Allen *et al.* 1998). In our laboratory, we have used the latter and have found that using PCR buffer, water and proteinase K in a small extraction volume works very well for shed hairs (see Jeffery 2007; 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 steps involving several centrifugation steps. We recommend using the QIAamp Stool mini kit (QIAGEN) which has given reliable results in primates (gorillas: Bradley and Vigilant 2002; orang-utans: Utami *et al.* 2002; Goossens *et al.* 2005); baboons: Bayes *et al.* 2000) and other mammals (black rhinoceros: Garnier *et al.* 2001; brown bear: Bonin *et al.* 2004; wolverine: Hedmark *et al.* 2004). Other methods have been described in the literature and include: 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 and Taylor 2003a). A pilot study is recommended as one extraction technique may work for some species but may not work for others. Extraction (and storing) methods will depend on the field conditions, location, season, size and age of the samples (see Taberlet *et al.* 1999; Piggott 2004). For bird faeces, Broderick *et al.* (2003) used a modification of Milligan's (1998) silica and guanidine isothiocyanate based protocol (for a detailed protocol, see Idaghdour *et al.* 2003).

Urine Protocols using the QIAamp DNA stool mini kit (GmbH, Hilden, Germany) to extract DNA from urine collected in snow are well described in Valière and Taberlet (2000) for canids and in Hedmark *et al.* (2004) for wolverine. For birds, a detailed protocol is described in Nota and Takenaka (1999).

Feathers Eguchi and Eguchi (2000) developed a simple method to extract DNA from bird feathers, and also from snake cast-off skin using collagenase. Jensen *et al.* (2003) describe a technique to extract DNA from feathers using Chelex-100® (also used by Morin *et al.* (1994a) for hornbills).

Wadges Different methods can be used to extract DNA from wadges and can be found in Takenaka *et al.* (1993 and in Hashimoto *et al.* (1996).

RECENT INNOVATIONS

Multiplex PCR (Henegariu *et al.* 1997) systems for comparative genotyping are well developed in human forensics (Wallin *et al.* 2002; Shewale *et al.* 2004) and are now developed for other animal species such as cervids (Eld deer and swamp deer, Rusa and Vietnamese sika deers: Bonnet *et al.* 2002;

roe deer: Galan *et al.* 2003); primates (orang-utans: Immel *et al.* 1999; Roeder *et al.* 2006), fish (great white shark (*Carcharodon carcharias*): Chapman *et al.* 2003). Piggott *et al.* (2004) developed a multiplex pre-amplification method to improve microsatellite amplification and error rates when using faecal DNA. Qiagen have developed a multiplex kit, which is commonly used for genotyping of DNA extracted from non-invasive samples such as faeces and hair in our laboratory with reliable results.

In addition, 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). Rönn *et al.* (2006) recently tested this approach to assess its efficacy on a variety of primate DNA, including non-invasively collected samples, and found broadly that MDA template DNA produced equivalent genotype accuracy as unamplified DNA.

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 in consideration. The two most commonly used markers used in non-invasive genetics today are mitochondrial DNA

and nuclear microsatellites. The specific attributes of these markers will not be discussed here, but their behaviour and likely information content in a non-invasive genetic analysis context will be alluded to. However, it is likely in the future that single nucleotide polymorphisms (SNPs) will become the genetic marker of choice to study the ecology and conservation of wild populations because they allow to access variability across the whole genome. Although examples remain scarce to date, in one study Seddon *et al.* (2005) addressed ecological and conservation issues in recolonized Scandinavian and Finnish wolf populations using 24 SNP loci. These loci were able to differentiate individual wolves and differentiate populations using assignment tests. Compared to microsatellites, SNPs allow the amplification of extremely small fragments, which makes them very useful for population and conservation genetics using non-invasive samples, and are much easier to automate, for example on microarrays (see chapter by Vernesi and Bruford, this volume). SNPs have the advantage that a range of different sequence types can be chosen, to give information on both neutral markers and those under selection (for example the major histocompatibility complex; Smulders *et al.* 2003).

Sex chromosomes in mammals (Fernando and Melnick 2001; Bryja and Konecny 2003; Hellborg and Ellegren 2003; Erler *et al.* 2004; Hedmark *et al.* 2004) and other vertebrates (birds: Griffiths *et al.* 1998; and fish: Matsuda 2003) can provide DNA sequence useful for the identification of an animal gender. Using both Y-chromosomal DNA and an autosomal or X-linked marker is useful in providing sex information (Griffiths and Tiwari 1993; Sloane *et al.* 2000; Huber *et al.* 2002). The amelogenin locus can also be used to identify gender, e.g. in great apes (Bradley *et al.* 2001; Matsubura *et al.* 2005), in bears (Yamamoto *et al.* 2002) and in felids (Pilgrim *et al.* 2005). In birds, for example, Sacchi *et al.* (2004) used the CHD (Chromo-Helicase-DNA-Binding) sex-linked gene and feathers to differentiate males and females for the endangered short-toed eagle (*Circaetus gallicus*). Russello and Amato (2001) described a PCR-based test, using feather DNA, to identify the sex in an endangered parrot species, *Amazona guildingii*.

Amplified fragment length polymorphisms (AFLPs) are dominant markers that can be used in parentage, population assignment (Campbell *et al.* 2003), gene flow and migration, although they are less adequate for reconstructing past events and historic patterns of variation (Wayne and Morin 2004; Bensch and Åkesson 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.

TECHNICAL CHALLENGES

Non-invasive genetic analysis, despite its obvious advantages for studying wild populations of elusive and endangered species, has its own limitations and pitfalls that must be seriously taken into consideration. Samples collected non-invasively are far less reliable than invasive samples such as blood and tissue biopsies. DNA can be highly fragmented and sometimes PCR may be inhibited by co-extracted compounds present in the material. Contamination from humans (particularly for primate species) and cross-contamination between samples are common and must be avoided. Therefore, precautions need to be taken such as using a laboratory room dedicated to non-invasive sample storage and handling. DNA extracted from non-invasive samples can be of low quantity and quality and therefore analyses need to be rigorously done and checked (Taberlet and Luikart 1999; Taberlet *et al.* 1999; Bonin *et al.* 2004; McKelvey and Schwartz 2004a). DNA extraction has to be highly efficient (quick and avoiding unnecessary steps) and several new methods and ever-sophisticated and high-yielding extraction kits are now available to expedite rapid extraction and minimal liquid handling. DNA extraction has also to be able to remove inhibitory material during purification (Eggert *et al.* 2005) and whereas this used to be a laborious process, required reagents are now included in many of the commercially available kits.

Low template DNA copy number and PCR inhibition have led to several phenomena being observed in non-invasive genotyping. First, PCR products may be extremely difficult to generate and the resultant fragments may not be sufficient for analysis. We advise to use more PCR cycles (up to 40–50) or a second round of PCR, using the fragments generated in the first to 'seed' the reaction. Decreasing the annealing temperature may also help. Increasing the number of cycles may, however, have a negative impact if a copying error is introduced, producing false polymorphisms. Therefore, replicate PCRs are imperative to confirm the results (see Taberlet *et al.* 1996; Goossens *et al.* 2000).

False data may occur in DNA sequences (artificial point mutations) or in microsatellite fragments (false allele lengths due to DNA polymerase slippage during PCR). DNA polymerase slippage is a general phenomenon in microsatellite PCRs, but can usually be compensated for by recording only the one (for homozygotes) or two (for heterozygotes) most intensely amplified fragments. False alleles may confuse this process, although such artifact fragments are usually weakly amplified, and should in any case be replicated (Bradley and Vigilant 2002). Further, the stochastic

non-amplification of one of the two potential alleles at a microsatellite locus can occur ('allelic dropout') because of low template copy number or DNA degradation. The latter may be a special problem for loci exhibiting a wide range of allele lengths, because longer alleles may not be amplifiable if their length exceeds the maximum fragment size present in the degraded template DNA. Repeated amplifications using several independent DNA extractions (see Navidi *et al.* 1992; Taberlet *et al.* 1996; Goossens *et al.* 1998b; Taberlet *et al.* 1999 for a review; Goossens *et al.* 2000) are a minimum requirement in such studies. Software such as GIMLET (Genetic Identification with MultiLocus Tags, http://pbil.univ-lyon1.fr/software/Gimlet/gimlet.htm) can assist in the identification of false homozygotes and false alleles by comparing the repeated genotypes and the associated consensus genotype for each sample (Valière 2002).

THE NEED FOR PILOT STUDIES

We strongly advocate carrying out preliminary experimental protocols and critical pilot data evaluation before starting a full-scale study on a new species or population. If working with faecal samples, an environmental decay experiment can be extremely useful to establish the likely success of DNA extraction from faeces found in field conditions. Fresh samples always produce better DNA, but sometimes these may be impossible to find. Piggott (2004) investigated the effect of sample age (and seasonality) on the amplification and genotyping reliability of microsatellite loci from faecal DNA of a marsupial herbivore (the brush-tailed rock wallaby, *Petrogale penicillata*) and a carnivore (the red fox). The author compared DNA profiles from 1 day to 6 months for both species and found that as the age of the samples increased there was progressively poorer quality DNA present on the surface of the faeces. This resulted in significantly lower amplification rates and higher genotyping error. This problem is most likely to be severe the case for tropical environments, where rainfalls are very strong and the risk of washing the DNA off the outer layer (mucus) of the faeces is high. Therefore, there is a need to know how DNA yield correlates with the age of your sample (decay rate experiment) and the weather conditions, such as rain. It is also important to consider the diet of the species studied (Murphy *et al.* 2003). Problems are often met with leaf-eater species, probably due to vegetal inhibitory material. We also suggest to liaise with biochemists for knowing, depending on some specific biological features of the studied species, which compounds are expected to be co-extracted with DNA from sources such as faeces and hair. This would

allow adopting more efficient procedures to get rid of these molecules thus improving the quality of the extracted DNA.

Quantitation of DNA in non-invasive genetic samples has often proved problematic by conventional means due to the degraded nature of the DNA present, contamination with exogenous DNA and the presence of RNA. One reliable quantitation approach, described by Morin *et al.* (2001), uses a quantitative PCR assay with appropriate standards. This method has proven reliable in samples such as DNA extracted from previously autoclaved fox teeth (Wandeler *et al.* 2003) and provides a major positive development in the field. Once the pilot data have been produced, the software GEMINI (Genotyping Errors and Multitube Approach for Individual Identification, http://pbil.univ-lyon1.fr/software/gemini.html; Valière *et al.* 2002) allows the user to evaluate and quantifying the effects of genotyping errors on the genetic identification of individuals. It also allows simulating the effectiveness of a specific multitubes approach to correct these errors.

REQUIREMENTS

To recap, before starting any non-invasive genetic study on a specific species, and especially when using faecal samples, there is a need to:

(1) identify the genetic markers that you will need (i.e. if you use microsatellites, check available markers published)
(2) carry out a pilot study on the effect of age and season on the reliability of microsatellite genotyping (particularly for tropical species and if you work with faeces)
(3) select the right sample preservation method (check the literature or test it if necessary)
(4) select the right DNA extraction method (check the literature or test it if necessary)
(5) test the effects of genotyping errors and multitubes approach using software such as GEMINI (Valière *et al.* 2002)
(6) during collection, try to sample the same faeces at least twice, and always sample the outer layer of the faeces (mucus).

ANALYSIS

Reliability

Different methods have recently been published to check the integrity of the genotypes produced during a study and to ensure that the multi-locus genotypes are correct. The method most commonly used so far is the

multi-tubes approach (originally developed by Navidi *et al.* 1992) formalized by Taberlet *et al.* in 1996. Since then, most studies using non-invasive DNA carry out three to seven replicate PCRs per sample for each locus. However, such a number of replicates considerably increases the cost and time of such studies. Therefore, the pre-screening methods described above (Morin *et al.* 2001) or computer packages (Valière 2002; Valière *et al.* 2002; van Oosterhout *et al.* 2004; Roon *et al.* 2005b) are highly recommended before starting any non-invasive work. MICRO-CHECKER (van Oosterhout *et al.* 2004, http://www.microchecker.hull.ac.uk/) is software that tests for genotyping errors due to null or false alleles and for allelic dropout. It can also be used to discriminate between Hardy–Weinberg deviations caused by null alleles and those caused by demographic factors such as consanguinity. More recently, Roon *et al.* (2005b) tested the effectiveness of several methods for error-checking non-invasive genetic data and cautioned against using non-comprehensive data filters in non-invasive genetic studies and suggested the combination of data filters with careful technique and thoughtful non-invasive study design. Wilberg *et al.* (2004) have produced software (GENECAP) to facilitate the analysis of multilocus gentypes data in non-invasive DNA sampling and genetic capture–recapture studies. It uses multilocus genetic data to match samples with identical genotypes, calculate frequency of alleles, identify sample genotypes that differ by one and two alleles, calculate probabilities of identity, and match probabilities for matching samples.

Demographic information

Alongside previously mentioned software, such as GIMLET (Valière *et al.* 2002), increasingly sophisticated approaches, such as the likelihood-based methods implemented in API-CALC 1.0 (Ayres and Overall 2004) allows the user to calculate probabilities of identity (individualize from non-invasive genetic data) allowing for complications such as genetic substructure, inbreeding and the presence of close relatives.

A large number of software packages have been designed in the last 10 years to assign parentage. The strengths and weaknesses of these methods have been reviewed by Jones and Ardren (2003). We strongly recommend assessing their merits before selecting any recent parentage software. There are four approaches to calculate parentage: (1) exclusion (based on the Mendelian rules of inheritance), which uses incompatibilities between offspring and parents to reject a particular parent/offspring pair and assumes all potential parents are samples and no genotype errors; (2) categorical allocation, which uses likelihood-based (LOD score) approaches to select

the most likely parent from a pool of non-excluded parents and allows the user to include a genotyping error rate and incomplete sampling of potential parents (Marshall *et al.* 1998; Slate *et al.* 2000); (3) fractional allocation, which assigns some fraction (between 0 and 1) of each offspring to all non-excluded candidate parents (see Devlin *et al.* 1988); (4) parental reconstruction, which uses the multilocus genotypes of parents and offspring to reconstruct the genotypes of unknown parents contributing gametes to a progeny array for which one parent is known a priori (Jones 2001). Table 8.2 provides a list of the most recent parentage software used in the literature, with a few examples of studies with, for most of the examples, implications in conservation. The most common software used for parentage analysis is CERVUS (Marshall *et al.* 1998).

A number of software packages can be used to estimate relatedness in wild populations. The most commonly used have been RELATEDNESS (Queller and Goodnight 1989) and KINSHIP (Goodnight and Queller 1999). RELATEDNESS estimates pairwise relatedness between individuals or average pairwise relatedness between groups using regression while KINSHIP tests pedigree relationships using likelihood methods. Another package, DELRIOUS (Stone and Björklund 2001), analyses molecular marker data and calculate delta and relatedness estimates with confidence. Finally, IDENTIX (Belkhir *et al.* 2002) tests relatedness in a population using permutation methods.

There are several packages available that allow the identification of a source population for a specific dispersing individual. Eldridge *et al.* (2001) used assignment tests using the programs STRUCTURE (Pritchard *et al.* 2000) and GENECLASS (Cornuet *et al.* 1999) to identify the source population of rock wallaby (*Petrogale lateralis*) individuals. Berry *et al.* (2004) examined the accuracy of assignment tests to measure dispersal in the grand skink (*Oligosoma grande*) and suggested the use of Bayesian assignment methods. Hansson *et al.* (2003) used GENECLASS software to assign immigrants of specific cohorts of great reed warblers (*Acrocephalus arundinaceus*) and revealed female-biased dispersal in that species. Möller and Beheregaray (2004) used GENECLASS and RELATEDNESS to identify male-biased dispersal patterns in bottlenose dolphins (*Tursiops aduncus*). Isolation by distance using Mantel tests (Liedloff 1999) can also be used to estimate dispersal in wild populations. Many examples now exist where these approaches have been used on invasive samples, but only a few studies have used non-invasive DNA sampling to assign gene flow or dispersal patterns in animal species (Broderick *et al.* 2003 in great bustard; Launhardt *et al.* 2001 in langurs (*Semnopithecus entellus*); Gerloff *et al.* 1999 in bonobos (*Pan paniscus*); Morin *et al.* 1994b and Gagneux *et al.* 2001 in chimpanzees).

Table 8.2 *List of the most recent parentage software used in the literature*

Software	Authors	Web site	Examples
Cervus	Marshall *et al.* (1998)	helios.bto.ed.ac.uk/evolgen/cervus/cervus.html	Baker *et al.* (2004) (red foxes); Garnier *et al.* (2001) (black rhinoceros); Utami *et al.* (2002) (orang-utans)
Famoz	Gerber *et al.* (2003)	www.pierroton.inra.fr/genetics/labo/Software/Famoz	
Gerud 1.0	Jones (2001)	www.bio.tamu.edu/USERS/ajones/JonesLab.htm	Chapman *et al.* (2004) (hammerhead shark, *Sphyrna tiburo*)
Gerud 2.0	Jones (2005)	www.bio.tamu.edu/USERS/ajones/JonesLab.htm	
Kinship	Goodnight and Queller (1999)	www.gsoftnet.us/GSoft.html	Clinchy *et al.* (2004) (brushtail possums, *Trichosurus vulpecula*);
Newpat	Worthington Wilmer *et al.* (1999)	www.zoo.cam.ac.uk/zoostaff/amos/newpat.html	Hoffman and Amos (2005) (Antarctic fur seals, *Arctocephalus gazella*)
Papa	Duchesne *et al.* (2002)	www.bio.ulaval.ca/louisbernatchez/	
Parentage 1.0	Emery *et al.* (2001)	maths.abdn.ac.uk/~ijw/downloads/download.htm	
Parente	Cercueil *et al.* (2002)	www2.ujf-grenoble.fr/leca/membres/manel.html	
Pasos	Duchesne *et al.* (2005)	www.bio.ulaval.ca/louisbernatchez/	
Patri	Signorovitch and Nielsen (2002)	www.biom.cornell.edu/Homepages/Rasmus_Nielsen/files.html	
Probmax	Danzmann (1997)	www.uoguelph.ca/~rdanzman/software/probmax/	
Relatedness	Queller and Goodnight (1989)	www.gsoftnet.us/GSoft.html	

Finally, and perhaps most excitingly, in the last 5–10 years, the use of non-invasive genetic sampling for capture–recapture population census studies on several animal species has started: for example in painted turtles (*Chrysemys picta*) (Pearse *et al.* 2001); whales (Palsbøll *et al.* 1997); bears (Woods *et al.* 1999; Dobey *et al.* 2005); African elephants (Eggert *et al.* 2003); coyotes (Kohn *et al.* 1999; Prugh *et al.* 2005) and in the development of new methods to estimate the size of populations using molecular mark–recapture data (Mills *et al.* 2000; Waits and Leberg 2000; Paetkau 2003; McKelvey and Schwartz 2004a, b; Paetkau 2004; Waits 2004; McKelvey and Schwartz 2005; Miller *et al.* 2005). There are a number of capture–recapture methods that are now available for use with non-invasive DNA-based capture–recapture data. These methods are highlighted in a recent review by Lukacs and Burnham (2005b). The most recent software (CAPWIRE: Miller *et al.* 2005) allows the application of a number of models of population aggregation and fecal deposition rates, and this has recently been applied with success, for example in giant pandas (*Ailuropoda melanoleuca*) (Zhan *et al.* 2006).

PERSPECTIVE

Non-invasive analysis is becoming the only acceptable way to retrieve genetic data from many endangered species. 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. However, care is still needed and experimental designs need to be implemented which allow full verification of the data, both by the researchers themselves and others wishing to replicate their work.

ACKNOWLEDGEMENTS

We would like to thank the Darwin Initiative for the Survival of Species (Defra, UK) for funding and Universiti Malaysia Sabah for facilities during the writing of this article.

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