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Identification of mustelid species: otter (*Lutra lutra*), American mink (*Mustela vison*) and polecat (*Mustela putorius*), by analysis of DNA from faecal samples

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Abstract

The abundance of mink, otter, and polecat may be inferred from records of their scats, and the feeding biology of the species is often studied by analysis of skeletal remains and other hard parts in faeces. However, in some situations it is difficult to distinguish between faeces from these three mustelid species. A method is described for assigning faeces to these three mustelid species, based on analysis of DNA extracted from their scats. Mustelid-specific primers were developed for PCR amplification of a part of the mitochondrial cytochrome *b* gene, and two restriction enzymes were found to detect species-specific sequence variation. Analysis of DNA from different faecal samples showed that the results were reproducible and that the approach provided an efficient method of species identification.

Key words: *Lutra lutra*, *Mustela vison*, *Mustela putorius*, DNA, faeces

INTRODUCTION

Carnivores living along rivers and lakes often include fish in their diets. The otter (*Lutra lutra* L.) is the main fish-eating species among European riverine mammals, and has traditionally been considered vermin, killing fish and competing with humans. During the past decades, however, the otter has declined severely all over Europe, and is now protected in most countries. In the same period the American mink *Mustela vison* Schreber has spread in several European countries having escaped from fur farms, and has bred in the wild. Mink often inhabit river and lake areas, and include fish in their diet along with mammals and birds (Erlinge, 1969; Wise, Linn & Kennedy, 1981; Dunstone & Birks, 1987). The increasing populations of mink have been reported to predate fish farms as well as taking native wildlife and free-range poultry (Lever, 1978; Chanin, 1985). Moreover, predation by mink on wild fish populations has become a matter of concern (Heggenes & Borgstrøm, 1988). In turn, predation by both otter and mink has the potential to affect fish populations. This may be an issue of concern, for instance when rehabilitating salmonid populations by means of restocking, because the effectiveness of local stocking may depend on mortality factors such as predation.

The abundance of mink and otter is often inferred from records of their scats and, in the otter, this type of monitoring plays an important role in conservation programmes (Green & Green, 1987; Madsen, Collatz-Christensen & Jacobsen, 1992). To make quantitative assessments of predation by these species on native fish and wildlife populations, two types of approach have been used. One consists of correlating visual observations of the activity of the species with changes in the mortality rates of their potential prey species. While this may be feasible in the mink (Heggenes & Borgstrøm, 1988), the shyness and nocturnal feeding habits of otter in most of Europe renders this an extremely difficult task. A more direct way of estimating relative predation pressures consists of analysing skeletal remains in faeces (Carss, Kruuk & Conroy, 1990). It has been demonstrated that this is a reliable source of information, both for identifying the prey species consumed and for estimating the size of the prey (Wise, 1980; Jacobsen & Hansen, 1996).

When scats are used for studying the abundance and feeding biology of mink and otter, it is essential to know which carnivore produced them. Both species may share the same habitat and marking places, but the sweet odour and the appearance of otter spraints normally makes it possible to distinguish them from mink faeces. Nevertheless, when both contain remains of the same food item, mostly fish, identification can be uncertain. Further, distinguishing mink faeces from those of polecat *M. putorius* L. is difficult if not impossible (B. Jensen, pers. comm.). Even though polecat is less associated with aquatic habitats than are mink and otter, and it probably only uses riparian habitats occasionally, it does eat fish (Weber, 1989). Misidentification of faeces could add a serious error to studies of abundance

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and diet of mink in areas that are also inhabited by polecat.

The purpose of the present study was to develop a method for distinguishing between scats of otter, mink and polecat. Faeces have been used as a source of DNA from both the defaecator as well as residues of food items remaining in the faeces (reviewed by Kohn & Wayne, 1997). Consequently, we focused on the possibility of extracting DNA and analysing species-specific DNA markers from scats of the three mustelid species. The protocol must be based on the polymerase chain reaction (PCR) in order to be able to analyse minute amounts of DNA. Furthermore, otter, mink and polecat are known to feed on a variety of prey (Wise *et al*., 1981; Mason & Macdonald, 1986; Weber, 1989). In order to avoid amplification of DNA from residues of prey in the faeces, it was necessary to design PCR primers specific to the three mustelid species that at the same time contained too many sequence mismatches to allow for amplification of DNA from the most common prey items (primarily fish, mice, amphibia and birds).

MATERIALS AND METHODS

Sampling and DNA extraction

DNA was analysed from ethanol preserved muscle tissue samples from otter, mink and polecat, which had either been killed by traffic (otter) or been shot by hunters (mink and polecat). Nine faecal samples were analysed, which had been collected at various localitites in Northern Jutland, Denmark. On the basis of smell and physical appearance these samples were categorized as mink or polecat scats, but not otter. A further 14 faecal samples were collected along a *c*. 10 km stretch of the River Trend in Northern Jutland. It was known from observations and previous surveys that otter inhabited the area, and mink were assumed to be present, perhaps as escapes from nearby mink farms. A few other faecal samples, some assumed to be from red fox (*Vulpes vulpes*) and others of unidentified but possibly mustelid origin, were also collected. Based on the freshness of the scats, we estimated that they were less than 1 week old at the time of collection.

DNA was extracted from tissue samples by ordinary phenol-chloroform extraction (Sambrook, Fritsch & Maniatis, 1989). Different protocols were tested for extraction of faecal DNA, including phenol-chloroform extraction and chelex extraction (Paxinos *et al*., 1997). However, the technique described by Gerloff *et al*. (1995) was the only one yielding DNA suitable for PCR amplification. Briefly, this protocol is based on lysis of cells in a buffer with a high concentration of guanidine-thiocyanate, a strong protein denaturant. The DNA is bound to diatomaceous particles, and after several washes with guanidine-thiocyanate buffer and ethanol the DNA is eluted from the particles. For further technical details, see Gerloff *et al*. (1995). We extracted DNA from approx. 50 mg faeces per sample. In order to test the reproducibility of results, DNA was extracted twice from 8 of the samples.

Design of PCR primers and PCR reaction conditions

We chose to focus on the mitochondrial cytochrome *b* gene, as in this segment there is usually some sequence variation between species, but little or no variation within species (Avise, 1994). Sequences for the mitochondrial cytochrome *b* gene from otter (GenBank accession no. X94923), mink (S73783), polecat (X94925), house mouse *Mus musculus* (J01420), frog *Rana japonica* (S76594), duck *Cairina* sp. (L07521), brown trout *Salmo trutta* (M64918), and European eel *Anguilla anguilla* (D28775) were aligned, using the program DNASIS 2.1 (Hitachi Software Engineering Co., 1995). The result of the alignment for 189 bases is shown in Fig. 1. On the basis of this alignment, a set of mustelid-specific PCR primers was designed (the priming sites are indicated in Fig. 1): *Mustcytb L* 5′ TTA GCC ATA CAC TAT/C ACA TCA GAC 3′ and *Mustcytb H* 5′ TCA TGT TTC G/TGT/G G/AAA TAT ATA A 3′. The latter primer was marked with CY5 at the 5′ end in order to allow for detection of fragments on an automated sequencer. The expected size of the amplified segment was 189 bp.

PCR reactions consisted of 5 μl 10X SuperTaq PCR buffer (HT Biotechnology), 0.2 μM of each primer, 0.2mM dNTP mix, 0.5 unit SuperTaq polymerase (HT Biotechnology) and 1 μl (DNA extracted from tissue) or 3 μl (excremental DNA) template DNA. Distilled water was added to a final volume of 50 μl. PCR amplification took place in a Techne Genius thermal cycler and consisted of 40 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 50 °C and 30 s extension at 72 °C.

Restriction enzyme analysis

A search for restriction enzyme recognition sites, using the program DNASIS, revealed that the enzyme *Taq* I detected restriction sites unique to both mink and polecat. Also, the enzyme *Nla* IV detected a restriction site unique to otter (see Fig. 1). Restriction digests took place according to the manufacturer's (New England Biolabs) recommendations in volumes of 14 μl, consisting of 8 μl PCR product, 1.4 μl restriction enzyme buffer, 4.6 μl distilled water and 2.5 units restriction enzyme. The digested DNA was analysed on an ALFexpress automated sequencer according to the recommendations of the manufacturer (Pharmacia). This allowed for detection of the fragment containing the CY5-labelled primer. The predicted size of the fragment was as follows: *Taq* I: polecat 127 bp, mink 101 bp, otter 189 bp; *Nla* IV: polecat 189 bp, mink 189 bp otter 80 bp. Aliquots of the two restriction digests for each sample were mixed and analysed in multiplex on the automated sequencer.

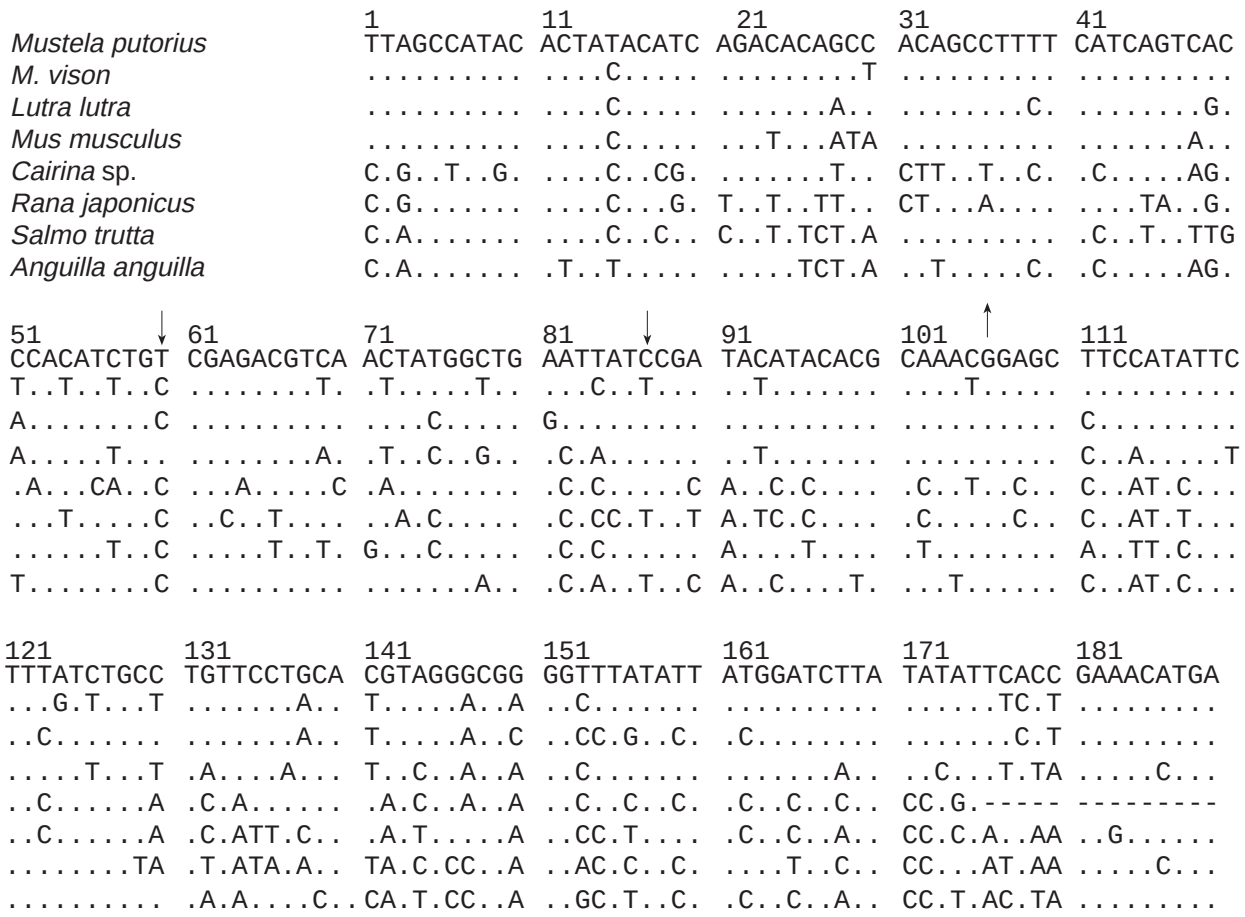

Fig. 1. Aligned sequences of a part of the mitochondrial cytochrome *b* gene from polecat *Mustela putorius*, mink *M. vison*, otter *Lutra lutra*, house mouse *Mus musculus*, duck *Cairina* sp., frog *Rana japonicus*, brown trout *Salmo trutta* and European eel *Anguilla anguilla*. Priming sites for the *Mustcytb H* and *L* primers are underlined. Restriction sites for the enzyme *Taq* I (recognition sequence TCGA) in polecat and mink are indicated by downward arrows, while the *Nla* IV restriction site (recognition sequence GGNNCC) in otter is indicated by an upward arrow.

RESULTS

As expected, a 189 bp segment was amplified, using DNA from tissue of otter, mink and polecat as template. The segment was successfully amplified from all 23 faeces samples originating from mustelids. No amplification was observed from red fox scat DNA. Similarly, no amplification was observed for the samples of unknown origin (as visualized under UV light on a 3% ethidium bromide stained agarose gel). This could mean that the faecal samples were not from mink, otter or polecat or that PCR amplification was unsuccessful for technical reasons, for instance due to highly degraded DNA. The quantities of PCR product varied among samples and were in some cases clearly lower than for DNA extracted from tissue.

The observed sizes of restriction fragments from the three species were as predicted, as determined by analysis of DNA extracted from tissue (i.e. otter, 80 and 189 bp; mink, 101 and 189 bp; polecat, 127 and 189 bp). All the faecal samples that were successfully amplified yielded fragment profiles characteristic of one of the three mustelids, and there was no indication of amplification of DNA from residues of prey. In all cases, double extraction and analysis of DNA from the same sample yielded identical profiles, which demonstrated that the results were reproducible. Examples of typical fragment profiles of the species are shown in Fig. 2.

On the basis of the DNA analyses, the nine faecal samples initially classified as coming from mink or polecat were found to have come from mink ($n = 3$), polecat ($n = 5$), and otter ($n = 1$). Thus, the analyses suggested that one sample of otter spraint had been misclassified as mink or polecat. Upon reinspection, this sample did appear to have a smell and texture characteristic of otter faeces. Of the 14 scats collected along the River Trend, 13 were classified as otter spraints and one as mink, confirming the presence of mink in the area.

DISCUSSION

Analysis of scat DNA (often referred to as molecular scatology) is a recent development that has been used

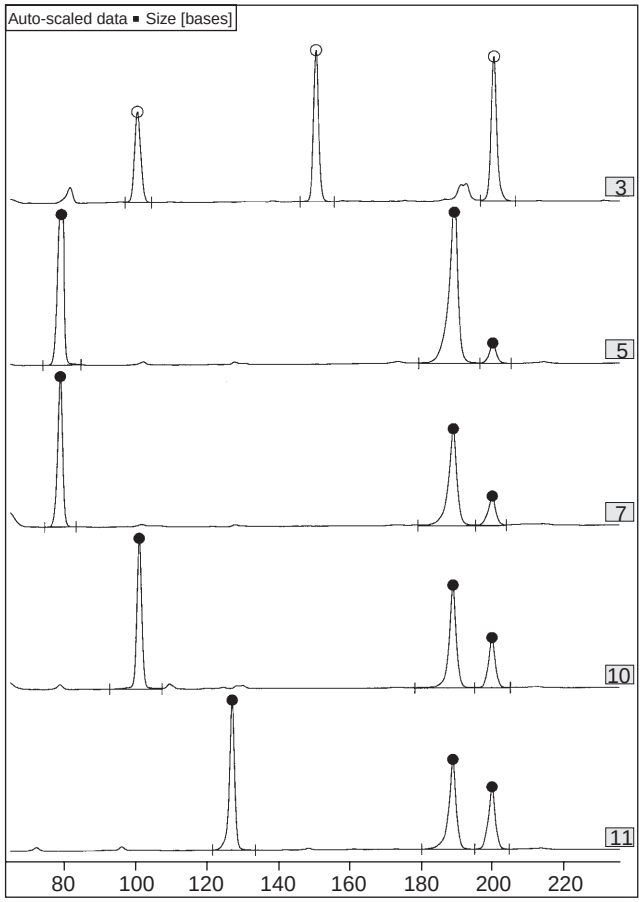

Fig. 2. Typical fragment patterns of the 189 bp cytochrome *b* mtDNA segment digested with *Taq* I and *Nla* IV restriction enzymes and analysed on a Pharmacia ALFexpress automated sequencer (the scale is in base pairs). Lane 3: 100, 150 and 200 base pair size marker. Lane 5: DNA extracted from otter tissue, 80 and 189 bp fragments and 200 bp internal size marker. Lane 7: Faecal sample, 80 and 189 bp fragments and 200 bp internal size marker, classified as otter. Lane 10: Faecal sample, 101 and 189 bp fragments and 200 bp internal size marker, classified as mink. Lane 11: Faecal sample, 127 and 189 bp fragments and 200 bp internal size marker, classified as polecat.

not only for studies of the genetic structure of populations of mammals (Höss, Kohn & Pääbo, 1992; Gerloff *et al*., 1995; Kohn *et al*., 1995), but also for assigning individual identity to faeces (Reed *et al*., 1997). This renders it possible to study the feeding habits of individual animals. Identification of species is a further application of molecular scatology and, apart from the present study, molecular markers have been used by Reed *et al*. (1997) and Paxinos *et al*. (1997) to distinguish between faeces from seal species and canid species, respectively. Our results demonstrate that analysis of scat DNA is useful also for distinguishing between mustelid species. PCR amplification of the targeted segment from excremental DNA was highly successful, the results were reproducible and no restriction digest patterns were observed that could not be accounted for by one of the three mustelids under investigation. Only in extreme situations, for instance if an otter preyed on a mink, which has been reported from Russia (Novikov, 1956), might problems occur and amplification of segments from both species could be envisaged.

The use of scats to monitor distribution and abundance of a mammal relies, of course, on correct identification of the species producing the scat. Unambiguous identification of mink faeces represents an important step towards assessing the abundance and, if combined with analyses of dietary choice, an important aspect of the environmental impact of this species. Reliable identification of faeces is even more important for an endangered species such as the otter. Confirmation of the presence of otters in an area might lead to conservation measures being taken, whereas missing a positive otter sign could lead to omission of protection measures that might be crucial to ensure the survival of populations. Analysis of species-specific DNA markers yields efficient species identification of faecal samples and circumvents subjective evaluation of smell and appearance of spraints. The finding of an otter spraint originally misclassified as mink or polecat scats, the success of distinguishing between polecat and mink excrement, and the confirmation of the presence of mink in the River Trend area demonstrate the relevance of applying analysis of faecal DNA in situations where accurate species identification is needed.

In general, analysis of faecal DNA opens the possibility of a number of new research opportunities related to both population genetics and ecology, the latter including studies of migratory behaviour and estimation of both census and effective population sizes (Kohn & Wayne, 1997). These applications must be considered particularly relevant to elusive species such as otter and other mustelids. Apart from estimation of genetic variation and other population genetic parameters, analyses of hypervariable nuclear DNA markers, such as microsatellites, have led to new insights into migratory behaviour and family relationships among individuals in mammal species (Prodöhl *et al*., 1998). However, particularly in the otter, collection of tissue samples has so far been restricted to individuals killed by traffic or drowned in fishing nets. Faecal samples represent an easy way of non-invasive sampling, potentially making large numbers of individuals available for analysis. Further, assuming that the microsatellite multilocus genotype of any particular individual is unique to that individual, spraints may be considered individual-specific genetic tags and represent information on ecological and behavioural parameters of individuals as well as the total population size within an area. Analysis of faecal DNA can in this way represent an important supplement to studies of the home range and migratory behaviour of otters, based on radio-tracking combined with radioactive marking of released animals (Green, Green & Jeffries, 1984; Sjöåsen, 1996). Moreover, analysis of faecal DNA can improve estimates of population sizes

by traditional survey methods, which are based on the combination of monitoring traces of the animal (spraints, footprints) and assumptions of density of individuals (Anon., 1984; Green & Green, 1987; Madsen *et al*., 1992). Thus, molecular scatology could add substantially to the scientific basis for conservation of this endangered species.

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