

Use of PCR amplification and restriction enzyme digestion of mitochondrial D-loop for identification of mustelids in Ireland

M. STATHAM, P. D. TURNER AND C. O'REILLY*

Department of Chemical and Life Sciences, Waterford Institute of Technology, Cork Road, Waterford, Ireland

The mustelids found in Ireland are *Martes martes* (pine marten), *Mustela vison* (American mink), *Lutra lutra* (otter), *Meles meles* (badger) and *Mustela erminea hibernica* (Irish stoat). A DNA test has been developed to unambiguously identify scat or hair samples by amplification and restriction enzyme analysis of the mitochondrial D-loop region. Sequencing revealed one haplotype for pine marten (Hap p), two for otter (Lut 1 and 2), three novel haplotypes for mink, and one for badger. Three haplotypes were identified for Irish stoat and phylogenetic analysis confirms the position of *M. erminea hibernica* within the species *M. erminea*.

The pine marten *Martes martes*, otter *Lutra lutra*, Irish stoat *Mustela erminea hibernica*, badger *Meles meles* and the introduced American mink *Mustela vison* are the only members of the family Mustelidae found in Ireland. The pine marten is thought of as rare in Ireland; this reputation is enhanced by its shy nature and although not exclusively nocturnal it is rarely seen. A survey of pine marten in Ireland in the early 1980s found evidence of pine marten in 97 locations scattered throughout the island but mostly in the mid-west region (O'Sullivan 1983). The preferred habitat for pine marten in other parts of Europe is mixed forest but in Ireland mixed forest is uncommon and little is known of the preferences of the Irish population. As forestry practice increasingly takes account of biodiversity issues the management of plantations for wildlife is increasingly important. There is therefore a need for more data on the distribution of pine marten in Ireland in order to formulate conservation plans.

As the pine marten is rarely seen its presence is usually determined by searching for faeces (scats). Scat identification is difficult and has recently been shown to be error prone even by experts (Davison *et al.* 2002). In particular many fox faecal samples are misidentified as pine marten (Davison *et al.* 2002). DNA analysis is a more reliable method for species identification and has been successfully used to identify mustelid species (Hansen and Jacobsen 1999; Davison *et al.* 2002). This is a particular problem in poorly established populations as seen in the study carried out in northern England (Davison *et al.* 2002) and in these cases a DNA test could be an invaluable means of obtaining reliable data. PCR amplification and restriction enzyme digestion of part of the mitochondrial cytochrome *b* gene has been used to differentiate between otter, mink and polecat (Hansen and Jacobsen 1999) while mitochondrial D-loop amplification and sequencing has been used for species identification (Davison *et al.* 2002) and pine marten phylogenetic studies (Davison *et al.* 2001). The aim of this study has been to develop an easy DNA test to identify pine marten from fox and other mustelid species found in Ireland.

* Corresponding author

Materials and Methods

Sample collection and DNA isolation

Fresh tissue from local roadkill animals was used for DNA isolation from pine marten (x 5), badger (x 2), mink (x 5), otter (x 4), fox (x 1) and Irish stoat (x 2). In addition DNA was isolated from three Irish stoat skins held at the Natural History Museum, Dublin. DNA was isolated from tissue using the Qiagen DNeasy[®] Tissue DNA extraction kit. DNA was isolated from pine marten scats using the Qiagen DNeasy[®] Plant system and 10-30mg of scat surface material. DNA was isolated from hair using the Chelex-100 method of Walsh *et al.* (1991) with some modifications as described by Goosens *et al.* (1998).

PCR Amplification

The PCR programme consisted of 40 cycles of 94°C for 60s, 50°C for 60s, 72°C for 60s followed by 5min final elongation at 72°C. The 50µl reaction mixture contained 10-100ng DNA, 2.5units *Taq* DNA polymerase (Promega) in 50mM KCl, 10mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.25mM MgCl₂, 0.2mM dNTPs and 0.5mM each primer. Restriction digestion was carried out overnight at 37°C with 10 units of *Mva* 1 (Promega) in manufacturer's buffer. Products of PCR amplification and restriction enzyme digestion were analyzed on 2 per cent agarose gels using standard methods.

DNA Sequencing

All products were sequenced commercially (Qiagen). Lasergene from DNASTAR Inc. and Phylip version 3.6a1 (Felsenstein 2002) was used for DNA sequence analysis. Novel sequences determined have been deposited in the European Molecular Biology Laboratory (EMBL) database with accession numbers AJ585349 (*M. meles*), AJ585350-AJ585352 (*M. vison*), AJ585353-AJ585356 (*M. erminea hibernica*), AJ585357 (*M. martes*) and AJ585358 (*Vulpes vulpes*).

Results and Discussion

Initial studies used primers LRCB1 (5'-TGGTCTTGTAACCAAAAATGG-3'), (Davison *et al.* 2001) and H16498 (5'-CCTGAAGTGAACCAGATG-3'), (Shields and Kocher 1991) to amplify a region of approximately 400bp of the mitochondrial D-loop from fox, pine marten and mink. Poor amplification sometimes occurred when using this primer set on fox DNA although pine marten and mink yielded good products routinely. Alignment of available D-loop sequences of *Vulpes vulpes* (AF098155), *Mustela erminea* (AB006733) and *Lutra canadensis* (AF418979) in the region homologous to H16498 indicated that H16498 differed at two residues to the two mustelid species and three positions to the fox sequence. A new primer, designated H169498M was designed to the consensus mustelid sequence (5'-CCTGAAGTGAACCAGATG-3', differences to H16498 are in bold) and this primer produced good products routinely with fox, badger, pine marten, mink, stoat and otter. The products were sequenced which revealed total product sizes (including primers) of 434bp for fox, 405bp for pine marten, 408bp for Irish stoat, 418bp for American mink, 409bp for badger and 411bp for otter. Davison *et al.* (2001) identified 25 *Martes martes* D-loop haplotypes. Sequence analysis indicated that all pine marten samples in this study (a total of 11 isolates) were haplotype *p* (AF336964) (Davison *et al.* 2001) as are all Irish pine martens sequenced to date. The five American mink samples tested represented 3 haplotypes which were all different to the one available haplotype (AB052720) in the databases.

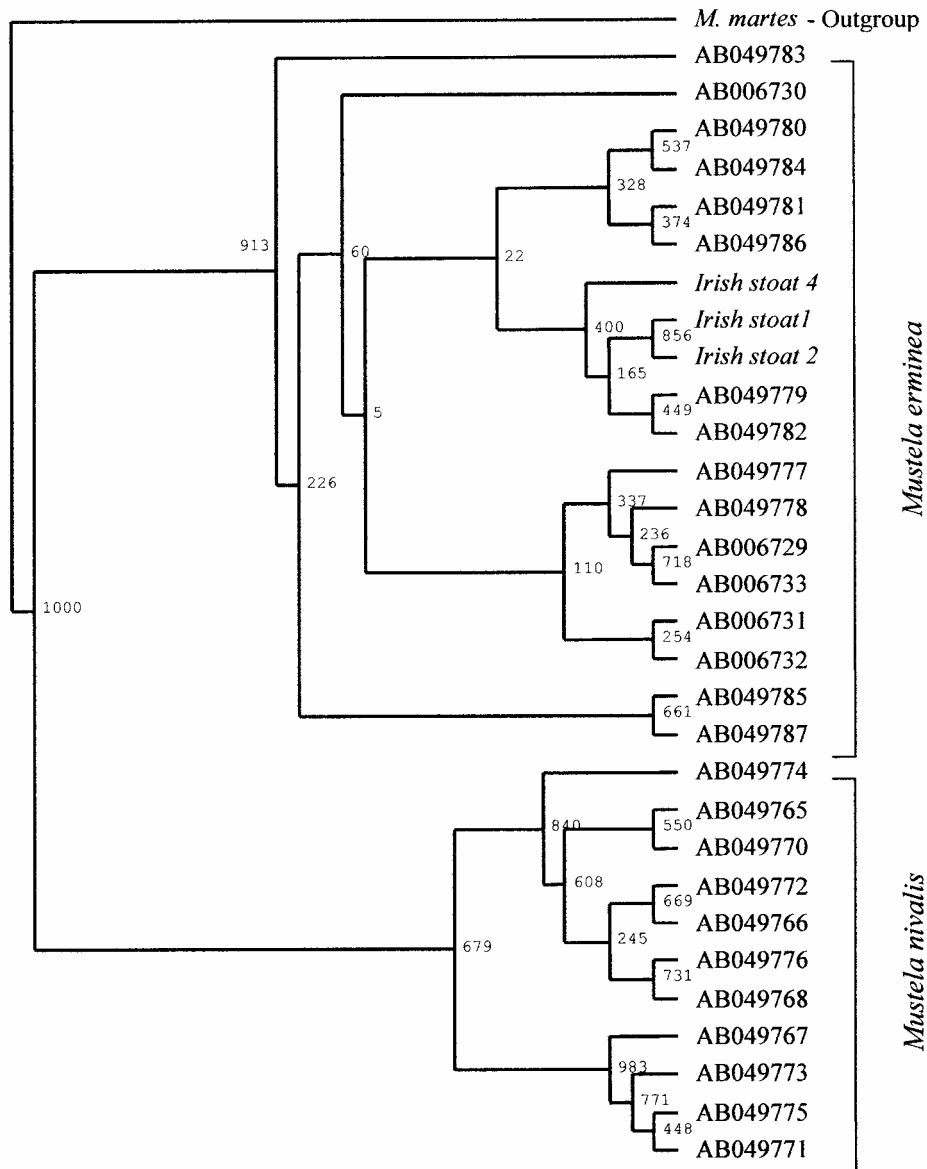


Figure 1. Phylogenetic tree of three Irish stoat haplotypes with other *Mustela erminea* haplotypes and with *Mustela nivalis* haplotypes. The tree was constructed using the maximum likelihood method based on a 269bp sequence of the mitochondrial D-loop region. Numbers on the internal branches are bootstrap values derived from 1000 replicates.

Table 1. Predicted sizes of fragments produced following *Mva* 1 digestion of PCR products produced using LRCB1 and H16498M. Fragments marked with an asterisk are too small to be seen in Plate 1.

Species	PCR product size (bps)	<i>Mva</i> 1 sites (bps)	Fragment sizes (bps)
Pine marten	405	292	292, 113*
Fox	434	None	434
Irish stoat	408	207, 384	207, 177, 24*
Mink	418	37, 395	358, 23*, 37*
Badger	409	386	386, 23
Otter	411	387	387, 24

Of the four otter isolates two were identical to *Lutra lutra* clone Lut 1 (AJ006174) and two were identical to clone Lut 3 (AJ006176) (Cassens *et al.* 2000).

Cassens *et al.* (2000) examined 129 D-loop sequences of otters from all over Europe and found only five haplotypes and found Lut 3 only in animals from Eastern Germany (a total of 42 isolates) and the authors argue that this is due to geographical isolation. The identification of the Lut 3 haplotype in Irish otters is very interesting due to the geographical separation of Ireland from Eastern Germany. Further analysis is required to determine the frequency of the *Lutra lutra* d-loop haplotypes in Ireland. Cassens *et al.* (2000) found Lut 1 to be the commonest haplotype (83/129 isolates). Lut 1 is found all over Europe and in particular is the dominant haplotype found in Scotland (12/14 isolates) (Cassens *et al.* 2000).

The mitochondrial D-loop sequence of Irish stoat *Mustela erminea hibernica*, and badger *Meles meles* had not previously been determined. The two badger d-loop sequences are identical. Database analysis using BLASTN indicated highest homology to the fisher, *Martes pennanti* (91%). Phylogenetic analysis confirms the relationship of *Meles meles* within the Mustelidae as determined from cytochrome *b* gene and IRBP gene analysis (Sato *et al.* 2003).

The Irish stoat, *Mustela erminea hibernica*, is one of 20 recognized subspecies of *Mustela erminea* (Thomas and Barrett-Hamilton 1895, King 1983, Sleeman 1987). The Irish stoat differs from stoats found elsewhere by having an irregular back-belly line. It is frequently, but not always, smaller than the stoat found in Britain and characteristically does not go white in winter (Fairley 1981, Sleeman 1987). It has been suggested that the Irish stoat has a number of characteristics of the weasel *Mustela nivalis* such as smaller body size and not turning white in winter (Thomas and Barrett-Hamilton 1895) but more recent work does not support this theory. It has been shown that there is a north-south cline in Irish stoat size with considerable overlap in size between British and Irish stoats (Sleeman 1987, Fairley 1981) and the lack of colour change in winter may reflect the mild climate with little snow (Sleeman 2002). Analysis of the d-loop sequence of five Irish stoats gave three different haplotypes. Database analysis with BLASTN indicated that these were novel haplotypes most closely related to *Mustela erminea* d-loop haplotypes. Phylogenetic analysis of these sequences with all *Mustela erminea* haplotypes and all *Mustela nivalis* haplotypes is shown in Figure 1. This analysis confirms the position of the Irish stoat within the *Mustela erminea* species and shows a close relationship among the Irish haplotypes.

DNA sequencing of amplified products for identification of species is slow, expensive and very dependent on the quality of the PCR product. Restriction enzyme digestion of amplified products is a cheaper and quicker alternative to sequencing and less sensitive to DNA quality. Analysis of the determined d-loop sequences for restriction enzyme sites

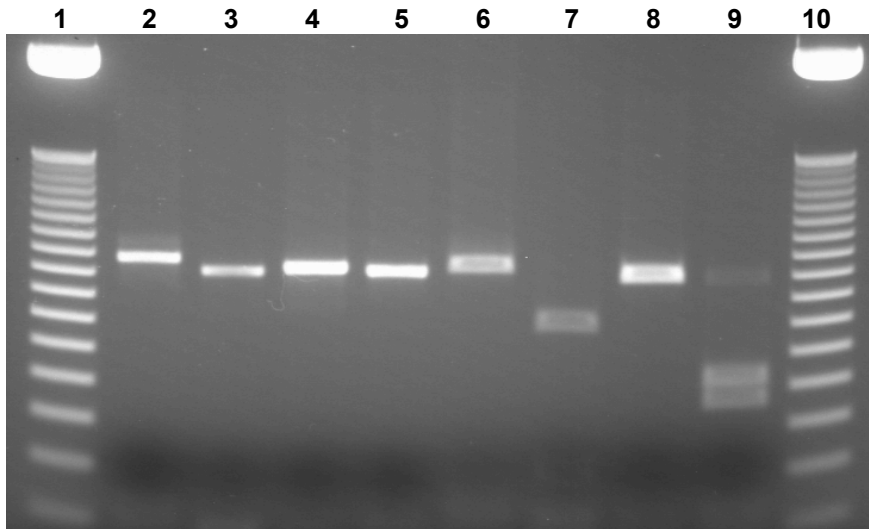


Plate 1. 2 per cent agarose gel of PCR products amplified with primers LRCB1 and H16498M and *Mva* 1 digestion of PCR products. Lanes 1 and 10 show the 50 bp DNA Step ladder size standard. Lanes 2-5 are PCR products, Lanes 6-9 are PCR products restricted with *Mva* 1. Lanes 2 and 6, *V. vulpes*, Lanes 3 and 7, *M. martes*, Lanes 4 and 8, *M. vison*, Lanes 5 and 9, *Mustela erminea hibernica*.

indicated that the enzyme *Mva* 1 (*Bst*N 1) would differentiate between the mustelids found in Ireland and importantly would also identify fox. The predicted product sizes from *Mva* 1 restriction digestion are shown in Table 1. Plate 1 shows a 2 per cent agarose gel of PCR products from fox, pine marten, mink and Irish stoat before and following digestion with *Mva* 1. The results clearly differentiate between pine marten and the other species with the differences in product sizes easily discernable on the gel. In particular the fox, which is the most likely faecal sample to be confused with pine marten, can easily be distinguished as the product has no *Mva* 1 site and the PCR product of 434bp is not affected by digestion.

In conclusion this study provides interesting data on mustelid species in Ireland and describes the development of an easy and inexpensive method for species identification.

Acknowledgements

The authors would like to thank Dr Paddy Sleeman, University College Cork and Mark Holmes of the Natural History Museum, Dublin for providing tissue samples, Dr Paddy Sleeman for useful discussions and John O'Halloran, Coillte for facilitating fieldwork. Ann Riddell, Wildwoods Trust, Herne Bay, Kent, U.K. for continued interest and help with our work. This work was funded, by the Heritage Council of Ireland, and by the Operational Programme for Development Graduate Training Programme.

References

- CASSENS, I., TIEDEMANN, R., SUCHENTRUNK, F. & HARTL, G. B. (2000) Mitochondrial DNA variation in the European otter (*Lutra lutra*) and the use of spatial autocorrelation analysis in conservation. *Journal of Heredity* **91**: 31-35.
- DAVISON, A., BIRKS, J. D. S., BROOKES, R. C., BRAITHWAITE, T. C. & MESSENGER, J. E. (2002) On the origin of faeces: morphological versus molecular methods for surveying rare carnivores from their scats. *Journal of Zoology* **257**: 141-143.

- DAVISON, A., BIRKS, J. D. S., BROOKES, R. C., MESSENGER, J. E. & GRIFFITHS, H. I. (2001) Mitochondrial phylogeography and population history of pine martens *Martes martes* compared with polecats *Mustela putorius*. *Molecular Ecology* **10**: 2479-2488.
- FAIRLEY, J. S. (1981) A north-south cline in the size of the Irish stoat. *Proceedings of the Royal Irish Academy* **81B**: 5-10.
- FELSENSTEIN, J. (2002) PHYLIP (Phylogeny Inference Package) version 3.6a3. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.
- GOOSSENS, B., WAITS, L. P. & TABERLET, P. (1998) Plucked hair samples as a source of DNA: reliability of dinucleotide microsatellite genotyping. *Molecular Ecology* **7**: 1237-1241.
- HANSEN, M. M. & JACOBSEN, L. (1999) Identification of mustelid species: otter (*Lutra lutra*), American mink (*Mustela vison*) and polecat (*Mustela putorius*), by analysis of DNA from faecal samples. *Journal of Zoology*, London **247**: 177-181.
- KING, C. M. (1983) *Mustela erminea*. *Mammalian Species* **195**: 1-8.
- O'SULLIVAN, P. J. (1983) The distribution of the pine marten (*Martes martes*) in the Republic of Ireland. *Mammal Review* **13**: 39-44.
- SATO, J. J., HOSODA, T., WOLSAN, M., TSUCHIYA, K., YAMAMOTO, M. & SUZUKI, H. (2003) Phylogenetic relationships and divergence times among mustelids (Mammalia: Carnivora) based on nucleotide sequences of the nuclear interphotoreceptor retinoid binding protein and mitochondrial cytochrome *b* genes 23. *Zoological Science* **20**: 243-264.
- SHIELDS, G. F. & KOCHER, T. D. (1991) Phylogenetic-relationships of North-American ursids based on analysis of mitochondrial-DNA. *Evolution* **45**: 218-221.
- SLEEMAN, D. P. (1987) The ecology of the Irish stoat. Unpublished PhD thesis. National University of Ireland.
- SLEEMAN, D. P. (2002) The Irish stoat - ancient and misunderstood. *Wild Ireland* **3**: 10-14..
- THOMAS, E. & BARRETT-HAMILTON, G. E. H. (1895) The Irish stoat distinct from the British. *Annual Magazine of Natural History* **15**: 374..
- WALSH, P. S., METZGER, D. A. & HIGUCHI, R. (1991) Chelex-100 as a medium for simple extraction of DNA for PCR- based typing from forensic material. *Biotechniques* **10**: 506-513.