SHORT COMMUNICATION

Non-invasive methods for genotyping of stoats (*Mustela erminea*) in New Zealand: potential for field applications

Dianne M. Gleeson[1*], Andrea E. Byrom[2] and Robyn L. J. Howitt[1]

[1] Landcare Research, Ecological Genetics Laboratory, PB 92170, Auckland, New Zealand
[2] Landcare Research, PO Box 40, Lincoln 7640, New Zealand
* Corresponding author (Email: gleesond@landcareresearch.co.nz)

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Abstract: Stoats (*Mustela erminea*) are a significant pest in New Zealand. A critical aspect of their management is the ability to identify individuals in order to estimate abundance or to determine the origin of residual animals after control, particularly as the trap-shy nature of stoats reduces the utility of trapping to gain this information. We investigated non-invasive 'capture' methods as an alternative to live-trapping or removal methods for estimating stoat abundance. First we determined whether sufficient variability exists at six microsatellite DNA loci to reliably identify individuals in the potentially bottlenecked, introduced stoat populations of New Zealand. In December 2001 we conducted a 7-night pilot field experiment using a modified hair-tube design, where we obtained a total of 64 hair samples. Sufficient DNA was extracted from 3–6 hair follicles to genotype a total of 51 samples. DNA quality declined if samples were left in the field for several nights before being collected, and daily checks proved best for maximising the quality of DNA obtained, while minimising the risk of multiple 'captures' of stoats. Conclusions were that non-invasive molecular sampling is likely to be a viable technique for estimating population density of stoats in New Zealand beech forest but that additional variable loci are required.

Introduction

Stoats (*Mustela erminea*) are a significant pest in New Zealand, with predation by these invasive mammals being blamed for the continuing decline of many native bird species (e.g. O'Donnell 1996). Identification of individuals in a population, enabling estimation of abundance and/or population density of stoats, is essential for three main applications. First, understanding the relationship between stoat population density and impact on particular native species is critical in developing models that guide conservation management decisions (Barlow & Choquenot 2002). Second, monitoring the relative abundance of pests before and after control operations is required in order to determine the effectiveness of a particular pest management strategy. Third, quantifying the genetic make-up of a population can help quantify reinvasion of stoats into control areas. However, obtaining absolute-density estimates of animals such as stoats using standard census methods can result in biased and/or imprecise estimates, as stoats tend to be elusive and trap-shy (King & Murphy 2005). Furthermore, estimates derived from live-trapping are very labour intensive and require large areas to be sampled in order to provide sufficient data to satisfy statistical requirements.

An alternative approach for estimating animal abundance is to use DNA profiling from non-invasive samples (e.g. hair or faeces). This method offers the potential of being able to identify individuals in a population without having to physically capture and/or mark animals. The application of microsatellite DNA markers in combination with novel sample capture techniques and traditional statistical approaches have now become standard methods for estimating population densities of several species (Lucchini *et al*. 2002; Mowat & Paetkau 2002; Eggert *et al*. 2003). Indeed, genotyping of individuals and analysis of the resulting data is becoming one of the most efficient and accurate methods for a range of applications in wildlife monitoring (Waits & Paetkau 2005). These methods have been shown to offer new options for accurate estimation of population abundance of species that are especially difficult to estimate due to their habitat and behaviour, such as stoats.

The aim of this research was to investigate the utility of obtaining DNA from stoats in the field using non-invasive methods, for estimation of abundance and detecting individual movements. Here we concentrate on (1) determining whether microsatellite DNA markers could identify individual stoats within a bottlenecked population and (2) developing hair collection protocols in the field. We then describe a pilot field experiment to determine whether DNA data have the potential to be used to develop a standardised mark–recapture method for population census of stoats.

Methods

Sampling – tail-tip tissue samples

To determine whether sufficient DNA variability exists within New Zealand stoats to assign individual genotypes, we collected tail-tip samples from 30 stoats trapped by the Department of Conservation for predator control at a site near Lake Rotoiti, Nelson Lakes, South Island, during December 2000 and January 2001. Tail tissue was either frozen at −20°C or stored in 95% ethanol before transportation to the laboratory.

Sampling – hair samples from captive stoats

To retrieve DNA from hair samples, we initially trialled hair-capture tubes on stoats held at the Landcare Research animal facility (Lincoln, New Zealand) to ensure the design had the potential to obtain sufficient samples in a field situation. Hair-tubes consisted of a 20-cm length of PVC pipe, 45 mm in diameter, with a rubber band covered in adhesive gel (Trappers Glue, USA) stretched across the aperture of each end of the pipe. Tubes were baited with a small piece of rabbit meat placed in the centre of the tube to encourage entry. In trials of captive stoats, tubes successfully removed hair with a sufficient number of follicles attached (>3 per individual) to enable duplicate PCR reactions and subsequent genotyping.

Sampling – hair samples from stoats in the field

A pilot field trial was then undertaken in red beech (*Nothofagus fusca*) forest at Matakitaki Station, near Murchison (42°00' S, 172°30' E) in the South Island, approximately 45 km from the Lake Rotoiti site. Hair-tubes were located at 'tube stations' spaced at 250-m intervals along lines 500 m apart, on a 3 × 3 km grid. The grid consisted of

seven lines each with 14 tube stations, resulting in a total of 98 tube stations. With this design we were able to offer all resident stoats an equal opportunity of capture, because the spacing between stations (250–500 m) was significantly smaller than the average home-range size of a stoat (approximately 1 km^2; King & Murphy 2005). The field trial was run over 7 nights from 15 to 21 December 2001. Hair-tubes were checked daily and the rubber bands removed and replaced with fresh glue if hair was present. The tubes were re-baited. At the end of the intensive week of daily checks, tubes were left in the field to be checked weekly for the three remaining weeks of the trial (22 December to 12 January 2002).

After being transported to the laboratory, hair samples were removed from the sticky surface of the rubber band using sterile tweezers and examined under a microscope. A 3-mm portion containing the hair follicle was excised using a sterile scalpel. In most instances follicles from six hairs were used per sample, although in a few samples 3–5 hairs were used depending on the actual number present.

DNA extraction

Tail tissue samples were subsequently dissected in the laboratory, where 50 mg of muscle tissue and caudal skin were removed. DNA was then isolated, using a Bio-Rad AquaPure Genomic Tissue Kit (Cat# 732-6343) following the manufacturer's protocol, and resuspended in 100 µl of supplied buffer.

DNA extraction from hair samples used a modified protocol following Walsh et al. (1991). Hair follicles were placed in an Eppendorf tube containing 100 µl of extraction buffer (5% chelex 10 mM Tris, 0.1 mM EDTA), followed by an addition of 1 µl Proteinase K (20 mg ml^{-1}) and 2.7 µl of 1 M DTT. Samples were incubated at 56°C for 2 h. A further 1 µl of Proteinase K was added and samples incubated an additional 2 h at 56°C, being tapped occasionally. Samples were then boiled for 8 min, vortexed at high speed for 15 s, and centrifuged (13 000 rpm) at room temperature for 3 min. The supernatant was transferred to a new tube with a wide-bore pipette tip, and stored at −20°C.

Microsatellite amplification and analysis

We used six microsatellite loci that were all di-nucleotide repeats with a CA repeat motif. Primers used were MER005, MER030, MER022, MER009, and MER082 developed from *M. erminea* (Fleming *et al*. 1999), and MVI057 developed from *M. vison* (O'Connell *et al*. 1996). PCR amplifications were performed in 25-µl reactions containing either 1 µl of DNA extract from tissue or 10 µl (c. 10%) of DNA extract from hair, 1× PCR buffer with $MgCl_2$ (50 mM Tris/HCl, 10 mM KCl, 5 mM $[NH_4]_2SO_4$, 2 mM $MgCl_2$, pH 8.3), 200 µM of each dNTP, 10 uM of each primer, and 2U of FastStart Taq DNA Polymerase (Roche Diagnostics). Amplification conditions on a GeneAmp PCR System 9700 thermocycler (Applied Biosystems) were: initial denaturation at 95°C for 4 min; 10 touchdown cycles of 20 s at 94°C, 20 s at 62°C – 58°C, 20 s at 74°C; 40 cycles of 20 s at 94°C, 20 s at 58°C, 20 s at 74°C, and a final extension of 40 min at 72°C. The 5'-end of the forward primer of each pair was fluorescently labelled with either 6FAM, NED, or VIC dyes (Applied Biosystems) and amplification products were separated using capillary electrophoresis (ABI PRISM 310). Alleles were sized relative to an internal size standard (GS-350 ROX) using GENESCAN 3.1 (Applied Biosystems).

The genotype profiles were analysed by eye and using the software GENOTYPER Version 2.5 (Applied Biosystems). Calling of peaks by each method was compared and, where ambiguities existed, primary profiles were re-examined by eye and/or reaction repeated. Microsatellite genotypes were analysed using GenALEx v. 6.2 (Peakall & Smouse 2006) to generate heterozygosity and P_{sib} scores (Table 1) from the Lake Rotoiti population.

Genotyping was carried out using a step-wise protocol of exclusion that has been shown elsewhere to ensure rigorous and conservative determination of identity (Paetkau 2003; Weaver *et al.* 2005). Two attempts were made to genotype all loci from each sample. If this was not successful for the four loci with the lowest P_{sib} scores (Table 1), then such samples were excluded from further analysis. We required a perfect match between the two amplifications in order to accept that genotype. Samples that differed at only one locus were checked for scoring or typing errors. If these differences were not able to be explained by errors in scoring/typing, samples were then subjected to a further round of PCR and scoring (Poole et al. 2001; Mowat & Paetkau 2002).

Results

DNA recovery from hair-tubes in the field

Servicing of hair-tubes in the field and removal of hair samples proved straightforward. A total of 40 samples were collected over the 7-night period when hair-tubes were serviced daily. Upon examination and comparison with a reference set of known mammalian pest hair samples, using Hair ID © Ecobyte Pty Ltd (www.ecobyte.com.au), the majority of the samples (around 98%) were found to be stoat hair. Our pilot trials from the captive animals showed that DNA could be successfully extracted from multiple hairs, which was assessed via the ability to amplify a microsatellite locus. However, the quantity of DNA obtained from fewer than three hairs limited the ability to conduct duplicate amplifications and still be able to repeat genotypes if required. Weekly checks of the tubes in the field yielded an additional 24 samples. Of the 64 samples in total, sufficient hairs (>3) were present from 51 to enable DNA to be successfully extracted and subsequently genotyped using duplicate amplifications for all loci.

Microsatellite amplification and genetic variability

All tail-tip tissue samples from the Department of Conservation sampling at Lake Rotoiti were successfully genotyped for all six loci. The number of alleles found at each locus ranged from two to eight, with a total of 33 alleles across all loci. There was no significant difference detected between observed and expected allele frequencies at each locus, and as such they were assumed to be at Hardy–Weinberg equilibrium and thus are unlikely to possess any null alleles (Table 1). Using all six loci, each individual could be discriminated as they each returned a unique genotype (between 3 and 8 pairwise allelic differences). We also tested the relative importance

Table 1. Genetic variability at each locus obtained from 30 individual stoats trapped at Lake Rotoiti, New Zealand, in December 2000 to January 2001.

Locus	Tag	No. alleles	Het_{obs}	Het_{exp}	Size range	P_{sib}[1]
MER005	6FAM	7	0.613	0.641	282–306	0.417
MER030	VIC	6	0.586	0.687	220–234	0.432
MER022	NED	8	0.666	0.741	242–270	0.387
MER009	NED	5	0.586	0.633	206–216	0.507
MER082	VIC	2	0.449	0.465	120–122	0.656
MVI057	6FAM	5	0.684	0.650	99–113	0.468

[1] The mean probability across 30 individual stoats that a full sibling would have the same genotype.

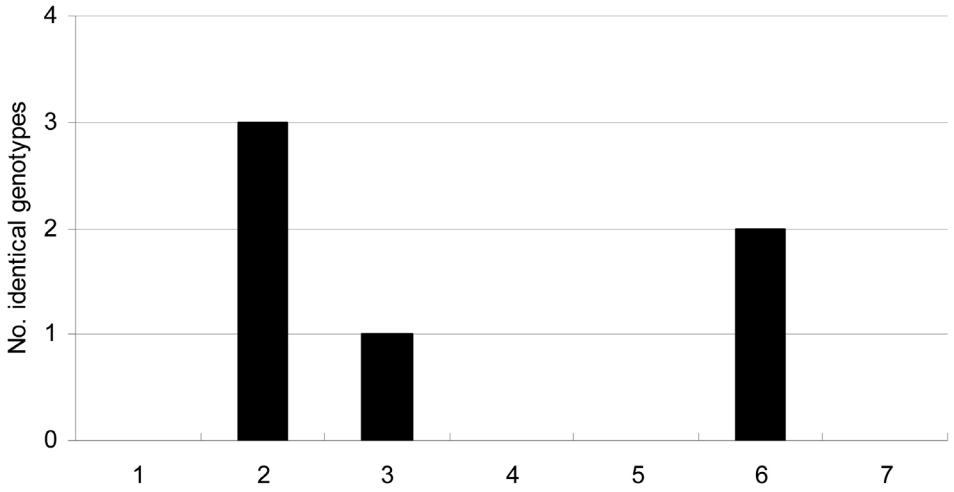

Figure 1. Relative contribution of each locus in identifying individual stoats from Lake Rotoiti. Position 7 is when all six loci are used; positions 1 through 6 show the effect of removing a single locus. The loci removed at each position are as follows: 1, MER005; 2, MER030; 3, MER022; 4, MER009; 5, MER082; and 6, MVI057.

of each locus discriminating genotypes by removing one from the analysis and observing the number of matching genotypes without that information. It was shown that MER030, MER022 and MVI057 were the most important loci in this particular population, in agreement with theoretical expectation (Fig. 1). The overall P_{sib} score was calculated as 0.011, which is slightly above the 0.01 reported as the upper limit required for population estimation (Mills et al. 2000).

Hair samples varied in the number of loci at which they could be successfully genotyped. Samples collected during the daily checks over the 7-night period resulted in 73% (29/40) being able to be genotyped for all six loci. Of the remaining 11 samples, two could be genotyped for at least the four loci with the lowest P_{sib} scores (MER005, MER022, MER030, and MVI057), two appeared to be a sample consisting of more than one individual, due to the presence of additional alleles at heterozygote loci, while seven could only be partially genotyped. Only 8% (2/24) of the samples collected weekly were able to be genotyped for all loci, whereas 83% (20/24) could be amplified for the four most informative loci. The remaining four samples appeared to contain more than one individual.

The average number of alleles per locus was relatively high (7.3), which enabled individual genotype profiles to be assigned even if only four loci could be amplified. From the total of 51 hair samples from both the daily and weekly sample sets, 34 unique genotype profiles were obtained, with 10 profiles being shared among up to four samples, indicating multiple resampling of individual stoats. From the samples obtained during the 7-night period of daily checks, 20 unique stoat profiles were obtained with eight shared genotypes, suggesting some individuals had been sampled multiple times in the week. Seventeen unique profiles were obtained from samples obtained during the weekly checks, with three shared genotypes within that sample period. The sample sets had three genotypes in common, which suggested that three individuals had been resampled at the field site over the one-month time period.

Discussion

Non-invasive molecular sampling proved to be a viable technique to identify individual genotypes, with the potential for estimating population density of stoats in New Zealand or for quantifying reinvasion of stoats into control areas. As this was the first application in a pilot field trial, the main focus of the study was on refining our techniques at each step (field collection protocols, extraction of DNA from hair follicles, and genotyping). Field collection of hair samples proved successful and efficient, as it was possible for one fieldworker to carry many (at least 50) tubes in a pack, and servicing about 100 tubes took two people one day at the scale of our trial grid (3 × 3 km). Stoats visited a large number of tubes on the grid, and these visits translated into several 'recaptures' of individuals.

There were two problems with sample quality. The first was sample degradation observed in the second set of hair samples that were derived from the weekly collection. In this set, only 8% of samples could be genotyped for all six loci. This was most likely due to prolonged exposure to unfavourable environmental conditions; there was approximately 75 mm of rain during that sampling period, and further periods of high humidity, which is consistent with previous studies showing degradation of DNA from hair exposed to environmental moisture (Lindahl 1993; Jeffery et al. 2007).

A second problem was the presence of mixed samples (i.e. more than one stoat entering a tube), which was identified on the basis of having three or more alleles at one or more loci. In order to reduce the rate of genotyping errors inherent in non-invasive DNA samples due to low DNA yield, pooling of hairs is necessary. However, this increases the risk of failing to identify mixed DNA from multiple sources, particularly if insufficient variable loci are used (Alpers et al. 2003). Our initial study using tissue samples from Lake Rotoiti was able to discriminate among individual stoats, although the power of the data to discriminate closely related individuals as measured by P_{sib} showed that this was marginal for all six loci (> 0.01).

For future application, it may be possible to eliminate the problem of mixed samples by designing a hair-tube that closes following hair removal, or a similar sticky device, thereby eliminating the chance of another stoat visiting before the sample has been collected. However, this would require field trials in order to ensure that stoats were not discouraged from revisiting hair-tubes following experience with a closure mechanism, which is particularly important for trap-shy species such as stoats.

Overall this pilot study has shown that non-invasive DNA typing of stoats in New Zealand could be a viable tool for applications such as population estimation or identifying individual movements, but that additional loci with greater variability would be required in order to achieve a more acceptable P_{sib} score.

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