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TECHNICAL NOTE

Efficient species identification of pine marten (*Martes martes*) and red fox (*Vulpes vulpes*) scats using a 5′ nuclease real-time PCR assay

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Abstract Monitoring wildlife species by DNA identification of samples collected non-invasively is an important tool in conservation management. DNA identification of species from faecal (scat) samples is problematic due to the small quantities and poor quality of the DNA isolated from such samples. This study demonstrates the use of real-time PCR technology in the identification of red fox (*Vulpes vulpes*) and pine marten (*Martes martes*). It is shown that real-time PCR can be used to identify fox and pine marten by either melting curve analysis (Tm determination) with SYBR Green 1 detection or by the use of species specific fluorogenic probes. The technique is shown to work efficiently with scat DNA.

Keywords Real-time PCR · TaqMan probes · Scat · Species identification · Fox · Pine marten

Species identification by PCR amplification of mitochondrial DNA is a well-established technique. The amplified DNA is traditionally species typed by DNA sequencing or RFLP analysis. A number of molecular methods have been used to identify pine marten, *Martes martes*, from other Mustelids and from fox (*Vulpes vulpes*) (Hansen and Jacobsen 1999; Davison et al. 2001; Davison et al. 2002; Statham et al. 2005). DNA can be isolated from a variety of sample types, such as faeces (scats), hair and urine, collected by non-invasive sampling. In this study the use of real-time PCR for differentiation of pine marten and fox from scat DNA was investigated. Real-time PCR uses fluorogenic dyes to monitor DNA synthesis during PCR. Here the use of both SYBR Green 1 and TaqMan MGB probes for the identification of pine marten and fox DNA from tissue and scat samples is reported.

Tissue samples were from local road kill animals. Species known pine marten scat samples were collected from captive animals at Wildwood Trust, Kent, UK and species known fox samples were from samples in our collection which had previously been identified by DNA sequencing. Other scat samples ($n = 213$) used in this study were collected from various locations in Ireland during 2005 and 2006. The scats collected varied greatly in age and in dietary content. Scats were collected in sealable plastic bags and were stored at –20°C on arrival in the laboratory. The samples analysed in this study had been stored for ≤6 months. DNA was isolated from tissue using the Qiagen DNeasy® Tissue DNA extraction kit. To isolate DNA from scat samples approximately 0.2 mg of scat material was transferred to 1 ml S.T.A.R ® buffer (Stool Transport and Recovery buffer, Roche), vortexed and allowed to stand for at room temperature for >30 min 100 µl chloroform was added to each sample, vortexed and centrifuged at 1,000*g* for 1 min. DNA was then isolated from 200 µl of supernatant using either the Sigma GenElute Mammalian Genomic DNA kit or Roche High Pure PCR Template Preparation Kit used according to the manufacturer's instructions for mammalian cells. In our hands the results

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obtained using this method are as good as those obtained using the specialised stool DNA isolation kits and the method involves less sample handling and is equivalent in price or less expensive depending on the kit used.

Primers and probes for real-time PCR were designed using Primer Express 2 software from Applied Biosystems. The sequences used for designing primers were fox (AJ585358) and pine marten (AJ585357) d-loop sequences (Statham et al. 2005). The optimum size for a PCR product using real-time PCR is 50–150 bp. Alignment of fox and pine marten d-loop sequences identified the region 313–333 (AJ585357) as a good region for probe construction as there are five base pair differences in this region between fox and pine marten (Fig. 1). Primers were selected to amplify this region based on Tm, product size, predicted Tm of amplicon and the ability of the primer set to amplify both fox and pine marten. The primers and probes are shown in Fig. 1. The primers designed were conserved in all available (from GenBank and our collection) fox and pine marten sequences.

TaqMan® MGB probes were labelled with 6-FAM (pine marten) and VIC (fox). Primers were purchased from MWG-Biotech AG and fluorescently labelled TaqMan® MGB probes from Applied Biosystems UK.

All real-time PCR experiments were carried out in an ABI Prism 7300 sequence detection system. The PCR program used was 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. PCR reactions with SYBR Green 1 were carried out using 12.5 µl SYBR Green JumpStart™ Taq Ready Mix™ (Sigma) with 0.5 µl ROX (Sigma) as a passive reference dye, 1 µl each primer (5 µM), 10 µl DNA (diluted to required concentration in distilled H_2O) to give a total volume of 25 µl. DNA melt curve analysis of the PCR products was carried using a dissociation step at the end of the PCR program (SYBR green experiments only). In the dissociation step the samples were slowly (ramp rate 1.75°C/min) heated from 60°C to 95°C and fluorescence data was collected continuously. PCR with Taqman MGB labelled probes was carried out with 12.5 µl TaqMan® Universal PCR Master Mix (Applied Biosystems), 1 µl each primer and probe (5 µM) and 8.5 µl DNA (diluted to required concentration in distilled H_2O) in a total volume of 25 µl All PCR reactions were carried out in Microamp® Optical 96-well reaction plates (Applied Biosystems). Applied Biosystems Sequence Detection Software (SDS1.2.3.) was used for data analysis.

Real-time PCR amplification was tested using fox and pine marten tissue DNA using SYBR Green 1 detection. Figure 2a shows the amplification curves of samples with serially diluted pine marten DNA and a standard curve derived from these data is shown in Fig. 2b. The results indicate that using real-time PCR as little as 0.4 pg of DNA can be detected. The results were similar for fox tissue DNA with Ct values of 18.78–32.68 for the dilution series (4,000 pg–0.4 pg) compared to 18.14–32.91 for pine marten. The primers were chosen to produce products from fox and pine marten that differed significantly in T_m. The Tm for pine marten was 81.13°C ± 0.21 for fox and 82.48°C ± 0.14 in 18 samples of each species in 3 independent amplifications. The Tm determination is useful in the determining the purity of the PCR product and could be used for species identification.

The 5′ nuclease, or Taqman, PCR assay uses fluorogenic probes to detect the PCR product in real-time PCR (Livak et al. 1995). In this study oligonucelotide probes were designed to discriminate between fox and pine marten d-loop sequences (Fig. 1). The probe sequences are conserved in all available fox sequences ($n = 28$) and in all except *hap* h and *hap* t pine marten sequences (17/19) (Davison et al. 2001). These haplotypes have only been found in Slovenia (*hap* h) and Latvia (*hap* t) to date. The probes were tested three times with identical results. The PCR primers PM-FOR and PM-REV were used in a duplex reaction with both probes added to the reaction mixture. In each experiment six fox, pine marten and mixed (1/1) DNA samples were amplified and in all cases the samples were correctly identified.

Species known scat samples were used to test the applicability of the 5′ nuclease probe assay to species identification. A preliminary analysis was carried on a selection of scats ($n = 9$) to determine the quantity of isolated DNA sample to add to the reaction. For each sample a dilution series was tested using SYBR Green 1 detection of amplified product. Results indicated that using >1 µl of purified scat DNA often inhibited the reaction. Subsequently 1 µl of scat DNA was used as the standard assay condition. Using Tm determination the fox samples gave a Tm = 81.03°C ± 0.21 (Ct range 21.01–29.07) and pine marten had a Tm = 82.52 °C ± 0.34 (Ct range 19.11–31.2) Only one of the 20 pine marten scats failed to give a successful amplification at but when this was retested at 10^{-1} dilution a positive result (Tm = 82.34°C; Ct = 32.25) was obtained. All species known scat DNAs were tested using the 5′ nuclease probe assay and all scats were correctly species identified.

The 5′ nuclease probe assay was used to identify 213 scats collected as part of a pine marten distribution survey

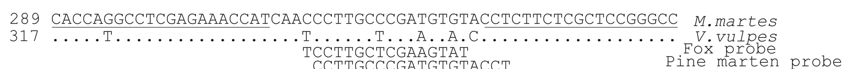

Fig. 1 DNA alignment of a short region of the mitochondrail d-loop sequences of fox (AJ585358) and pine marten (AJ585357) showing the position of primers (indicated by arrows) and probes used in this study

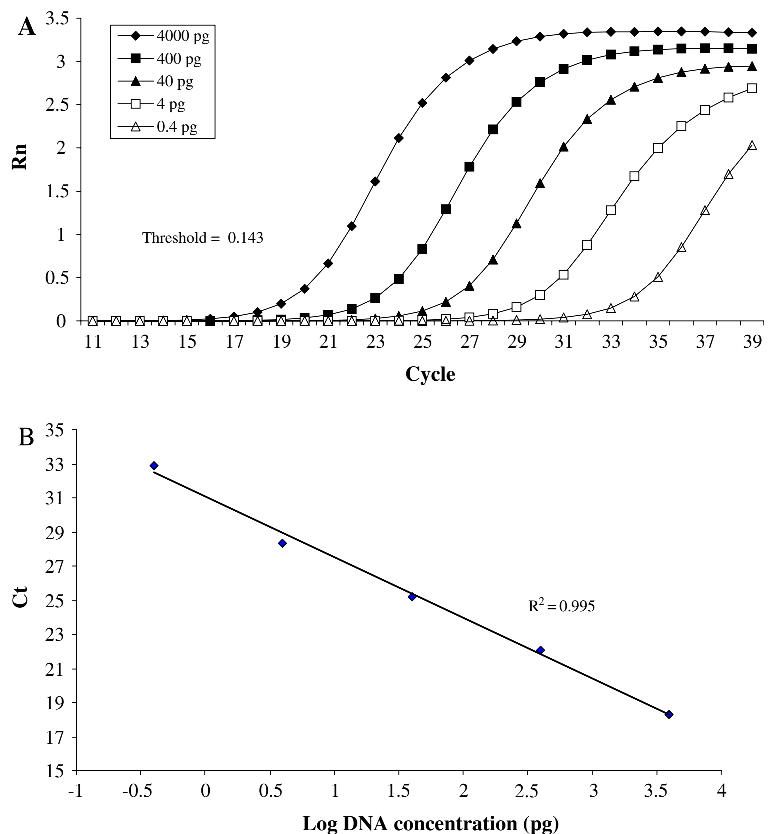

Fig. 2 Quantification of mitochondrial DNA using real-time PCR with SYBR Green detection. (**A**) Amplification plots of serially diluted pine marten tissue DNA. R_n = ratio of the fluorescence emission intensity of the detector dye (SYBR Green 1) to that of the passive reference dye (ROX). ΔR_n measures the change in the detector dye fluorescence from the baseline during the PCR reaction. The threshold was set automatically by the SDS software and indicates the start of the exponential phase of the reaction. (**B**) Standard curve derived from amplification data. The Ct (Treshold cycle) value is the cycle number at which the detector fluorescence passes the threshold

of Ireland. 63.4% of the scats were identified as pine marten, 19.7% as fox. 16.9% of the scats yielded no result with the 5′ nuclease assay. These samples (n = 36) were assayed using SYBR green detection which indicated that 24 of these scats contained good quality amplifiable DNA (Ct ≤ 30) but that the scats were neither pine marten nor fox. Twelve samples, representing 5.6% of the total showed no amplification in the SYBR green assay indicating poor quality DNA or a species that cannot be amplified with primers PM-FOR and PM-REV.

In conclusion this study shows that real-time PCR can be successfully used to species type pine marten and red fox from tissue or scat samples. The application of this technique requires careful analysis of the d-loop sequences of local animals to determine if the primers and probes used in this study are appropriate. Slight redesign of primer/probe sequences may be required to cover the location specific haplotypes. The 5′ nuclease assay could be applied to any species by careful primer and probe design. The advantages of the method outlined in this study are emphasized by consideration of the amplification success rate. In a previous, similar, study (Davison et al. 2001) analysed 163 scats using conventional PCR based methods 53% of scats yielded successful PCR amplification (n = 163). The corresponding rate in this study was 94.6% (n = 213). The increased success rate can be explained by the greater sensitivity of the probe assay and the small size (60 bp) of the amplified product. The ease of the technique and the reduced sample handling greatly facilitates rapid analysis of large numbers of samples. The cost of the analysis (<€1/sample) compares favourably with other molecular methods for species identification. Although real-time PCR equipment may not be available in many molecular ecology laboratories such equipment is becoming much more generally available in molecular biology laboratories. The ease and speed of the technique and its increased sensitivity make real-time PCR analysis for species identification a very valuable tool in molecular ecology.

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