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# Identifying species from pieces of faeces

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Endangered populations are complicated to study due to low densities. However, faeces are frequently used as a source of information in conservation to confirm presence and diet. Moreover, advances in molecular biology permit researchers to analyse DNA from faeces rendering information on populations, home ranges, genetic variation and phylogenetic relationships (Kohn and Wayne 1997).

To complete such studies, however, the faeces need to be correctly assigned to the species in question. This can sometimes be difficult through conventional methods (Reed et al. 1997; Farrell et al. 2000). Davison et al. (2002) showed that expert naturalists fail to distinguish faeces from martens (*Martes martes*) and red foxes (*Vulpes vulpes*). Several molecular methods have been developed for species identification of faeces. A direct approach involves amplification and sequencing of DNA extracted from the faeces (Höss et al. 1992; Farrell et al. 2000). This approach is straightforward but time-consuming and expensive. Therefore, several studies have instead employed species-specific restriction enzymes (Paxinos et al. 1997; Hansen and Jacobsen 1999), but if only one restriction site is used, a failure in the reaction would lead to false positives. In a recent study, faeces from Iberian lynx (*Lynx pardinus*) were identified by use of species-specific primers (Palomares et al. 2002). However, in this study a failed amplification could be due to low DNA content of the sample or the faeces originating from another species, thus creating false negatives. Mills et al. (2000) analysed hair samples and used a combination of felid-specific primers and several restriction enzymes that solves these two problems.

In this paper we describe a simple six-hour method for faecal samples that handles the problem of false negatives. The method is designed to separate arctic fox (*Alopex lagopus*), red fox and wolverine (*Gulo gulo*), since faeces from these species are difficult to distinguish. The method has been implemented in the management of the endangered Fennoscandian arctic fox. Our method is based on a multiple primer system, from now on referred to as Rapid Classificatory Protocol PCR (RCP-PCR). One of the primers is designed to anneal to all species, whereas the others are species-specific. The specific primers bind at different distances from the general primer. Hence, the use of all primers in a single tube PCR results in fragments of different size depending on which species the faeces originates from (Figure 1).

We designed species-specific primers (Table 1) *Pex1F* (arctic fox: 5'-TAAACTATTCCCTGATACTC-3'), *Vul1F* (red fox: 5'-TCAATCCTTGCTCGAAG TA-3') and *Gulo1F* (wolverine: 5'-AGTATGTACCC TTTTCCTCC-3'). The primer *H3R* (5'-CCTGAAGT AGGAACCAGATG-3') was chosen as reverse primer since it binds to most mammals (e.g., humans, elephants, beavers, horses, cows, arctic foxes; *personal observation*). The species-specific primers had a 100% match to 27 red fox and 44 wolverine control region sequences on the genbank database. Further, we have sequenced *c.* 200 arctic foxes from all over the world using *Pex1F*, and never had a failed amplification. It is therefore unlikely that a polymorphism would generate false negatives.

We initially extracted DNA from muscle tissue of two wolverines, three arctic and three red foxes using Qiagen's Dneasy tissue kit (Qiagen). The primers were first tested alone with *H3R* in a PCR, and then together in a single tube PCR. All reactions yielded single fragments of the expected size when scored on a 1.5% agarose gel.

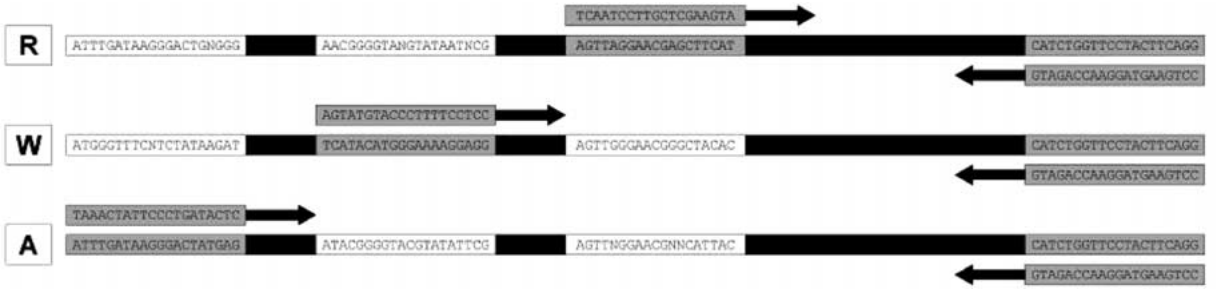


*Figure 1.* Illustration of the concept of RCP-PCR. One of the three species-specific primers will react with the general primer. The resulting fragment size depends on whether red fox (R), wolverine (W) or arctic fox (A) DNA is present in the extract. Intra-specific variable sites in the template are are shown as (N).

*Table 1.* Results from the RCP-PCR. The values in brackets were muscle samples whereas all other were from faeces

| Sample | Results from the PCR | | | | |
|---|---|---|---|---|---|
| | Arctic fox (332bp) | Red fox (100bp) | Wolverine (242bp) | Failed | TOTAL |
| Known AF | 11+(3) | 0 | 0 | 0 | |
| Known RF | 0 | 11+(3) | 0 | 0 | |
| Known W | 0 | 0 | 2+(2) | 0 | |
| Unknown | 40 | 57 | 2 | 29 | 128 |

Faeces with known origin were collected at a zoo (Skansen, Stockholm) and in the field from dens with known inhabitants (Table 1). DNA was extracted using Qiaamp DNA stool kit (Qiagen), and was subsequently subjected to a four-primer single tube 25 $\mu$l PCR as follows: 2 $\mu$l DNA extract, 0.2 mM of each nucleotide, 2.5 mM $MgCl_2$, 0.1 mg/ml BSA, 0.5 $\mu$M of each primer, 10x PCR Gold Buffer and 0.75 units of AmpliTaq Gold polymerase (Perkin Elmer Cetus). The cycling parameters for the PCR-reaction were: 94 °C denaturation for 10 min, followed by 35–40 cycles of 94 °C denaturation for 20 s, 55 °C annealing for 30 s, and 72 °C extension for 20 s, followed by a single 7-min final extension at 72 °C.

We also analysed 128 faeces of unknown origin collected during field surveys all over Fennoscandia. The sampling protocol was designed to minimise the possibility that several samples were taken from the same individual. The samples were gathered in plastic jars containing silica pellets (Wasser et al. 1997) and stored at –80 °C pending extraction. We used approximately 200 mg of faecal material for each extraction and analysed as described above. We also sequenced one fragment from each species. Sequences were obtained using a CEQ 2000XL automated sequencer (Beckman Coulter) according to the manufacturer's instructions. All extractions were performed in a physically isolated laboratory, dedicated to DNA extractions. We used one extraction blank for every ten samples extracted.

The sequence from the 332 bp fragment was identical to a haplotype observed in wild Scandinavian arctic foxes (Dalén et al. 2002). The 242 bp fragment gave a wolverine sequence. The 100 bp fragment gave a fox sequence. This 100 bp region is identical in some red and arctic fox haplotypes. However, since the sequence obtained did not correspond to any of the arctic fox haplotypes observed in Scandinavia it presumably came from a red fox.

Samples of known origin consistently gave the results expected, as did the sequences from the PCR products. This suggests that RCP-PCR provides an effective method for species identification on tissues of unknown origin. There is a possibility that amplification from non-target species would lead to false positives. However, faeces from non-target sympatric species, such as small mustelids and wolves (*Canis lupus*), are easy to separate by eye from the target species. Furthermore, since this approach allows identification of all potential defecators, it bypasses the problem of false negatives discussed above. Of 128 unknown faeces, 99 were successful (Table 1) but 29 did not yield any PCR-product, probably due to low DNA content as samples collected in summer failed more frequently (40%) than in winter (9%; $\chi^2 = 15.8$, $p = 0.0001$).

We have applied this method in the conservation of the arctic fox in Fennoscandia to implement conservation actions such as supplementary feeding and red fox control.

RCP-PCR is both cheaper and more rapid than methods previously used to identify the origin of unknown faeces. The total time for an analysis, including extraction, was less than six hours. This method can be used for identification of unknown tissues between any species, provided that enough variable sites exist for species-specific primers to be designed.

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