SHORT COMMUNICATION

Use of forensic genetics to detect a potential incursion of the brushtail possum onto Great Barrier Island

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Published online: 7 November 2013

Abstract: The brushtail possum (*Trichosurus vulpecula*) is a widespread introduced pest in New Zealand. Some hair and faecal remains suspected to be from a possum were found on a vehicle transport barge in port at Great Barrier Island in the Hauraki Gulf (North Island, New Zealand), an island that has historically remained possum free. So that appropriate action could be taken, we used forensic genetics to confirm the species, number, and sex of the individuals that may have disembarked at the island. We concluded that forensic samples were attributable to a single male possum that did not disembark on the island, hence no eradication response was put in place. This case study illustrates how forensic DNA analysis of wildlife remains can assist in the response to a potentially disastrous invasive event by providing information in a timely and cost-effective manner.

Keywords: faecal DNA; pest detection; *Trichosurus vulpecula*

Introduction

The common brushtail possum (*Trichosurus vulpecula*) has become a major animal pest in New Zealand since it was introduced from Australia in 1837 to establish a fur industry (Cowan 1990; King 2005). This species, now widespread throughout the mainland and also on many offshore islands, has had a negative impact on New Zealand's natural ecosystems. This has been mainly by affecting forest composition and competing for food with native fauna (Cowan 1992; Gormley et al. 2012); but also by preying on bird nests (Innes et al. 2004), and transmitting diseases (Montague & Warburton 2000). More recently, eradication operations have successfully removed possums from various offshore islands, but these incur considerable costs (Cowan 1992). Consequently, it is essential that possum-free locations remain so, given the significant resources required for their management.

Genetic techniques have previously been applied to the detection and management of vertebrate pests (Darling & Blum 2007; Rollins et al. 2009). Mitochondrial DNA barcoding has been used to confirm or identify vertebrate pest species (Berry et al. 2007; Darling & Blum 2007). DNA profiling using microsatellites (STRs) has been applied to determine whether rats found on islands after eradication were either surviving residents or reinvaders (Abdelkrim et al. 2007; Fewster et al. 2011). More recently, population genetics has been used to determine the origin of a stoat incursion on Secretary Island, New Zealand (Veale et al. 2012) and also to estimate the number of stoats on Resolution Island (Clayton et al. 2011). Latest advances in DNA recovery, coupled with better analytical capability, have resulted in the increased use of genetics in pest management to inform appropriate action. (See Rollins et al. (2006) for a comprehensive review of genetic analysis tools used in pest management.)

DNA-based methods have conventionally been used to prevent incursions through the early detection of invasive invertebrates, especially of larval stages (Darling & Blum 2007; Bott et al. 2010), but are not yet fully exploited as a tool for detecting incursions or investigating early stages of vertebrate pest invasions. Forensic DNA methodologies can enable early detection through trace samples such as saliva, hair or faecal remains. Such samples can provide information resulting in timely management actions, which are critical if pest-free, and in this example possum-free, areas are to be maintained. Although forensic DNA methodologies have been employed for monitoring vertebrates pests (Berry et al. 2007; Vargas et al. 2009; Harrington et al. 2010), to the best of our knowledge they have not been reported for early detection and surveillance of a vertebrate pest incursion.

Great Barrier Island, in the Hauraki Gulf offshore from Auckland, is New Zealand's fourth largest island (285 km^2) and is free of some key introduced pests, including possums. Suspected possum faecal pellets and a lock of fur were found in October 2010 on a digger that had just arrived via a barge that had departed from Half Moon Bay marina, eastern Auckland. This case study describes the use of forensic DNA analyses to assist in the response to a potentially disastrous invasive event, by providing information regarding the species, sex and most likely number of individuals represented in the samples found.

Materials and methods

DNA was derived from the faecal and fur samples and individually genetically profiled in order to confirm species, determine sex and whether more than one individual was involved. A total of nine samples were received for genetic analysis (Table 1). These comprised one unidentified lock of fur and two unidentified sets of faecal pellets found on the digger; a third faecal conglomerate that had been squashed by cars was also found a day later on the barge. In addition, trapping was undertaken on the mainland, in Manukau City (Auckland) where the digger originated, to determine if any of the trapped animals matched the genetic profile of these remains as it was

Table 1. List of analysed samples and results. Lower-case letters denote subsamples.

Sample	Type of sample	Sample group	Molecular sex determination	Individual assignment
1a	Fur	Unknown		A
1b	Fur	Unknown	Male	A
2a	Scat	Unknown	Male	A
2b	Scat	Unknown	Male	A
2c	Scat	Unknown	Male	A
2d	Scat	Unknown		A
3a	Scat	Unknown		A
3b	Scat	Unknown		A
4a	Scat	Unknown		A
4b	Scat	Unknown		A
5	Tissue	Reference	Female	B
6a	Tissue	Reference	Female	C
6b	Scat	Reference		C
7	Tissue	Reference	Female	D
8a	Tissue	Candidate	Male[1]	A
8b	Fur	Candidate		A
9a	Scat	Unknown		A
9b	Scat	Unknown	Male	A

[1]Sample sexed by morphological observation.

possible the possum did not stay on the digger during the transportation. Three reference tissue samples were obtained from the trapping event (one of them also with some faecal material). Ultimately, a week later, a live possum was found on the barge and trapped. A tissue sample and some fur from this possum (candidate) and some more faecal material that was most likely from the latter since it was found at the same time were subsequently genetically identified. Two replicate extractions were performed for the unidentified fur, and from two to four replicates (subsamples a, b, c, d in Table 1) for the unidentified pellet-like faecal samples (scats), depending on the number of pellets that comprised each set.

Three hairs with visible follicles were used for each fur extraction. For each faecal subsample three faecal pellets were gently rubbed with a sterile swab (COPAN^{TM} plastic applicator rayon swab) soaked in DXT lysis buffer (Qiagen®) based on the method deployed by Ball et al. (2007). The swab tip was removed and used as the starting material for subsequent DNA extraction. All samples were digested in 500 µl of DXT buffer and 5 µl of DX digest enzyme (Qiagen®), followed by overnight incubation at 56°C. Each sample was then extracted by using 220 µl of the lysate in an automated extraction machine (QIAxtractor®, Qiagen®) following the manufacturer's instructions. DNA was eluted in 70 µl of DXE (Qiagen®) and then stored at 4°C for immediate use.

At least one subsample for the unidentified fur and faecal samples was first subjected to species identification analysis by amplifying the cytochrome b (CytB) mitochondrial region using primers CB-J-10612 (5'-CCATCCAACATCTCAGCTGATGAAA-3') and CB-N-10920 (5'-CCCTCAGAATGATATTTGTCCTCA-3') (Kocher et al. 1989). Samples were then genotyped using eight possum-specific microsatellite loci: Tv16, Tv19, Tv27, Tv53, Tv54, Tv58, Tv64 and TvM1 (Taylor & Cooper 1998; Lam et al. 2000) in a multiplex polymerase chain reaction (PCR). The multiplex PCRs were performed in a final volume of 10 µl reaction containing 1× QIAGEN® Multiplex PCR Master Mix, 0.2 µM of each primer and 3 µl of faecal and hair DNA or 1 µl of DNA from tissue samples, following Qiagen-recommended cycling conditions (annealing temperature 60°C). Amplified fragments were analysed in a 3130xl Applied Biosystems Genetic Analyser and scored using Genemapper v 4.0. In addition to this, 23 possum samples from Coatesville (Auckland, North Island) were also subjected to the same genotyping protocol to build an allelic frequency dataset within the Auckland Region for statistical comparison. GeneAlEx v6.4 (Peakall & Smouse 2006) was used to test for matching genotypes and probability of identity of the samples.

Sex determination was attempted on the samples shown in Table 1 following the protocol developed by Eckery et al. (2002), by amplifying a possum SRY fragment using forward (5'-TCCGTGAGAAGTGGATCAAGCA GTACA-3') and reverse primers (5'-GGGTATTCTTCT CTGTGTTTAGCACGC-3'), together with the possum GnRH receptor fragment [as a positive control for females] using forward (5'-ATGGCAAACAGA GCCTACCTTGAGCAG-3') and reverse primers (5'-AGCGTACCACTGCACGGTCACATTCCA-3'), and using 1 µl of DNA for tissue samples and 3 µl for faeces and fur. Sex determination PCR reactions were also carried out in a final volume of 10 µl containing 1× PCR Buffer with 2 mM $MgCl_2$ (Roche, Germany), 0.5 µM of each primer, 2 mM dNTP, 0.4 µg ml^{-1} BSA, and 0.8 U of Faststart Taq polymerase (Roche, Germany). Cycling conditions were: 94°C for 5 min followed by 40 cycles of 30 s of denaturation at 94°C, 45 s at 54°C, and 45 s at 72°C; and 10 min of final extension at 72°C. Known-sex samples were included in the reaction as positive controls. Amplicons obtained in the sexing determination were visualised in an ethidium-bromide-stained 2% (w/v) agarose gel and typed as females when a single product, derived from the GnRH-receptor gene, was obtained and as males when both GnRH-receptor and SRY genes were visualised, as in Eckery et al. (2002).

Results

All the samples assayed using CytB were found to have identical DNA sequences (GenBank Accession number JX968802) and were positively identified as brushtail possum, *Trichosurus*

vulpecula, using the NCBI BLAST algorithm with an 100% identity. Genotyping tests were only performed as single-tube reactions, due to the urgent requirement for results. Single-tube genotyping performance was sufficient, as opposed to the standard multi-tube genotyping procedure for low-copy DNA (Taberlet et al. 1999), as there were extraction replicates and the chromatograms were all of good quality. Complete profiles of all samples and subsamples (tissue, hair and scats) were obtained with the exception of three faecal samples (3b, 6b and 9b) from, respectively, one of the unidentified specimens, a reference specimen, and the candidate. The three partial genotypes matched other genotypes tested in this study. No further amplifications were performed for these partial genotypes. None of the unknown samples showed more than two alleles, thus there is no evidence of their being from more than one individual.

A week later, a live possum (candidate) was found on the barge when it arrived back into port in Auckland. Material from this possum was used to compare with the previously collected samples, with all fur and faecal samples (labelled from 1 to 4 in Table 1) matching the profile of the possum, with a genotype probability of identity of 3.8×10^{-7}. Probability of identity was also calculated for two populations in the Auckland Region (Table 2). The genotypes of the three individuals trapped at Manukau do not group with any of the unknown samples or with the samples taken from the later barge-found possum. The genotype of the individual found on the barge groups with at least one subsample of the unknown samples. The rest of the subsamples match the genotype with scarce occurrence of allelic dropout. In addition to this, the individual on the barge to Great Barrier Island and its matching samples were all sexed as males.

Discussion

Given the high diversity of North Island possums (Taylor et al. 2004) and the probability of identity obtained for the possums tested in this investigation, all unknown samples of this study were considered to belong to the possum candidate specimen that was caught in the barge a week later. Therefore, no possums were thought to have disembarked on Great Barrier Island and no pest management measures were required to be put in place there. Moreover, the only individual proven to have made it onto the barge was a male, so even if it did disembark, the biosecurity threat would have been minimal as no reproduction event could have happened.

The use of faecal DNA is usually challenging due to the presence of PCR inhibitors, low concentrations of DNA, and expensive and time-consuming laboratory procedures (Fernando et al. 2003). However, we have shown that the combination of swabbing the samples to retrieve the epithelial mammal DNA, along with the use of high-throughput extraction machines (a modified technique based on the one deployed by Ball et al. (2007)), can overcome the above challenges. Proof of this is the fact that in all cases a single PCR amplification replicate was sufficient to provide reliable and consistent results across extraction replicates, which is opposite to the standard multi-tube approach normally required when typing nuclear faecal DNA. This was an advantage in this particular case where promptness was crucial. The simple practice of swabbing the scats maximises the recovery of the epithelial cells from the digestive system and minimises the likelihood of non-target DNA being yielded (e.g. non-digested material) as it only gently wipes the outermost layer of the sample.

The size of the fragment amplified in the sex test (~330 bp) is evidence that good quality nuclear DNA was recovered, demonstrating that the technique can overcome the usual forensic-related challenges such as co-purified inhibitors and degradation. Although some co-purification inhibition from the non-digested material is thought to be carried through with the extracted DNA, no further conclusions can be made as to whether there was less co-purification of inhibitors using the swabbing technique with the tests implemented for this event.

The results of this DNA analysis provided information that was critical for undertaking appropriate management action in a timely and cost-effective manner. By showing there was most likely only a single animal, and that it was a male, this meant there were no unnecessary resources expended in undertaking an eradication operation. However, if the possum had been identified as a female, then this would have necessitated an eradication response on the chance it was either pregnant or carrying a pouch young. The subsequent discovery of the possum on the barge was further confirmation that these methodologies were highly accurate and in any future similar scenario could be relied upon to inform appropriate management response. The methods that have been developed through this work can be applied to larger scale faecal DNA monitoring work. This can include detecting elusive species, enumerating individuals at a location, or general environmental monitoring.

Acknowledgements

We thank the Auckland Council and Weihong Ji for their contributions. Additional thanks to Rob Cruickshank, Hannah Buckley, Jo Monks, and one anonymous reviewer for their valuable input and suggestions.

Table 2. Probability of identity (PI) of two populations from the Auckland Region for eight loci.

	Population		
	Manukau[1] ($N = 4$)	Coatesville ($N = 23$)	Auckland ($N = 27$)
PI	8.2×10^{-6}	5.6×10^{-7}	5.6×10^{-8}
PI_{sibs}	5.2×10^{-3}	2.3×10^{-3}	1.3×10^{-3}
Expected no. individuals with same genotype (PI/N)	3.3×10^{-5}	1.3×10^{-5}	1.5×10^{-6}
Expected no. individuals with same genotype based on PI_{sibs} (PI_{sibs}/N)	2×10^{-2}	5.2×10^{-2}	3.6×10^{-2}

[1]The Manukau population consists of the three reference individuals and the possum from the barge (Individuals A, B, C and D in Table 1).

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Editorial Board member: Hannah Buckley
Received 31 January 2013; accepted 30 June 2013