

Faecal DNA detection of invasive species: the case of feral foxes in Tasmania

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Abstract. Early detection of biological invasions is critical to reducing their impact, but because invading organisms are initially at low densities, detection and eradication can be challenging. Here, we demonstrate the utility of faecal DNA analysis for the detection of an elusive invasive species – the red fox, *Vulpes vulpes*, which was illegally introduced to the island of Tasmania in the late 1990s. Foxes are a devastating pest to both wildlife and agriculture on the Australian mainland, and would have a similarly serious impact in Tasmania if they became established. Attempts to eradicate foxes from Tasmania have been hampered by unreliable distribution data derived mostly from public sightings. In response, we developed a highly accurate and reliable DNA-based PCR-multiplex test that identifies foxes from field-collected faeces. We also developed a sexing test, but it was reliable only for faeces less than three weeks old. Faeces are a useful target for DNA-based diagnostics in foxes because they are deposited in prominent locations and are long-lasting. The species identification test formed a key component of a Tasmania-wide detection and eradication program. In all, 1160 geo-referenced carnivore scats were analysed; of these, 78% contained DNA of sufficient quality for species identification. A single scat from the north-east of the island was identified as belonging to fox, as was a nine-week-old roadkill carcass from the north coast, and a blood sample from near Hobart, triggering increased control and surveillance in these regions. The accuracy, reliability, and cost-effectiveness of non-invasive tests make them a critical adjunct to traditional tools for monitoring cryptic invasive species that are at low density in the early stages of invasion and when eradication is still an option.

Introduction

Biological invasions are a major cause of world-wide species endangerment and extinction (Caughley 1994) and also contribute massive economic costs (Perrings *et al.* 2002). Early detection of invading organisms is a key to their effective management, by enabling better targeting of control measures (Myers *et al.* 2000; Simberloff 2003). However, invasive species are typically scarce and difficult to locate during the early phases of an invasion, making eradication problematic (Mack *et al.* 2000).

Exemplifying this situation, the red fox, *Vulpes vulpes*, was introduced to the island of Tasmania in the late 1990s. Foxes are a devastating pest to both wildlife and agriculture on the Australian mainland (Saunders *et al.* 1995), costing AU\$227.5 million annually in biodiversity and economic losses (McLeod 2004). In contrast, the native fauna of Tasmania, a large (64332 km²) island south of the Australian mainland, is relatively unchanged by European settlement, in large part because of the absence of the fox (Short and Smith 1994).

Three fox carcasses have been discovered in Tasmania since 2001, and further evidence has suggested that these individuals were part of a deliberate illegal introduction of between 12 and 19 individuals of unknown sex to the island in *c.* 1999/2000 (Saunders *et al.* 2006). The threat that this invasion poses led to

the establishment of a major control program centred on widespread baiting with the poison 1080 (sodium fluoroacetate). 1080 is toxic to native wildlife, and to minimise non-target deaths, baits must be set by hand, which is expensive and time consuming. A baiting program has focused on regions where sightings of foxes have been most concentrated. Over 1000 sightings have been reported by the public since 2001 (Fig. 1). However, most have been difficult to verify, and many are believed to be erroneous (Saunders *et al.* 2006).

To bolster evidence from sightings, earlier phases of this program employed analysis of faecal (scat) morphology and hair to monitor fox distributions. This approach makes use of the tendency of foxes to defaecate in prominent locations such as tracks and track junctions (Macdonald 1980), and the potentially long life of canid scats (weeks or months: Kohn *et al.* 1999). Scats are regularly sampled by ecologists because they provide a non-invasive and practical monitoring tool for cryptic animals (e.g. Triggs 1996; Sadler *et al.* 2004). However, in many cases definitive diagnosis can be difficult and lead to ambiguous results of limited usefulness (Davison *et al.* 2002).

DNA analysis of scats provides a solution to this problem, by combining the benefits of sampling offered by scats with the

robust detection provided by approaches based on the polymerase-chain-reaction (PCR) (Kohn and Wayne 1997). It potentially also enables more sophisticated data to be obtained, such as the determination of sex ratios (Dallas *et al.* 2000), identification of individuals (Piggott *et al.* 2006), and analysis of diet (Farrell *et al.* 2000). These approaches have been primarily used to study the ecology of native wildlife. However, the same principles could equally be applied to the detection and distribution mapping of elusive invasive species, such as the feral fox. With appropriate verification of the methodology (Taberlet *et al.* 1999), non-invasive DNA-based methods could provide the high-quality distribution data that is required for effective control.

Here, we describe our approach to determining the extent of the fox incursion in Tasmania by developing, testing, then applying a PCR-based species-identification test to 1160 putative fox scats collected from throughout the island between 2003 and 2005, as part of the fox-eradication program. The identification of positive fox traces triggers management action such as extra baiting, scat collections, and spotlighting surveys. Such extra actions are expensive, so it is critical that any markers developed are reliable and accurate. We show our method to be highly accurate and more reliable than morphological analyses of scats. In addition, as an aid to determining the likelihood of fox reproduction in Tasmania we developed a sex-diagnostic DNA test. We identify a single fox scat from north-east Tasmania, and positively identify highly degraded roadkill remains from the north coast of Tasmania, as well as a blood sample from near Hobart, enabling control and surveillance to be intensified in these regions.

Methods

Principles of marker and assay development

Given the potentially large number of scats that would have to be screened to detect foxes in Tasmania, our focus was on developing rapid and cost-effective tests, rather than more involved DNA sequence analysis (e.g. Adams *et al.* 2003). Our preferred method involved species-specific PCR, which generally permits amplification of shorter PCR fragments than restriction digestion methods (e.g. Paxinos *et al.* 1997), and requires minimal processing of samples, thus increasing PCR robustness and reducing opportunities for sample mix-ups.

Multiplex PCR fox-diagnostic test

Primers were designed to amplify a 134-bp PCR product in the presence of fox DNA only. To ensure species-specificity we aligned the entire mitochondrial cytochrome *b* sequences of the seven mammalian carnivores that produce morphologically similar scats to those of foxes (fox, *Vulpes vulpes*, GenBank Accession X94929; domestic dog, *Canis familiaris*, GenBank Accession X94920; domestic cat, *Felis catus*, GenBank Accession AB004238; eastern quoll, *Dasyurus viverrinus*, GenBank Accession U07582; spotted-tailed quoll, *Dasyurus maculatus*, GenBank Accession M99461; Tasmanian devil, *Sarcophilus harrisi*, GenBank Accession M99465; and thylacine, *Thylacinus cyanocephalus*, GenBank Accession M99452), and designed primers that maximised mismatches between the fox and the remaining species (9–11 mismatches per species). Primer sequences are: *VV-cytb F* GACCTTCCCGCACCATCAAATAT, and *VV-cytb R* GAA-AGCAGTAGCTGTGTCAGA. These primers have no mis-

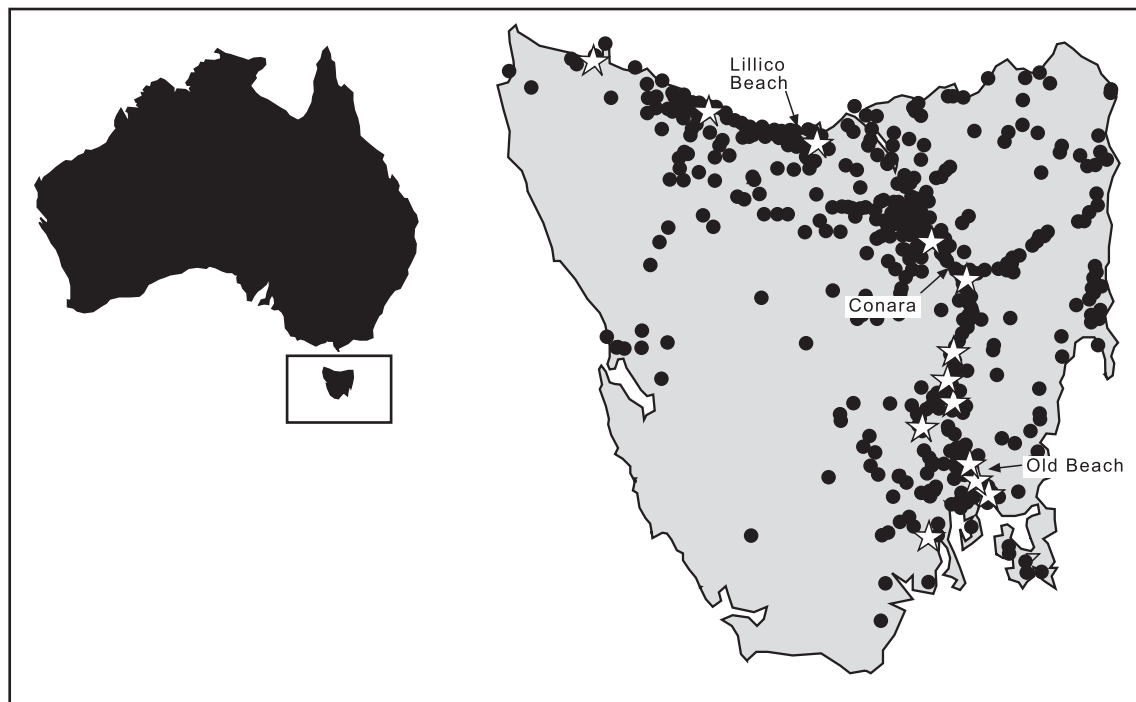


Fig. 1. The location of putative sightings of feral foxes reported in Tasmania between 2002 and 2005 (black circles), and areas where intensive scat searches took place (stars). Arrows mark localities referred to in the text. Data source: Fox-Free Tasmania Taskforce.

matches with western European *V. vulpes* sequences on GenBank (including samples from The United Kingdom, Sweden and Spain).

We also designed 'universal' primers that would serve as a positive amplification control and amplify a PCR product in the presence of any of the candidate mammalian carnivores and also co-amplify with the fox-specific primers (Fig. 2). To do so we aligned sequences from the mitochondrial 12S ribosomal gene from the candidate carnivore species (GenBank accession numbers: Y08508, Y08507, AY012149, U87402, U87401, AF009893, U87405) and designed primers GAGGGT-GACGGGCGGTGTGT (coinciding with the position of primer H1478: Kocher *et al.* 1989), and 12SL-1 CATAAA-AAMGTTAGGTCAAGGTGT, which produce a 183-bp PCR product.

We combined these primer pairs in a 10- μ L PCR multiplex reaction as follows: PCR Buffer (160 mM $(\text{NH}_4)_2\text{SO}_4$, 670 mM Tris-HCl pH 8.0, 0.1% Tween-20; Bioline), 2.5 mM MgCl_2 , 0.4 mg mL^{-1} bovine serum albumin, 0.2 mM each dNTP, 0.5 U Taq (Bioline RedTaq), primers (all at 0.4 μM), and 20 ng DNA template. The reaction was cycled through 94°C for 2 min (94°C for 30 s, 58°C for 20 s, 72°C for 15 s) \times 30, and 72°C 2 min. The specificity of these primers was confirmed by running a series of PCRs with 20 ng of genomic DNA from 10 individuals of each other candidate species sourced from across their geographic range or from multiple breeds (except thylacine). In addition, we trialed the test on DNA obtained from multiple foxes from each Australian state in which they are present ($n = 26$ overall, $n = 3-7$ per state). All amplifications were successfully diagnostic for fox. Finally, as a test of the risk of scoring error, we conducted a blind trial involving 10 fox, 5 dog and 5 cat samples.

Multiplex PCR sex-diagnostic test

We also used a multiplex-PCR approach to determine the sex of foxes from their scats. To do so we obtained sequences from the fox testis-determining *SRY* gene (deposited in GenBank ac. AY600298), and designed primers *VV-sry F* and *VV-sry R* within and adjacent to the HMG box. These primers maximised mismatches with prey or other species that may potentially contaminate scats (mice, rats, humans, dasyurid marsupials, macropod marsupials), and amplified a 78-bp PCR product. We confirmed the specificity of the *SRY* primers by attempting PCR amplification from genomic DNA of multiple male rats and mice ($n = 2$ each). As an amplification control we designed primers to the sequence of the *HPRT* gene of the domestic dog (Meyers-Wallen *et al.* 1995), which produces an X-linked product of 150 bp: *CF-hprt F* AGTCAACGGGGGACATAAAAG, *CF-hprt R* ACCATTTTGGATTACTGC. The PCR set-up for the sexing multiplex was the same as for the species-identification test, except that the *HPRT* primers were at a final concentration of 0.2 μM and the *SRY* primers were at 0.8 μM .

Robustness of the test under field conditions

To determine the robustness of our test under field conditions we conducted a scat ageing trial during winter in Canberra, ACT. We collected 200 fresh scats from captive foxes over a 14-day period and stored them at -80°C for 1-3 weeks until the ageing trial began. The scats were then randomly allocated

to one of five ageing treatments (40 scats per treatment: 0 days, 1 week, 3 weeks, 6 weeks and 12 weeks) and were placed in a grassed outdoor enclosure. In addition, we allocated an equal number of male and female fox scats to each ageing treatment.

Effect of different DNA extraction methods

Because of the potentially high cost and effort involved in extracting DNA from many scats (Piggott and Taylor 2003), we conducted a field experiment to examine the performance of these tests when using DNA extracted by two methods: one involving a commercial DNA extraction kit, which is labour intensive and costly, and the other a relatively rapid and inexpensive chelex-based technique. At each time interval (ageing treatment), scats were collected, split roughly in half (to enable direct comparison of PCR success between methods) and each half placed into a different plastic bag ready for DNA extraction and stored at room temperature until extraction.

DNA extraction protocols

DNA extractions took place within two days of collection. All scat extractions took place in a fume cupboard within a laboratory that had not previously been used for processing fox tissue or DNA, and all pipetting was done with aerosol-resistant tips. Extraction blanks were run with each extraction at the ratio of 20 samples to each blank. DNA was stored at 4°C until PCR analysis (usually <2 weeks). The commercial kit protocol involved gently washing scats in SLP buffer (500 mM Tris-HCl pH 9.0, 50 mM EDTA, 10 mM NaCl; Deuter *et al.* 1995) to release epithelial cells, followed by overnight digestion and purification with a Qiagen DNeasy kit (see Banks *et al.* 2002 for details). The second chelex-based method involved an initial wash with PBS buffer (pH 7.4) as above, then 500 μL of supernatant was combined with an equal volume of SET buffer (10 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 0.2% SDS) containing 5% chelex 100 beads (Biorad), and digested overnight with 0.25 mg proteinase K at 55°C. The tubes were then boiled for 5 min and centrifuged at 13 000 rpm for 5 min before 200 μL of supernatant was removed and stored at 4°C.

PCR amplification, scoring of results and data analysis

PCRs for scat DNA were as described above except that 2 μL of scat extract was used as template, and they were run for 35 cycles. PCR products were visualised on 3% high-resolution TBE agarose gels (Amresco 3:1 HRB™) after running for

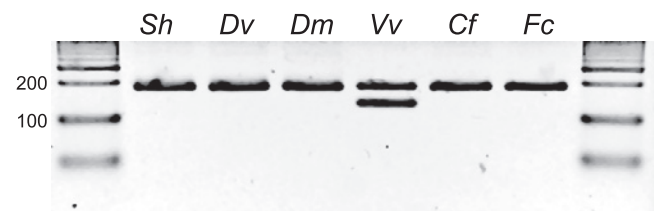


Fig. 2. Agarose gel showing diagnostic banding pattern for the fox (*Vv*) and other candidate carnivores present in Tasmania. *Sh*, Tasmanian devil; *Dv*, eastern quoll; *Dm*, spotted-tailed quoll; *Cf*, domestic dog; *Fc*, domestic cat. The left and right lanes are 100-bp-size standards.

35 min at 90 V, and then stained with ethidium bromide. For the fox-diagnostic PCR, we loaded 2.5 μ L of PCR product and for the sexing protocol, 10 μ L of PCR product. Negative controls (no template added) were run in each PCR and size standards of known DNA concentration were run on each gel (Hyperladder II™; Biorad). Gel images were captured on a Geldoc 2000 with QUANTITYONE software (Biorad). We used the volumes tool from the QUANTITYONE software to determine the average intensity of PCR products in optical density units. Intensity was corrected for differences in staining between gels by reference to the 200-bp standard band (=20 ng DNA). Repeatability of intensity was high (data not shown).

The effect of scat age and method of DNA extraction on species and sex diagnostic PCR

All species-diagnostic PCRs were successful on known fox scats ($n = 199$ for each extraction method). Therefore, we tested whether the age of scats or the method used to extract DNA from scats had an effect on the amplification efficiency (intensity) of the universal PCR product with generalised linear modelling implemented with SAS (SAS Institute, NC, USA). We also tested whether the method of DNA extraction or age of the scat affected the amplification success of the sexing multiplex (correct versus null result or incorrect) using log-linear models (Sokal and Rohlf 1995).

Tasmanian samples

Possible fox scats were collected opportunistically in Tasmania by members of the Fox-Free Tasmania Taskforce during routine trapping and searches for foxes in areas where sightings were most common. Because carnivore scats are commonly misidentified (Davison *et al.* 2002), and fox scats are morphologically similar to those of at least five other medium-sized carnivores present in Tasmania (Triggs 1996), the scats collected comprised all medium-sized carnivore-like scats.

Most were collected from the north-east of Tasmania, where sightings have been most common (Fig. 1). Scats were given a unique identity number, a GPS coordinate was obtained, and they were then sealed in a paper bag, and dried at room temperature before being sent by mail for analysis. Following the success of the field trials (see Results), DNA from all scats collected from Tasmania was extracted using the chelex method in a laminar-flow hood that had not been used to process any fox DNA, scats or tissue. The work area was subject to UV irradiation between extractions to destroy any potential residual DNA. PCRs were run with a positive and negative control.

In addition to scats, roadkill remains that were suspected to be fox were collected from a locality in northern Tasmania (Lillico Beach) (Fig. 1), and a blood sample was collected from a wooden surface adjacent to a chicken shed near Old Beach, north of Hobart. Both samples were subjected to the species-identification and sexing tests following DNA extraction via standard phenol-chloroform methods (Sambrook *et al.* 1989). The roadkill remains were collected at least nine weeks after they were first sighted. They consisted of skin and bone fragments, and their poor state of preservation meant that their species of origin could not be identified from gross morphology.

Results

Test verification: viability of scats in ageing trial

Scats persisted for extended periods in the field, with 100% of scats lasting for 12 weeks of the field trial ($n = 40$) and all but one lasting until collection (i.e. 1–6 weeks). The average weight of scats declined from 10.4 g (± 0.86 , s.e.) at Time 0 to 3.04 g (± 0.30 , s.e.) at 12 weeks.

Test verification: fox-diagnostic testing of scats of different ages

All of the samples used in the blind trial were correctly identified as either fox or non-fox (cat or dog). All known fox scats were correctly identified, regardless of the method of DNA extraction or their age, and in all cases both the universal and fox-specific PCR products were amplified ($n = 199$ for each extraction method). However, the intensity of PCR products declined significantly with scat age ($F = 46.58$, d.f. = 4, $P < 0.01$), and DNA extracted with the Qiagen method, on average, produced significantly more intense PCR products than chelex-extracted scats of the same age ($F = 32.01$, d.f. = 1, $P < 0.001$).

Test verification: sex diagnostic testing of scats of different ages

All sexing tests on scats that were no more than one week old were successful if DNA was extracted using the Qiagen kit, while tests using chelex extracts were ~10–25% less robust (Fig. 3). There was a decline in the ability to sex fox scats as they became older – at six weeks 60–70% of scats could be sexed correctly and at 12 weeks only 25% of scats could be accurately sexed. The main reason for failure of the sexing test was null results, where no diagnostic PCR products were produced, but up to 5% of tests identified sex incorrectly in each ageing treatment (Fig. 3). Log-linear modelling showed a significant three-factor interaction term, indicating that the difference in amplification success between Qiagen and chelex extracts also

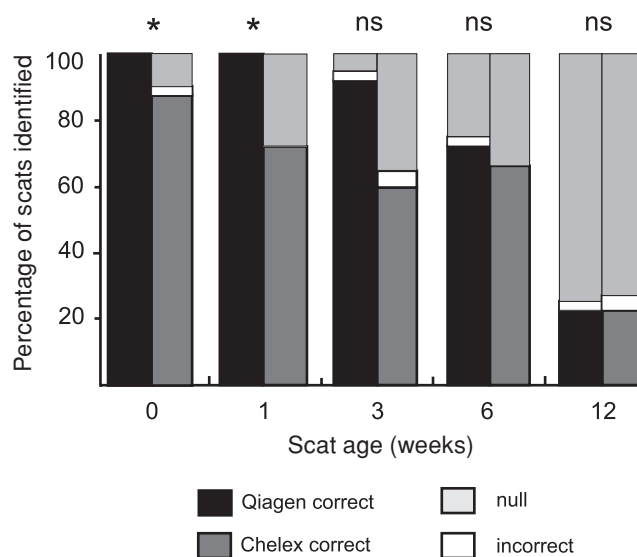


Fig. 3. Percentage of sex-diagnostic PCR tests that produced correct sex, incorrect sex, or null results (no amplification). The asterisk indicates that DNA-extraction methods were significantly different.

depended on scat age ($G = 17.38$, d.f. = 4, $P < 0.05$). Separate G-tests for scats of each age showed that Qiagen extracts were significantly more likely to produce a correct sex diagnosis than chelex extracts, if the scats were 0, 1 or 3 weeks old ($P < 0.05$), but neither method was significantly better at 6 or 12 weeks old. G-values for each time are 7.26, 13.62, 12.56, 0.96 and 0.00 for scats of 0, 1, 3, 6 and 12 weeks of age respectively, and the critical $\chi^2_{0.05,1}$ value is 3.841. G-tests also showed that the age of the scat had a significant effect on the success of sex-diagnostic PCR for both chelex and Qiagen extracts (Qiagen $G = 101.81$, chelex $G = 43.54$, d.f. = 4, $P < 0.05$).

In practice: testing of possible fox scats from Tasmania

In total, 1160 carnivore scats were collected from throughout Tasmania. The 'universal' PCR product was successfully amplified from 78% of scats, indicating that sufficient DNA was present for species identification. Most carnivore-like scats amplified the 'universal' PCR product only, indicating that they were not from fox. However, a single scat from Conara in the north-east of the island produced the diagnostic fox PCR product (Fig. 1). This result was replicated by PCR three times on two independent DNA extractions, and confirmed through direct sequencing of the fox-diagnostic PCR product, which showed a perfect match to *Vulpes vulpes* cytochrome *b* sequence (GenBank ac. X94929). The sexing test did not amplify DNA from this scat. Two independent DNA extractions from the roadkill remains and blood samples were identified as fox, and this diagnosis was confirmed by sequencing of the cytochrome *b* PCR product. The sexing test did not amplify any PCR products from either the roadkill or the blood sample.

Discussion

Early detection of an invasive species is a key to successful eradication (Myers *et al.* 2000). Species with low dispersal capabilities or strong affinities to particular habitats are typically good candidates for eradication because they are readily observed (e.g. Kuris and Culver 1999). Mobile, adaptable species such as the red fox, which typically occurs at low densities (Saunders *et al.* 1995), require novel detection strategies. Illustrating this, in Tasmania the analysis of DNA from carnivore scats has provided robust evidence for the presence of at least one fox, whereas the evidence from public sightings and morphological analysis of scats has often been ambiguous.

Foxes are present in Tasmania

Our aim was to identify fox scats in Tasmania with high accuracy and so provide a robust trigger for expensive and time-consuming control effort. Better targeting of baiting should reduce both expense and the poisoning of non-target native species. The number of field-collected scats that we processed (1160) is one of the largest such samples that we are aware of, and produced a single positive result from north-east Tasmania, in an area where sightings have been common. The significance of these results are twofold. First, in the context of growing public scepticism and political pressure to downscale control operations (Saunders *et al.* 2006), our results provide independent evidence for the presence of foxes in Tasmania, and given the potentially massive biodiversity and agricultural costs, reaffirms the importance of an eradication program. It also has enabled control

operations to be better targeted in this region (C. Parker, TDPIWE, pers. comm.).

Second, our results emphasise the large effort required to survey adequately for widely dispersed and mobile invasive species (cf. Sadlier *et al.* 2004). Undertaking such a large screening project requires a cost-effective protocol that is both highly accurate and reliable, and this is best achieved through extensive field-testing before implementation. Our field trials provided three significant results. First, fox scats are surprisingly persistent in the field, which, coupled with a method for species identification, greatly increases the opportunity to 'sight' animals and to take management action. This is important in that little was previously known of the persistence of canid scats in the field (but see Kohn *et al.* 1999). Second, sufficient DNA is contained within scats for 100% accuracy in species identification, even after three months of weathering, and probably longer, even though their external morphology was often greatly modified. Reliability, measured as the proportion of fox scats for which identity can be diagnosed, was also 100%. Third, DNA-based species identification is robust, no matter what method is used to extract DNA from scats. This is important, because DNA extraction with the commercial kit was the most costly (about AU\$6.0, Euro 3.6, US\$4.4 per sample in consumables) and time-consuming aspect of scat processing (compared with less than AU\$0.10 per sample for chelex). Use of a cheaper and more straightforward extraction protocol places fewer constraints on the number of scats that could be processed. In combination, these findings enable a high level of confidence to be placed on the analysis of field-collected scats from Tasmania. This credibility has real political and management consequences in situations such as the fox in Tasmania, where public scepticism and political pressure to abandon control programs are high (see Lapidge and Berry 2004; Saunders *et al.* 2006).

The value of extensive validation of the DNA-based method of testing is highlighted by earlier morphological analyses of carnivore scats from Tasmania, which was of limited usefulness as a monitoring tool, because a large proportion of scats could not be definitively identified (N. Mooney TDPIWE, pers. comm.), a finding that mirrors experience elsewhere (Davison *et al.* 2002). Indeed, several of the scats that we processed and identified as non-fox were independently identified as 'possible fox' by morphological analysis (L. Overend, TDPIWE, pers. comm.). Without recourse to a second method of diagnosis, this tentative appraisal could result in significant effort being wasted. Moreover, the scat that yielded a positive fox result via the DNA-based test was not initially identified as fox on the basis of hair and morphology.

In addition to species identification, DNA analysis of scats can reveal additional ecological information about invasive species that cannot be obtained from morphological analysis. For example, knowing the sex of feral predators could enable the estimation of sex ratios (Dallas *et al.* 2003) and the likelihood of reproduction. In the case of the fox in Tasmania, until a female fox was discovered dead in late 2003, there had been some evidence that only male foxes were introduced (N. Mooney, TDPIWE, pers. comm.). As expected from the results of Frantzen *et al.* (1998), our field trials demonstrated that such information is more difficult to obtain than species identifica-

tion from all but the freshest scats. Yet, success was still reasonably high from fresh scats (≤ 3 weeks old, 60–90%), and can be maximised by use of a more expensive and labour-intensive DNA-extraction protocol. If acquiring such information were particularly important for control of an invasive species, a two-tier process, where initial rapid and inexpensive screening for species is followed by the more involved sexing protocol, would provide the best scaling of costs and effort. However, as our results from the fox-positive scat, the roadkill and blood sample show, where the age of the sample is unknown and the degree of degradation is high, obtaining such results is not guaranteed.

In conclusion, we developed and tested a DNA-based method of species and sex identification for the feral fox, and applied it to an extensive field collection of carnivore scats from Tasmania, as well as some highly degraded roadkill remains and a blood sample. We demonstrated that scats are useful for monitoring elusive invasive species because they persist for long periods in the field and contain sufficient DNA for accurate and reliable species identification. Significantly, we identified a single fox scat from north-east Tasmania – a result that other detection approaches have been unable to achieve, and also positively identified roadkill remains and a blood sample from widely separate localities. Because foxes are likely to continue to be introduced to Tasmania both deliberately and accidentally (Saunders *et al.* 1995, 2006), this non-invasive approach should remain an important part of the ongoing management program.

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