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NON-INVASIVE GENETIC SAMPLING OF THE EURASIAN OTTER (*LUTRA LUTRA*) USING HAIRS

HEATHER M. ANDERSON[1], DOMINIC J. MCCAFFERTY[2a], ILIK J. SACCHERI[3], ALAN E. MCCLUSKIE[1]

[1] Natural Research Ltd., Banchory Business Centre, Banchory, AB31 5ZU, Scotland, UK
[2] Department of Adult & Continuing Education, University of Glasgow, 11 Eldon Street, Glasgow G3 6NH, Scotland, UK ([a] Author for correspondence: Tel.: +44 141 330 1803; E-mail: d.mccafferty@educ.gla.ac.uk
[3] School of Biological Sciences, University of Liverpool, Biosciences Building, Crown Street, Liverpool L69 7ZB, England, UK

ABSTRACT - The material for the genetic characterisation of wild Eurasian otters (*Lutra lutra*) has previously been derived from carcasses and spraints. Hair samples however have proved to be a much more reliable source of DNA than spraints, and offer the opportunity of sampling the living population non-invasively. Until now there has been no research into methods of sampling hairs from wild otters or on the DNA extraction efficiency from these hairs. A hair trap was therefore developed and tested on otters in captivity. The success rate of the trap was 0.71 samples collected per trap night. The suitability of genetic analysis from otter hairs was examined using paired samples of hair and tissue taken from 15 individual otters recovered from road mortalities. DNA was extracted from the tissue samples using a Proteinase K digestion in a PCR compatible buffer. This process had a 100% success rate. Individual root hair segments were treated by Chelex Ionic bead resin treatment and Proteinase K digestion in a PCR compatible buffer. The Chelex method gave a 55% amplification success rate while the Proteinase K method gave a much higher amplification success rate of 87%. The DNA extracts were typed for 9 microsatellites using the latest versions of the primers. Proportions of allelic dropout and false allele detection associated with hair DNA extracts were estimated by comparing the genotypes of hair extracts with the genotypes from tissue. Preliminary attempts to develop a ZFX/Y assay to sex otters identified polymorphisms between ZFX and ZFY sequences, but typing based on restriction digests requires further optimisation. The use of recovered DNA from hair offers a step forward in the study of Eurasian otter populations as its continuing endangered status in many countries creates legal and ethical constraints on capturing animals for marking or radio tracking.

Key words: Otter, *Lutra lutra*, DNA, hair, genotyping, molecular sexing

RIASSUNTO – ***Campionamento genetico non-invasivo della Lontra* (Lutra lutra) *mediante l'uso dei peli***. La caratterizzazione genetica di lontre selvatiche (*Lutra lutra*) fino ad ora è stata ottenuta da carcasse e feci. Campioni di peli tuttavia forniscono DNA più affidabile, ed offrono la possibilità di campionare le popolazioni mediante metodi non-invasivi. Fino ad ora non sono state sperimentate metodologie per il campionamento e l'estrazione di DNA da campioni di peli ottenuti da lontre selvatiche. Perciò abbiamo costruito un model-

lo di trappola per peli che è stato sperimentato in lontre in cattività. Il tasso di cattura della trappola utilizzata è stato di 0,71 campioni per notte-trappola. L'efficacia delle analisi genetiche da peli è stata esaminata comparando i risultati ottenuti da campioni di peli e tessuti prelevati da 15 carcasse di lontre raccolte da incidenti stradali. Il DNA è stato estratto dai tessuti tramite digestione con Proteinasi K in un tampone compatibile con la PCR. Questo protocollo ha avuto successo nel 100% dei casi. Radici di singoli peli sono state trattate con Chelex ed estratte con il metodo della Proteinasi K in un *buffer* compatibile con la PCR. Le estrazioni col Chelex hanno avuto successo nel 55% dei casi, mentre il protocollo con la Proteinasi K ha prodotto un successo molto più alto dell'87%. I campioni di DNA sono stati genotipizzati con 9 microsatelliti utilizzando le più recenti versioni dei *primers*. Le percentuali di *dropout* allelico e falsi alleli associati con le amplificazioni del DNA estratto dai peli sono state stimate in confronto con i genotipi ottenuti dai tessuti. Tentativi preliminari di sviluppare un metodo di sessaggio molecolare hanno identificato polimorfismi tra sequenze ZFX e ZFY, ma la genotipizzazione basata sui siti di restrizione richiede ulteriori ottimizzazioni. La possibilità di utilizzare DNA estratto da peli rappresenta un progresso nello studio delle popolazioni di Lontra, poiché il loro status a rischio pone, in molti paesi, limitazioni legali ed etiche alla cattura di esemplari ai fini di marcatura o di *radio-tracking*.

Parole chiave: Lontra, *Lutra lutra*, DNA, peli, genotipizzazione, sessaggio molecolare.

INTRODUCTION

Over the past 50 years the Eurasian otter (*Lutra lutra*) suffered a widespread and rapid decline in numbers and a reduction in range throughout most of Europe. In the UK this decline has been attributed to the introduction of organochlorine pesticides, which led to the contamination of aquatic food chains. This occurred when the otter population was already stressed by persecution through organised hunts and game keeping control. In spite of this, the Eurasian otter is now showing a recovery in the UK and increasing populations are now of international importance (see review in Chanin, 2003).

In recent years a great deal of time and effort has been devoted to studying the Eurasian otter. A high proportion of this work has been concerned with diet, distribution and status (e.g. Kruuk, 1995). However, research on behaviour, especially in freshwater habitats, such as foraging, population structure and territory movements are relatively few in number.

The continuing endangered status of the otter in many countries creates legal and ethical constraints on capturing animals for marking or radio tagging. This is an additional factor that inhibits the study of otter behaviour, particularly in areas where the need for conservation is greatest as a result of past population declines. Non-invasive genetic sampling may therefore be a different approach to studying free ranging populations. Individual identification via non-invasive sampling has now become of prime importance in conservation and ecology, as it allows for genetic studies of wild animals without having to trap or observe them (Taberlet and Luikart, 1999).

Previously the material for the genetic characterisation of wild otters was derived from carcasses, which were mainly road casualties (Dallas *et al.*, 1999). Consequently the sampling used in these studies is opportunistic and mainly limited to areas where otter habitat and roads overlap. The discovery that DNA from cells lining the gut of an animal could be recovered from faecal material opened up a wide range of opportunities for ecologists by enabling them to record locations visited by individual otters over a period of time without any direct interference with the animals themselves and also to monitor the activities of a considerable number of individuals (Chanin and Coxon, 2000, Dallas *et al.*, 2003).

The DNA is amplified using the Polymerase Chain Reaction (PCR) and a genetic fingerprint or DNA profile for the individual is obtained. DNA fingerprinting is based on the fact that the chromosomes carry sections of DNA which do not form part of the genes but consist of short sequences of DNA repeated few to many times. These are known as satellites (micro-satellites if less than ten base pairs per repeat). The most significant features of these are that the numbers of repeats are variable giving rise to the equivalent of gene alleles, and that each is flanked by unique regions to which PCR primers can usually be designed (Chanin and Coxon, 2000). Microsatellites have many advantages as genetic markers. Firstly they are locus specific, in contrast to multi locus markers. They are co-dominant, meaning that heterozygotes can be distinguished from homozygotes. They are PCR based which means they can be analysed form small amounts of tissue and the process can work with degraded or ancient DNA. Finally they are highly polymorphic, which provides considerable information with which to differentiate individuals, social groups and populations at a range of spatial scales.

Dallas and Piertney (1998) identified 13 microsatellites in otters and in preliminary laboratory trials they were able to successfully extract intact otter DNA from the spraints of captive animals. However they also discovered that the DNA was rapidly broken down once the spraint had been deposited indicating that only spraints collected within hours of deposition could be used. Furthermore, a pilot study conducted by Chanin and Coxon. (2000) found that, of 600 spraints collected early in the morning, usable DNA was extracted from only 20%. Moreover, Dallas *et al*. (2003) found that the proportion of wild collected spraints yielding genotypes was low, and that the failure of most spraint DNA extracts to yield PCR products was due probably to DNA degradation, not PCR inhibition (Dallas *et al.*, 1999). This implies that to genetically characterise individual otters using spraints would require large efforts in field sampling and DNA typing (Dallas *et al.*, 1999).

In addition to these analytical problems, spraints are an inherently difficult indicator of otter presence, or population size, because as scent marks, their deposition will be affected by several behavioural and individual differences. During studies of otters using a combination of radio-telemetry and radionuclide recovery (Kruuk *et al.*, 1993), it

was found that there were large variations in the recovery rate of individuals' spraints, with some otters rarely sprainting on land making recovery impossible. This type of analysis could therefore lead to a bias towards the section of the otter population that are sprainting more frequently at the time of survey.

Hair samples have proved to be much more reliable in genetic analysis, and have been used in the study of several species of mammals. For example, DNA amplified from hair has been used to assess patterns of kinship (Morin *et al.*, 1994), as the foundation of large scale mark recapture studies (Woods *et al.*, 1999) and most recently to estimate population size (Frantz *et al.*, 2004). However the use of hairs does have many of the same drawbacks as faecal material. DNA extracts can be low in concentration, degraded or mixed with PCR inhibitors. These factors can lead to reduced amplification success or unacceptable genotyping error rates (Goossens *et al.*, 1998), making studies unfeasible or biasing results (Taberlet *et al.*, 1999). Due to these factors, it is of utmost importance to develop an effective extraction process, thereby reducing possible errors. In recent years several studies have looked at field collection of hairs for use in genetic analysis. In one such study of genetic diversity in black bears, Boersen *et al.* (2003) lured the bears into bated hair trap enclosures lined with barbed wire. Although this method is only suitable for large mammals there has been no research into methods of sampling hairs from wild otters or on the DNA extraction efficiency from these hairs.

Molecular sexing has found wide application in a number of fields including field biology and conservation. Two approaches using PCR have been used for molecular sexing mammals: a) amplification of Y-specific loci and b) differentiation of homologous fragments present on both X and Y chromosomes (Fernando and Melnick, 2001). The first of these methods is PCR typing for the male specific mammalian SRY gene. Using this method positive amplification indicates male identity. However non amplification of the target fragment does not indicate female identity as PCR amplification may fail for many reasons (Fernando and Melnick, 2001). Co-amplification of a mitochondrial or single copy nuclear gene fragment has been used to address this problem. However, the sensitivity and optimal conditions of different primer sets are unlikely to be identical and external controls have been found to be unreliable, especially when amplifying from sub optimal sources of DNA such as hair or faecal matter (Fernando and Melnick, 2001). It has also been found that when using this method, samples can become contaminated with DNA from male collectors (Dallas *et al*., 2000). As far as we are aware the second method has not been tried on the Eurasian otter.

While amplification of the X and Y homologous fragments with a single primer set should be more reliable, discrimination of the X and Y fragments requires the presence of a Y fragment unique restriction site. Therefore to adapt this system to a new species, Y specific restriction sites that differentiate the X and Y fragments of that

species have to be identified.
The aims of this research were to develop an efficient method for obtaining hair samples in the wild and to determine the DNA extraction efficiency from collected hairs. This information will allow the development of suitable methods for studying the population density and movement patterns of otters within freshwater habitats.

METHODS

1. Hair trap design

The first hair trapping method used was adapted from a baiting station previously used to monitor pine martens (*Martes martes*) (Messenger and Birks, 2000). The bait station took the form of a wooden tunnel constructed from four 46 cm long sections of treated soft wood. This produced a tunnel of internal dimensions of 15 cm diameter. At each end galvanised zinc plated extension springs (Eliza Tinsley & Co Ltd., Cradley Heath, UK) were attached horizontally that allowed the coils to remain apart. One end of the spring was fixed using a metal staple and the other end rested on the point of an L-shaped hook constructed from a 3 mm brass rod. The hook and spring were set so that the spring was dislodged as an animal moved through the tunnel so that guard hairs could be trapped as the coil closed. This was tested (with two box traps) in an enclosure with one male and one female otter at the Chestnut Centre, Chapel en le Frith, Peak District (Grid Reference: NH 064809)
The second method used Velcro® attached to the inside of two artificial tunnels (Proton Supplies, Essex) to capture hairs and was tested in an enclosure with two male otters at the Highland Wildlife Park, Kingussie, Scotland (Grid reference: NH 809037).

Following the initial field trials on the captive otters, a new design of the traps using clay pipes were constructed (Fig. 1) and the cylindrical geometry of the pipes increased the probability of the otter brushing against the Velcro® straps. Holes were drilled through the top of the pipes and a plank of wood was fitted to the inside of the upper surface with two bolts and wing nuts. The plank was covered with Velcro® and the bolt fittings allowed the plank to be removed and a new one fitted without removing the whole trap from the field. To eliminate any possibility of genetic contamination, the Velcro® covered wood was collected with latex gloves and placed into a sterile bag and taken to the lab for inspection.

2. Genetic Analysis

a) Sample Collection

Genetic samples were obtained from 15 road killed otters, stored at –20 ºC. Paired samples were taken from each otter to allow an assessment of the reliability of the hair extraction techniques and genotyping success. A 1 cm^2 tissue sample was obtained from the interdigital webbing from either fore or hind foot using a farm ear punch (Agrihealth Ear Notcher, Farmrite Ltd, Portadown, UK) between the digits. The sample was stored in 100% ethanol. A hair sample was taken by gripping several guard hairs from the underside of the body and pulling the hairs backwards against their natural direction. These were stored in labelled bags with silica desiccant beads (RS Components Ltd. Corby, UK).

b) DNA extraction from tissue.

DNA was extracted from the tissue samples using a standard Proteinase K/high salt procedure. The tissue sample was dried thoroughly with a sterile towel to remove all

Figure 1 - Latest design of hair trap used to obtain guard hairs from otters. The hair is caught by a Velcro® patch fitted to the upper surface of the tunnel.

ethanol (which is a PCR inhibitor), and 0.5 cm^2 of the sample was chopped finely with a sterile scalpel and placed in a labelled eppendorf tube. 500 µl of TNES buffer and 35µl of Proteinase K were added and placed in a 60 ºC water bath overnight. To this 170 µl of 6M NaCl was added, mixed by inverting and microfuged at 14,000 rpm for 5-10 minutes. The supernatant was then transferred to a new labelled 1.5ml eppendorf tube. The DNA pellet was precipitated using 800µl ice-cold 100% ethanol. The

sample was centrifuged at 14,000 rpm for 10-20 minutes. Without dislodging the pellet of DNA the supernatant was removed. The pellet was then rinsed with 70% ethanol, the ethanol removed and the tube left to air dry for 10-30 minutes. As soon as the sample was dry the DNA was re-suspended in 100-200 µl of sterile water.

c) DNA Extraction from hair

Individual root hair segments approximately 3mm in length were treated by two different methods: (1) Chelex Ionic bead resin treatment and (2) Proteinase K digestion in a PCR compatible buffer. For method 1 a binocular microscope was used to select a hair with an intact follicle. Five hairs were cut 0.5 cm from the follicle, with a sterile scalpel, and placed in a 1.5ml eppendorf tube. 150 µl of 6M NaCl was added and the tube vortexed for 15 seconds. The tube was then centrifuged for 10 minutes at 11,000 rpm. Following this the supernatant was poured off and 75 µl 10% Chelex solution was added. This was then incubated for 10 minutes at 99 ^{o}C. Finally the tube was placed on ice for 2 minutes and the supernatant decanted into a clean eppendorf tube.

For method 2 a hair was selected from storage with an intact follicle as before. The hair was placed in a 1.5ml eppendorf tube with 50µl of TNES buffer and 10µl of Proteinase K. The tube was then vortexed for 10 seconds, microfuged for 1 second and incubated overnight at 55 ^{o}C in a water bath. The extract was then incubated at 94 ^{o}C to denature the enzyme and allowed to cool to room temperature.

The two extraction processes were carried out on each of the fifteen hair samples. Success of the extractions was assessed from PCR amplification of ten micro-satellite loci: Lut 435, 457, 615, 701, 715, 717, 818, 832, 833, 902.

d) DNA amplification

A standard three-step cycle was implemented on a Peltier Thermal Cycler (MJR): initial 94 ^{o}C for 2 min, followed by 30 cycles of 94 ^{o}C for 30s, (annealing temperature 53 - 57 ^{o}C) for 30s and 72 ^{o}C for 1 minute, with a 5min final 72 ^{o}C extension. Each 20 µl PCR reaction contained 5µl of DNA extract, 10µl PCR Reddy Mix (1.5mM $MgCl_2$, Bioline), 0.5µM of forward and reverse primers and 3µl of H_20. The products were then electrophoresed in a 1-1.5% ethidium bromide stained agarose gel, and visualised with UV light. The products were electrophoresed against a DNA ladder of known size fragments to compare with those derived by PCR.

e) Microsatellite analysis

The DNA extracts were genotyped for 9 microsatellites Lut 435, 457, 615, 617, 715, 832, 833, 835 and 902 using the latest versions of the primers (Dallas *et al.*, 2002, Huang *et al.*, 2005). To 5µl of PCR product, 2µl of EXOSAP was added and incubated at 37 ^{o}C for 15 minutes followed by, an incubation at 80 ^{o}C for 15 minutes. For the cycle sequencing reaction set up, 1µl of this product was transferred to an ABI sequencing plate. Added to this was 1µl of buffer, 2µl Terminator Ready Reaction mix, 2µl primer (forward and reverse in separate reactions) and 4µl of H_20. The plate was placed in a Peltier Thermal Cycler and set on the sequencing programme. Following this 1.5µl of 3M sodium acetate, 31.25µl of ethanol and 7.25µl of deionised water was added to each well for the cycle sequencing reaction clean up. The plate was vortexed briefly on the agilent vortexer and left at room temperature for 15 minutes to precipitate the extension products. The plate was then spun in the centrifuge for 45 minutes at 3100 rpm. The supernatant was discarded by inverting the plate over a paper towel

and the pellet was further cleaned by adding 150 µl of 70% ethanol. This was spun in the centrifuge for 10 minutes and spun briefly upside down over a paper towel to remove all of the ethanol. Finally the pellet was re-suspended in 10 µl of formamide and vortexed. The products were then resolved on polyacrilamide gels using an ABI 377 automated sequencer and analysed using GENESCAN software (Applied Biosystems, Foster City, USA).
Proportions of allelic dropout and false allele detection associated with hair DNA extracts were estimated by comparing the genotypes of the hair extracts with the genotypes from the tissue extracts from the same otters. The proportion of allelic dropout was estimated as the proportion of all PCR assays of hair DNA in which a fragment observed in the tissue of a given individual was undetected in the hair DNA from the same individual. The proportion of false allele detection was estimated as the proportion of all PCR assays of hair DNA in which a fragment observed in hair DNA of a given individual was undetected in the tissue sample from the same individual.

f) Molecular sexing

DNA was extracted from 4 male and 4 female otter tissue samples using the Proteinase K digestion protocol described. PCR amplification of ZFX – ZFY fragments was performed using primers P1-3EZ and P2–3EZ, following the protocol of Fernando and Melnick (2001). PCR products were sequenced in forward and reverse directions using ZFSEQ and P1–3EZ. It was noted that in the protocol of Fernando and Melnick (2001), the reverse primer was mislabelled as P2- 3EZ. Sequences were analysed with ABI 377 automated sequencer, and aligned and edited using BioEdit software (BioEdit, Carlsbad, USA).
The PCR amplification was successful for all individuals and approximately 400 bp of the amplified fragments were used in the analysis. As both the X and Y chromosome fragments were amplified from male nuclear DNA, positions polymorphic between the X and Y sequences were represented by double peaks in the chromatograms of both forward and reverse directions. Comparison of the female (which only have the X copy) and male sequences enabled us to deduce the sequence of the Y fragment using the programme BioEdit. The sequences were aligned by sight and positions that were polymorphic in the male sequences were identified and a specific restriction enzyme was chosen that digested the X and Y fragments in different places. The enzyme chosen was Xmn1 and the positions of its restriction sites were at 150 and 330 bp on the X fragment.
For the enzyme digestion 10 µl of PCR product was placed in an eppendorf tube with 1 µl of Xmn1, 2 µl of NEB buffer and 7 µl of H_20. This was then incubated at 37 ^oC for 2 hours and the digested products electrophoresed on an agarose gel stained with ethidium bromide.

Results

1. Hair traps

a) Method 1

On the first night of the trial, the two boxes minus the springs were set out in the enclosure to allow the animals to adjust to changes in their surroundings. The female and the male otter were not obviously affected by the traps and engaged in play activity close to them. On the second evening, the springs were set and the female otter entered the box and retreated suddenly as the

spring coiled. She did not re-enter the box in the further 12 trap nights. The male otter had observed the behaviour of the female and also did not re-enter the box. Finally, the boxes were baited daily with their normal food of day-old-chicks (*Gallus domesticus*) and anchovy paste however, the otters continued to avoid entering the boxes and therefore no hair sample was collected.

b) Method 2

The two males involved in this trial were initially wary of the boxes and would not approach them. After two unsuccessful nights the boxes were baited with chicks. However the otters were observed to seize the chicks and retreat quickly to a safe place to eat them. This occurred for the first week until the otters finally accepted them as safe objects and began to venture through them. Although the Velcro® also collected other debris, hairs were found to be attached five nights out of seven or a success rate of 0.71 samples collected per trap night.

c) DNA extraction and amplification

The Proteinase K digestion used for the tissue extraction gave a 100% success rate. As the DNA concentrations for the tissue samples were very high, the samples were diluted 1/100 for PCR and the optimum conditions for the primers were tested using these extractions. The Chelex hair extraction method gave a 55% amplification success rate. The Proteinase K method gave a much higher amplification success of 87%.

d) Microsatellite analysis

The proportions of PCR errors in typing hair DNA extracts for the nine microsatellites, estimated by comparing genotypes of the nine microsatellites from the tissue samples, were 0.033 for allele dropout and 0.011 for false alleles.

f) Molecular sexing

The enzyme, Xmn 1 was found to be unsuccessful at molecular sexing the Eurasian otter. Several different proportions of enzyme to product and buffer were tried but the digest did not consistently produce the expected fragments.

DISCUSSION

1. Non-invasive sampling using hairs

Hair samples have been used in the study of various mammal species and several sampling methods have been tested (e.g. Boersen *et al*., 2003). However, none of these sampling methods were considered suitable for the study of the Eurasian otter. The ‘spring’ method that previously sampled pine marten hairs (Messenger and Birks, 2000) was not successful in capturing hairs from captive otters. Although this method has advantages over adhesives that lose their stickiness quickly or become fouled with dust and other debris, the action of the spring alarmed the otter when entering a trap. The second method that was tested in this study may be particularly useful in the field as the design is in keeping with the natural environment and can be incorpo-

rated easily into otter paths and the entrances to holts. The Velcro® covered planks can also be removed easily from the field without the disturbance of the whole trap. The only other attempt to collect hairs from otters was using artificial log pile holts (Cowell *et al.*, 2001). Nineteen artificial log pile holts were set up and dismantled prior to reconstruction. The bedding was analysed and 13 contained otter guard hairs.

Clearly, field collection of hair for non-invasive genetic sampling studies involves significant investment of time and energy, hence maximising the success of the genetic analysis is essential. Sample storage method has been shown to influence success and error rates (Roon *et al.*, 2003). In a recent evaluation of methods for preserving hair no significant difference in amplification success was found between freezing at -20 ^{o}C and silica desiccation, and the latter has advantages in field collection and transportation of samples (Chanin and Coxon, 2000, Dallas *et al.*, 2003) Hence desiccation with silica beads was chosen as the sample storage method. The time between collection and extraction is also though to affect success and error rates as DNA degrades with time particularly when exposed to moisture or UV light. The samples in this study were stored for a maximum of 1 month to reduce any associated time degradation errors.

It is possible that the reactions may have failed not due to the low amount of initial template but due to the presence of PCR inhibitors. Vigilant (1999) showed that for naturally shed hairs, it was possible to amplify a mtDNA target from 70% of samples. However, other reported success rates using Chelex are often substantially lower. The Proteinase K method gave a much higher amplification success rate of 87%. This is only slightly higher than the observed result of 85% by Vigilant (1999). The results from our study represent only one attempt with a single sample, the percentage of successful samples could increase slightly with repeated testing.

2. DNA extraction and amplification

Extraction of DNA from hairs using Chelex resin is the method most commonly used with field collected hair (Taberlet *et al.*, 1997); however in a recent evaluation of extraction methods, Proteinase K digestion also gave a high success rate (Vigilant 1999). Hence a comparison of the two methods was made. The Proteinase K digestion was the simplest method and also gave the highest success rate. The use of this procedure in which the root hair is digested in a PCR compatible buffer allows the processing of the hair with no chance of loss of any sample material. It has been observed that components that co-purify with the Chelex extracted DNA can inhibit subsequent analysis and possibly cause DNA degradation during storage, making the alternative of an enzymic digestion without further purification particularly useful (Vigilant, 1999). The success rate of this extraction process suggests that hair is indeed a useful source of DNA.

Genotyping errors in this study were

relatively high. The proportions of allelic dropout and false allele detection were similar to that found by Dallas *et al.* (2003) using DNA extracted from spraints.

However there are several strategies of limiting errors, which were not used in the methodology due to time and budget constraints. One strategy is to obtain more DNA by extracting more material. However, for this method using more than one hair collected from the tunnels could be risky as there is a chance they may originate from more than one individual. Increasing the amount of material can also increase the amount of PCR inhibitors in an extract which can prevent genotyping even if sufficient quantities of DNA are obtained.

Alternatively the quality of samples can be greatly improved by collecting the hairs just after the animal has left them behind. If the samples remain in the field for several weeks before collection then the DNA will be degraded and be difficult to amplify. This would mean checking the traps every 2-3 days for the best results. Another option is to multiplex (co-amplify) several loci during PCR, allowing more efficient use of limited amounts of DNA. However this approach would require time consuming adjustments that could be technically difficult when the quantity and quality of the DNA is low.

The most conservative method of obtaining reliable genotypes from small quantities of DNA is to repeat each DNA amplification independently for each locus several times (the multi-tube approach). However, this solution is time consuming, and more expensive than single tube amplification. The purpose of the multi-tube approach is to provide reliable genotyping when using very dilute DNA samples. It consists of repeating PCR experiments using aliquots of the same DNA extract. The genotype is then deduced by analysing the whole set of experiments. This method is the best for the detection and monitoring of the three main possible errors: (1) allelic dropout, (2) false alleles and (3) sporadic contamination. Clearly this would have been the best approach for this study and appears to be the most widely applied.

3. Contamination control

Contamination of genetic samples can be a problem in field collection and particularly in the laboratory. Along with the possibility of detecting a single target molecule there is also the possibility of detecting a single contaminant molecule (Taberlet and Luikart, 1999). Extreme care was taken to avoid contaminations, either by PCR products, or by concentrated genomic DNA. Several laboratory rules had to be adhered to, the first being physical separation of the laboratory rooms where pre and post PCR experiments were carried out. Other procedures included the avoidance of concentrated DNA extracts in the extraction room, the use of pipettes with aerosol resistant tips and continuous monitoring of all reagents for DNA contamination (use of negative controls).

4. Molecular sexing

A simple method for identifying positions that are polymorphic between X

and Y sequences is described by Roon *et al.* (2003). The positions are identified by the presence of double peaks in the chromatogram upon direct sequencing of PCR products from a male enabling the recognition of unique restriction sites on the Y fragment. This study has identified three fixed substitutions between X and Y copies of the ZF gene that allow categorical sexing from hairs by sequencing. Our experiment suggests that restriction with Xmn1 does not provide a robust assay for differentiating the polymorphisms. The restriction enzyme *Hph*1 is predicted to cut an alternative polymorphic site but was restrictively expensive. Development of X and Y specific PCR may be the most promising option for a sexing assay that avoids sequencing.

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REFERENCES

Boersen M.R., Clark J.D. and King T.L. 2003. Estimating black bear population density and genetic diversity at Tensas River, Lousiana, using microsatellite DNA markers. *Wildlife Society Bulletin*, 31(1): 197-207.

Chanin P. 2003. Ecology of the European otter. Conserving Natura 2000 Rivers. Ecology Series 10. Peterborough, English Nature.

Chanin. P and Coxon K. 2000. DNA fingerprinting of the otter spraint. In Conroy J.W.H., Yoxon P. and Gutleb A.C. (eds), Proceedings of the first otter toxicology conference. Broadford, Skye, UK. *Journal of the International Otter Survival Fund*, 1: 167-172

Cowell D., Thomas G., Liles G., Bradshaw A., Midgley L. and Slater F. 2001. Monitoring the use of artificial log pile otter holts using hair analysis from bedding. *IUCN Otter specialist group Bulletin*, 18: 6-10.

Dallas J.F. and Piertney S.B. 1998. Microsatellite primers for the Eurasian otter. *Molecular Ecology*, 7: 1247-1251.

Dallas J.F., Bacon P.J., Carss D.N., Conroy J.W.H., Green R., Jeffries D.J., Kruuk H., Marshall F., Piertney S.B. and Racey P.A. 1999. Genetic diversity in the Eurasian otter, *Lutra lutra*, in Scotland. Evidence from microsatellite polymorphism. *Biological Journal of the Linnean Society*, 68: 73-86.

Dallas J.F., Carss D.N., Marshall F., Koepfli P.F., Kruuk H., Piertney S.B. and Bacon S.B. 2000. Sex identification of the Eurasian otter *Lutra lutra* by PCR typing of spraints. *Conservation Genetics*, 1: 181-183.

Dallas J.F., Coxon K.E., Sykes T., Chanin P.R.F., Marshall F., Carss D.N., Bacon P.J., Piertney S.B. and Racey P.A.

2003. Similar estimates of population genetic composition and sex ratio derived from carcasses and faeces of Eurasian otter *Lutra lutra. Molecular Ecology*, 12: 275-282.

Dallas J.F., Marshall F.H., Piertney S.B., Bacon S.B. and Racey P.A. 2002. Spatially restricted gene flow and reduced microsatellite polymorphism in the Eurasian otter *Lutra lutra* in Britain. *Conservation Genetics*, 3: 15-29.

Fernando P. and Melnick D.J. 2001. Molecular sexing Eutherian Mammals. *Molecular Ecology Notes*, 1: 350-353.

Frantz, A.C., Schaul M., Pope L.C., Fack F., Schley L., Muller C.P. and Roper T.J. 2004. Estimating population size by genotyping remotely plucked hair: the Eurasian badger. *Journal Applied Ecology*, 41:985-995.

Goossens B., Waits L.P. and Taberlet P. 1998. Plucked hair samples as a source of DNA: reliability of dinucleotide microsatellite genotyping. *Molecular Ecology*, 7: 1237-1241.

Huang C., Hsu Y., Lee L.L. and Li S.H. 2005. Isolation and characterization of tetramicrosatellite DNA markers in the Eurasian otter (*Lutra lutra*). *Molecular Ecology Notes*, 5(2): 314-316.

Messenger J.E. and Birks J.B.S. 2000. Monitoring the very rare pine marten populations in England and Wales. In Griffiths H.I. (ed.),. Mustelids in a modern world. Backhuys Publishers, Leiden, The Netherlands, 217-230

Morin P., Wallis J., Moore J., Woodruff D. 1994. Paternity exclusion in a community of wild chimpanzees using hypervariable simple sequence repeats. *Molecular Ecology*, 3: 469–478.

Roon D.A., Waits L.P. and Kendall C. 2003. A quantitative evaluation of two methods for preserving hair samples. *Molecular Ecology Notes*, 3: 163-166.

Taberlet P. and Luikart G. 1999. Non invasive genetic sampling and individual identification. *Biological Journal of the Linnean Society*, 68: 41-55.

Taberlet P. Waits L. and Luikart G. 1999. Non invasive genetic sampling: look before you leap. *Trends in Ecology and Evolution*, 14: 323-327.

Taberlet P., Camarra J.J., Griffin S., Uhres E., Hanotte O., Waits L.P., Dubois-Paganon C., Burke T. and Bouvet J. 1997. Non invasive genetic tracking of the endangered Pyrenean bear population. *Molecular Ecology*, 6: 869–876.

Vigilant L. 1999. An evaluation of techniques for the extraction and amplification of DNA from naturally shed hairs. *Biological Chemistry*, 380: 1329-1331.

Woods J.G., Paetkau D., Lewis D., McLellan B.N., Proctor M. and Strobeck C. 1999. Genetic tagging of free-ranging black and brown bears. *Wildlife Society Bulletin*, 27: 616-627.