

NON-INVASIVE RECOVERY AND DETECTION OF POSSUM (*Trichosurus vulpecula*) DNA
FROM BITTEN BAIT INTERFERENCE DEVICES (WaxTags[®])

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Running Title: Non-invasive detection of possum DNA

ABSTRACT

The brushtail possum is a major agricultural and ecological pest in New Zealand. A novel non-invasive DNA sampling tool for detecting its presence (WaxTags[®], or WT) was tested. DNA was recovered from saliva left on WT, and two lengths (407 and 648 bp) of the Cytochrome c Oxidase I (COI) barcoding region were amplified by Polymerase Chain Reaction (PCR). PCR products were considered (+) when a DNA band was clearly visible by electrophoresis. Different factors that might affect PCR (+) were investigated with captive possums: 1) both extraction protocols of the Qiagen DNeasy Blood and Tissue Kit, 2) effect of an overnight or longer delay of up to three weeks prior to DNA extraction on both COI amplicons, and 3) effect of the individual, order and magnitude of the bite. Extraction protocols were not significantly different. The effect of the overnight delay was not significant, and amplification of the short amplicon was significantly higher (100%) than for the long fragment (48%). After a two or three-week delay, the short amplicon had 94% and 56% PCR (+), success rates, respectively. Individual, order and magnitude of a bite had no significant effect. The delay trial was repeated with WT from the wild, for which PCR (+) rate of the short amplicon was 63%, regardless of freshness. Four microsatellites were amplified from captive WT samples. We conclude that DNA from saliva traces can be recovered from WT, a potential new tool for non-invasive monitoring of possums and other wildlife.

INTRODUCTION

Non-invasive DNA sampling is a valuable tool for studying cryptic or rare vertebrate species (Piggot and Taylor 2003). It has been used for a broad range of wildlife studies, increasing understanding of gene-flow, migration, hybridisation, paternity and social structure (Kohn *et al.*, 1999; Waits 2004). Non-invasive DNA sampling may be a useful tool for monitoring abundance of the introduced brushtail possum (*Trichosurus vulpecula*), a major predator of New Zealand (NZ) native birds and vegetation (Montague, 2000). The brushtail possum is also a natural wildlife reservoir of *Mycobacterium bovis*, the pathogenic agent of Bovine Tuberculosis (Tb) and, as such, its monitoring is crucial in controlling the transmission of Tb to farmed deer and cattle (De Lisle *et al.*, 2002). Monitoring possum density is especially important after a poisoning operation to control possum numbers, as only a few surviving possums can maintain a reservoir of Tb for subsequent re-infection of livestock (Fraser *et al.*, 2002). Given these possum impacts, there has been a sustained effort to reduce possum numbers throughout NZ, but this nocturnal marsupial can be difficult to detect with traditional leg-hold trap-catch methods when at low densities (Warburton, 2000; Forsyth *et al.*, 2005).

Although genetic tagging of marsupials from faeces has been successful (Piggot, 2004), and microsatellites have been isolated for the brushtail possum (Taylor and Cooper, 1998; Lam *et al.*, 2000; Cowan *et al.*, 2002; Taylor *et al.*, 2004), preliminary studies using possum faeces in NZ have highlighted difficulties in finding the stools (Morgan *et al.*, 2007). While hair follicles are another good DNA source for some animals (Taberlet *et al.*, 1993; Frantz *et al.*, 2004; Fickel and Hohmann, 2006), possum underfur is often released without a follicle (Gleeson *et al.*, 2003).

One recently developed index method for detecting possum presence through bait interference in NZ consists of leaving small wax blocks called WaxTags[®] (WT, Patent No. 516900,

2002) in the wild to be bitten (Thomas *et al.*, 2003). A WT (Figure 1) consists of a 12 cm³ block of wax attached to a white triangular plastic tag. The tag is attached to a tree and can be combined with a visual or olfactory lure, such as a luminescent strip and/or a flour-icing sugar mix. A national protocol for their use was developed in 2005 and recently updated (National Possum Control Agencies, 2005, 2008). WT have the potential to be more sensitive than leg-hold traps for low-density possum populations as they can be placed in high numbers over large areas (Thomas *et al.*, 2003). WT pose no animal welfare or environmental issues as they are non-toxic, safe, and less costly to use than leg-hold traps, which must be checked daily to comply with the NZ Animal Welfare Act, 1999. Also, as traps can cause suffering to animals, they are not always accepted by animal welfare advocates internationally (National Animal Welfare Advisory Committee, 2000). An advantage of WT over faecal samples is that the age of the sample is known, as well as its location. It is known that the longer animal faeces are in the field, the less DNA can be recovered from them (Nsubuga *et al.*, 2004; Piggot, 2004; Santini *et al.*, 2007). WT have the advantage of being attached to a tree, above the ground, as opposed to faeces, which are in contact with the humidity of the ground. It has been reported that DNA extracts made from wolf faeces that were left for 14-21 days above the ground, on stones, had significantly higher positive PCR rates than extracts from faeces left sitting directly on the ground (Santini *et al.*, 2007).

Figure 1. A bitten WaxTag[®] (photo by Mike Bowie).



While the numbers of bitten WT can be used to calculate an indirect index estimate (Thomas *et al.*, 2003, 2007), individual identification of possums interfering with WT would allow a more robust estimation of population size with the application of ‘mark-recapture’ techniques (Lancia *et al.*, 1994). Mark-recapture has been attempted using leg-hold traps (Warburton, 2000), however, this requires both capture and tagging, which is labour-intensive and expensive. Non-invasive DNA samples from possums would provide the opportunity to genetically tag individual possums. A genetic tag could also provide additional information such as gender, relatedness (Bayes *et al.*, 2000), and possibly Tb presence.

For over a decade, epithelial buccal cells in saliva have been used as a source of DNA, and they are an important source of forensic evidence. Thanks to the Polymerase Chain Reaction (PCR)

(Saiki *et al.*, 1985) very small quantities of DNA can be detected, amplified and analysed, allowing the analysis of DNA from buccal cells in saliva and other body fluids. For example, saliva on chewed food has been used for genetic studies in chimpanzees (Hashimoto *et al.*, 1996; Inoue *et al.*, 2007; Sugiyama *et al.*, 1993; Takenaka *et al.*, 1993), saliva from predatory wounds on sheep has been used to identify the predator species (Williams *et al.*, 2003), and human saliva has been recovered from victims (Sweet and Shutler, 1999), drinking containers (Abaz *et al.*, 2002), stamps and envelopes (Sinclair and McKechnie, 2000) and partially consumed food (Sweet and Hildebrand, 1999). Accordingly, it seems possible that possum saliva found on WT could yield good quality DNA.

Genetic tagging of possums from bitten WT left in the wild would add greatly to the value of WT as a research tool for estimating, monitoring and studying low-density possum populations in NZ. This study tested the feasibility of recovering DNA from bitten WT in sufficient amounts for future genotyping, by extracting DNA from WT bitten by captive or wild animals and amplifying a 648 bp and a 407 bp fragment of the Cytochrome c Oxidase I (COI) gene. Different factors that might affect the successful amplification of DNA in sequenceable amounts were investigated with captive possums: (1) extraction protocol, (2) the effect of an overnight or longer delay of up to three weeks before DNA extraction, and (3) PCR recipe. In addition, the effects of the individual, order, and magnitude of the bite on the success of DNA amplification were investigated to detect possible DNA shedding differences, as has been reported for humans (Lowe, 2002). Following captive studies, field trials were carried out, resulting in amplification of long and short COI fragments from DNA extracts of wild-bitten WT. Species identification by BLASTing was tested with even shorter COI fragments (50 or 100bp). Once a sampling and DNA extraction methodology were

selected, microsatellite amplification was trialled for four microsatellites, in preparation for future genotyping of WT samples bitten by possums.

MATERIALS AND METHODS

Between July 2006 and January 2008, WT were individually presented to captive possums at Landcare Research, Lincoln, and Pest Control Research Ltd. (both located in Christchurch, Canterbury). All captive possums had been caught from the wild in Canterbury for other projects. The field trials were carried out between August 2006 and September 2007 in exotic pine forest and farmland near Lincoln University, Canterbury. The range of protocols that were implemented across different trials is described below. Each trial specifies which variation of any protocol was used.

Sampling of WT and blood from captive possums

WT were presented to individual possums. Within two hours, bitten WT were taken to the laboratory in a manner that prevented cross-contamination between samples. Blood samples from nine different captive animals were donated by Landcare Research, to compare the quality of this DNA source with the DNA recovered from bitten WT. All blood samples of captive animals were taken while the animals were anesthetized with Isoflourane (Attane[®]); these were subsamples of blood taken for a different research project (Animal Ethics Committee Permit Code # 04/12/02), from which post-mortem ear tissue samples were also donated. Blood samples were placed in collection tubes containing the anticoagulant EDTA and kept at 4°C until extraction on the same day. Ear tissue samples were of 10 captive possums that had bitten WT included in this study. Tissue samples were frozen in ethanol at -20 °C until DNA extraction.

DNA extraction from bitten WT, blood and tissue samples

DNA from bitten WT was extracted using the DNeasy Tissue and Blood kit from Qiagen. The wax block was cut off the plastic tag and rinsed with either 1.6 or 15 ml of Phosphate Buffered Saline (PBS) (50 mM potassium phosphate, 150 mM NaCl, pH 7.2) and the saliva/PBS mixture was transferred to a 15 or 1.5 ml centrifugation tube, respectively. Centrifugation of 1.6 ml samples was for 1150 g for 1 min and, for 15 ml samples, 1500 g for 10 min. For both sample volumes, the supernatant was removed by aspiration, leaving the bottom 200 μ l of concentrated buccal cell/PBS mix. The resulting 200 μ l sample was vortexed (and, if its original volume was a 15 ml, transferred to a 1.5 ml microcentrifuge tube), and DNA was extracted.

Both extraction protocols from the DNeasy kit were trialled with WT. The ATL extraction protocol is based on the manufacturer's tissue protocol. 180 μ l of buffer ATL and 20 μ l of Proteinase K were added to the 200 μ l of saliva/PBS mix and mixed by vortexing. The mixture was incubated in a dry oven at 56°C for 16 hours on a rocking platform, after which 200 μ l of buffer AL and 200 μ l of ethanol were added to the sample and vortexed, precipitating the DNA. The mixture was transferred to a spin column included in the kit and centrifuged at 6000 g for 1 min, binding the DNA to the filter of the spin column.

The AL extraction protocol is based on the manufacturer's blood and cultured cells protocol. 20 μ l of Proteinase K and 200 μ l of buffer AL were added to the 200 μ l saliva/PBS mix, and mixed by vortexing. The mixture was incubated in a water bath at 70°C for 10 minutes to promote cell lysis (this step has since been removed by the manufacturer). Following incubation, 200 μ l of ethanol (99%) was added to the sample and vortexed, precipitating the DNA. The mixture was transferred to a spin column included in the kit.

The remainder of both protocols followed the manufacturer's instructions, with two washes, the first with 500 μ l of buffer AW1 followed by centrifugation at 6000 g for 1 min, and the second with 500 μ l of buffer AW2 and centrifugation at 18400 g for 3 min, with flow-throughs being discarded with their respective spin collection tubes. The spin column was then placed in a new 1.5 ml spin tube, and the DNA was eluted by adding 50 μ l of distilled water to the spin column, releasing the DNA from the filter. Centrifuging at 6000 g for 1 min transferred the DNA extract as flow-through into the new spin tube, which was stored at -20°C .

DNA extraction of blood samples was carried out with the manufacturer's blood protocol. Blood samples contained 10 μ l of whole blood in EDTA and the elution volume was 50 μ l of water. Tissue samples were extracted with the manufacturer's tissue protocol and eluted in 200 μ l of Buffer AE.

Amplification and quantification of DNA from WT, blood and tissue samples

The DNA barcoding region of the mitochondrial Cytochrome c Oxidase I (COI) gene was amplified from WT and blood extracts using a MasterCycler (Eppendorf) (denaturation at 94°C for 2 min, then 33 cycles of denaturation at 92°C for 30 sec, annealing at 45°C for 30 sec and extension at 72°C for 90 sec, followed by 72°C for 5 min). Two PCR recipes were trialled for the COI amplicons. Standard PCR reactions of 25 μ l contained 2.5 μ l of DNA extraction, 2.5 μ l of x10 Buffer (Qiagen) (final MgCl_2 concentration of 1.5 mM), 0.2 mM of each dNTP (Qiagen), 1.2 μ l of each primer from a 10 μ M stock (Invitrogen) (Table 1), 0.2 μ l of Taq DNA polymerase (5 u/ μ l) (Qiagen) and 14.9 μ l of water. Improved PCR reactions had 2.5 mM of MgCl_2 per reaction (25 mM MgCl_2 stock from Invitrogen), and 0.25 mM of each dNTP, the rest of the recipe was identical to the standard recipe. All PCRs had negative and positive controls.

Table 1. Primers used for amplifying cytochrome c oxidase I (COI) from DNA recovered from bitten WaxTags[®]

COI amplicon	Primers (5'-3' direction)	Reference
648 bp	Forward primer LCO1490: GGTCAACAAATCATAAAAAGATATTGG-	Folmer <i>et al.</i> , 1994
	Reverse primer HCO2198: TAAACTTCAGGCTGACCAAAAAATCA	
407 bp	Forward primer MF1: GCTTCCACGAATAAATAATA	Hajibabaei <i>et al.</i> , 2006a
	Reverse primer as above	

Possum microsatellites were amplified by a preamplification followed by a reamplification, as suggested by Piggot *et al.* (2004) and Bellemain and Taberlet (2004) for increasing microsatellite amplification success of DNA in limiting conditions, with the primers indicated in Table 2.

Preamplifications had denaturation at 94°C for 2 min, then 30 cycles of denaturation at 92°C for 30 sec, annealing as in Table 2 for 30 sec and extension at 72°C for 90 sec. Reamplifications had 95°C for 4 min, then a touch down (TD) of 10 cycles of at 94°C for 30 sec, annealing as in Table 2 for 45 sec and extension at 72°C for 45 sec, followed by 35 cycles with 94°C for 30 sec, annealing at the coolest reached TD temperature as in Table 2 for 45 sec, 72°C for 45 sec, and a final extension at 72°C for 5 min.

Preamplification reactions of 20 µl had 4 µl of DNA extract, 2 µl of x10 Buffer (Intron) up to 0.8 µl of MgCl₂ 25 mM (Intron) (final MgCl₂ concentration of 1.5-2.5 mM), 0.25 mM of each dNTP (Intron), 0.5 pmoles of each primer (Invitrogen) (Table 2), 0.16 µl of iTaq DNA polymerase 5U/µl (Intron) and water. Reamplification reactions of 10 µl had 1 µl of 2% preamplification PCR product diluted in water, 1 µl of x10 Buffer (Intron), up to 0.4 µl of MgCl₂ 25 mM (Intron) (final

MgCl₂ concentration of 1.5-2.5 mM), 0.25 mM of each dNTP (Intron), 5pmoles of each primer (Invitrogen) (Table 2), 0.16 µl of iTaq DNA polymerase 5U/µl (Intron) and water.

Table 2. Microsatellite primers and annealing temperatures.

Locus	Primers (5'-3')	Annealing temp. (°C)
TV58 Preamplification	F*: GCACCCAAGGACCCCAAGA TV58_pre R: CCATCTCACTTTTGTTCCTG	50
TV58 Reamplification	TV58_post_F: TAGTCTTCCTGCACCGTTAATC R*: CCATATCACAGTGCTTGGCG	TD 65-55
TV53 Preamplification	F*: GGGAGTAGTTGTCTGAGTTCCC TV54pre R: CAAACGGAATGTGACCCAGAAC	60
TV53 Reamplification	TV53post_F: TGAATAGCAGGGATAAGCCTAGG R*: CCCTGGAGTTTGACAACCTG	TD 70-60
TV54 Preamplification	TV54pre_F: ATGCTTGATAAGGGCATAAGGGAG TV54pre R: GGCTTTTCCTGATATCGCTCAC	60
TV54 Reamplification	F*: GGGAGGCATAAGTGCCAGA R*: TGACCGACACTGACGACCCC	70-60
TV5.64 Preamplification	F _ψ : TTTATCCCTACTAGAGGTAGGT R _ψ : CCCTCTCCATCTGCTCCTC	50
TV5.64 Reamplification	F _ψ : TTTATCCCTACTAGAGGTAGGT R: ATTAGCGCTTACCAGAGTGC	TD 62-52

*Taylor and Cooper, 1998, ^ψ Nicola Aitken (pers. comm, 2006).

Evaluation of amplification success

The success of each PCR reaction was evaluated by running 5 µl of PCR product by electrophoresis at 80V for 45 min on a 1.5% agarose gel made with SYBRSafe (Invitrogen) with a 100 bp ladder (Tridye, New England Biolabs), then visualizing bands with the program GenSnap (Syngene, Synoptics Ltd, Cambridge). Where indicated, PCR products were quantified by

comparison with 2 μ l of DNA Low Mass Ladder (Invitrogen) with the program GenTools (Syngene, Synoptics Ltd, Cambridge). Clearly visible DNA bands (i.e. those that had >10 ng per band) were considered as PCR positive (PCR (+)).

In order to test if allelic size differences across individuals can be detected, 6 μ l of a mix of 7 μ l PCR product and 2 μ l loading dye of reamplification PCR product of the microsatellite TV5.64 (a (GATA)₁₄ repeat ranging in size from 91-173 bp) were loaded into a 2.5% high resolution Metaphor agarose gel (FMC Bioproducts) made in 1 x TBE. Electrophoresis was in 1 x TBE running buffer at 200V for 1.3 hr. The marker was 1.8 μ l of a 50 bp ladder (0.3 μ g/ μ l) (New England Biolabs). After electrophoresis, the gel was stained for 40 min in a solution of 1mg EtBr/L in 1 x TBE and destained in water for 20 min and DNA bands were visualized and photographed with GenSnap.

DNA clean-up and sequencing

DNA sequencing was performed for randomly selected PCR (+) products to confirm that the DNA belonged to a possum. Sequencing reactions contained 0.5 μ l of Big Dye Terminator (v3.1) and 2 μ l of x10 sequencing buffer (both from Applied Biosystems' Cycle Sequencing Kit), 0.8 μ l of primer (from a 10 μ M stock), and 1-3 μ l of PCR product (1 μ l if the PCR product had >10 ng DNA/ μ l, 2 μ l if it had 6-10 ng DNA/ μ l or 3 μ l if it had 2-6 ng DNA/ μ l) to have 6-20 ng of DNA per reaction, and water to complete 10 μ l. If PCR products had weak bands (\leq 10 ng in 5 μ l of PCR product), 10-15 μ l of PCR product was cleaned with the Qiagen QIAquick PCR purification kit, eluted in 30 μ l of elution buffer, of which 3.85 μ l was used in a sequencing reaction. The sequencing reaction was 96°C for 1 min, followed by 25 cycles of 96°C for 10 sec, 50°C for 5 sec

and 60°C for 4 min. After a magnetic clean-up (CleanSeq, Agencourt Bioscience Corporation) sequencing reactions were run on a 3100 Avant Genetic Analyser (ABI Prism) sequencer (Applied Biosystems).

Species identification of WT and blood extracts

Sequences were viewed with the program 4Peaks (Griekspoor and Groothuis, 2005), edited and aligned in the program Se-Al (Rambaut, 1996) and identified using the Basic Local Alignment Search Tool (BLAST) from PubMed (<http://130.14.29.110/BLAST/>) to compare the sequences with those in GenBank. WT and blood samples were also aligned and compared with a published possum mitochondrial genome sequence (Phillips *et al.*, 2001).

TRIALS

1. Captive possums: comparison of ATL and AL extraction protocols

During July 2006 to November 2007, WT were presented to 57 possums at Landcare Research, Lincoln and Pest Control Research Ltd. WT were then rinsed with 1.6 (14 ATL extractions and 16 AL extractions) or 15 ml (25 ATL and 17 AL) of PBS. The numbers of animals and tags used for each protocol are shown in Table 3. Samples were sequenced with the LCO1490 primer.

2. Captive and wild possums: effect of an overnight delay prior to extraction on PCR success according to the length of the amplified region

Captive possums: A total of 45 WT (20 for no delay, and 25 for overnight delay) were presented to a total of 32 captive animals at Landcare Research during July-September 2007. Overnight delay samples ($n=16$) were extracted after one indoor overnight delay at room temperature (RT) sorted on a rack placed in the transporting box. All WT had their DNA content extracted with the ATL protocol (15 ml PBS wash) and both lengths of COI fragments were amplified with the standard PCR recipe.

Wild possums: WT ($n=77$) were stapled or nailed individually on trees in commercial pine forests in Burnham, Christchurch, or on willows along the “LII” river and at a nearby farm, during the day. A flour/icing-sugar blaze (one part icing sugar to five of white (National Possum Control Agencies, 2008)), was sprinkled on the tree trunk before stapling on a clean WT. WT were checked the next day between 9-10 AM (to avoid unnecessary exposure of the DNA to the elements) and taken to the laboratory, keeping the samples separate. Extraction (AL or ATL protocol, with 1.6 and 15 ml PBS rinse respectively) took place within two hours of the samples being collected ($n=52$), except for the 25 WT that were kept overnight. Both lengths of COI fragments were amplified with the standard PCR recipe.

Captive and field WT combined: In order to determine how the origin of the sample affects PCR (+), and the overall effect of the overnight delay, the two datasets were analyzed together.

3. Captive possums: effect of a delay of one to three weeks prior to extraction on PCR success according to the length of the amplified region and PCR recipe

Two trials were carried out with four animals from Landcare Research (2006) and four animals from Pest Control Research Ltd. (2007). In each trial, four bitten WT from each possum were used to investigate the effect of a delay (of zero, one, two, and three weeks) indoors at RT before DNA extraction on PCR (+) ($n=32$ WT).

All WT were washed with 1.6 ml PBS and extracted with the AL or ATL protocol ($n=16$ with each protocol). Amplification of both COI fragments was made with the standard PCR recipe, followed by amplification with the improved PCR recipe.

4. Captive possums: effect of the individual and bite order on PCR success

WT ($n=30$) were offered to 10 captive animals (three WT each animal) at Pest Control Research Ltd in November 2007. The interval between the three WTs offered to any individual was 30 min. All WTs were washed with 1.6 ml of PBS and extracted with the ATL protocol. Amplification of the 648 bp long COI region was made with the standard recipe. PCR products were viewed and DNA was quantified. In order to observe any trends in bite order, the number of nanograms (ng) per bite per possum was graphed.

5. Captive possums: effect of the magnitude of the bite on PCR success according to the length of amplicon

All WT bitten by captive possums with quantified PCR product produced with either the standard or improved PCR recipe were weighed to determine the effect that removal of wax from the bite had on the amount of amplified DNA of the long and short COI fragments. The dataset

consisted of 102 long fragments obtained with the standard PCR recipe, 32 long fragments obtained with the improved PCR recipe, and 29 short fragments obtained with the improved PCR recipe. These samples were extracted with the ATL or AL protocol, and some had an overnight delay prior to extraction. The data were normalised using a reciprocal transformation (as they were positively skewed) and analysed by ANOVA, adjusted for the weight of the WT as a covariate.

6. Minimum length of COI sequence required for possum barcoding

BLAST searches on GenBank with 50 or 100 bp long segments of 600 bp of the 648 bp long COI fragment were performed and the maximum identity scores of the matches were recorded for the first and second best matches respectively.

7. Amplification of possum microsatellites

Microsatellites were amplified from DNA extracts of up to 31 WT bitten by different captive possums (extracted with ATL or AL protocols) which had been PCR (+) for the short COI amplicon. In addition to the captive possum DNA extracts, five WT bitten by wild possums (with no delay prior to extraction) were used as templates for the amplification of TV53, TV54 and TV58. TV5.64 was amplified from the 10 WT and respective tissue DNA extracts.

Statistical Analysis

For each PCR product the presence or absence of a notably visible (PCR (+)) band in the electrophoresis gel was recorded. A Generalized Linear Model (GLM) with a Bernoulli Distribution was then fitted to the percentage of WT that yielded PCR (+) per treatment. In the zero to three

week delay trial, a GLM was first run to detect any effect of year on PCR (+) before combining the 2006 and 2007 data for the complete GLM analysis (not significant).

The lengths (i.e. number of bp) of edited sequences from different treatments were compared using two-sample *t*-tests, assuming equal variances. When DNA was quantified (ng), ANOVA was used to quantify any treatment effect. Where ANOVA indicated significant differences, pairwise comparisons were made using Fishers LSD (Least Significant Differences) test.

RESULTS

1. Captive possums: comparison of ATL and AL extraction protocols

Both ATL and AL protocols recovered the long amplicon from approximately 56% of bitten WT, with no significant difference in the percentage of PCR (+) per protocol ($\chi^2_{1,7}=0.01$; $P=0.92$) (Table 3).

Table 3. Success of ATL and AL DNeasy Blood and Tissue Kit (Qiagen) extraction protocols with WT bitten by captive possums.

Protocol	Possums (<i>n</i>)	WaxTags [®] (<i>n</i>)	PCR(+) % (mean ± SEM)	Sequences (<i>n</i>)	Sequence [#] length (mean ± SEM)
Tissue (ATL)	31	39	56 ± 10	5	476.2 ± 50
Blood (AL)	33	33	55 ± 12	16	529.7 ± 22

The number of WT and animals involved, PCR success rate (PCR (+)), number of sequences obtained, and length of sequences for cytochrome c oxidase I DNA from bitten samples is indicated. DNA sequencing of successful PCR products was random and not extensive. # Unidirectional COI sequence.

There was no effect of the volume of the PBS wash buffer on PCR (+) ($\chi_{1,71}=1.269$, $P=0.26$) or length of the centrifugation time ($\chi^2_{1,71}=0.099$; $P=0.75$).

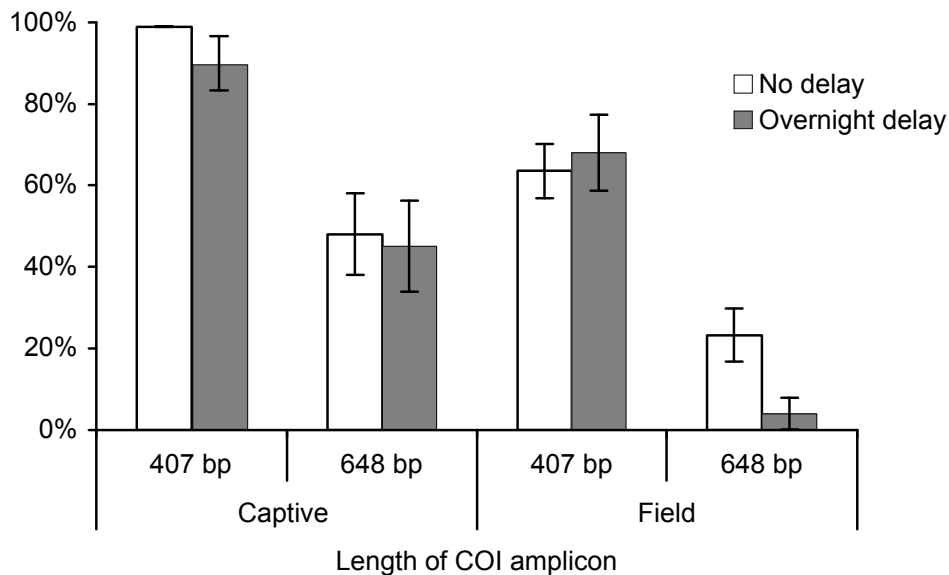
The nine blood sample sequences had a mean length of 572.1 ± 10 SEM bp. The length of the 21 sequences obtained from bitten WT using either the ATL and AL protocols was not significantly different from those obtained from blood samples ($t = -1.70$, d.f. = 28, $P = 0.099$).

2. Captive and wild possums: effect of an overnight delay prior to extraction on PCR success according to the length of the amplified region

Captive possums: the effect of an overnight delay on PCR (+) was not significant ($\chi^2_{1,87}=0.65$; $P < 0.422$) for any amplicon size; however, the overall effect of the size of the COI fragment on fresh or delayed WT was significant ($\chi^2_{1,87}=29.66$; $P < 0.01$), with success rates of 48% and 100% for the long and short amplicons respectively, regardless of freshness (Figure 2).

Wild possums: the effect of an overnight delay on PCR (+) was not significant ($\chi^2_{1,141}=0.87$, $P = 0.351$). PCR (+) rates from field samples were significantly affected by the length of the COI amplicon ($\chi^2_{1,141}=37.39$, $P < 0.001$), with the short amplicon having a significantly higher success rate (63%) than the long amplicon (23%). The interaction between the effects of the overnight delay and the length of the amplicon was significant ($\chi^2_{1,141}=4.44$, $P = 0.035$) (Figure 2).

Figure 2. Effect of an overnight delay before extraction on Polymerase Chain Reaction (PCR) success (PCR (+)% \pm Standard Error of the Mean) according to the length (long, 648 base-pair (bp), and short (407 bp)) of the Cytochrome Oxidase c I (COI) amplicon, with WaxTags[®] bitten by captive (no delay ($n=20$), overnight delay ($n=25$)) and wild possums (no delay ($n=52$), overnight delay ($n=25$)). The COI region was amplified with the standard PCR recipe.



The lengths of sequences obtained with WT from the field are given in Table 4. When BLASTed, each sequence matched a section of the complete mitochondrial genome of *Trichosurus vulpecula* (GenBank accession number AF357238). The sequences obtained from wild possum WT DNA samples matched exactly the sequences previously obtained from 10 µl blood samples of captive possums (Trial 1). Six field samples with strong DNA bands were sequenced, as were 16 field samples with weak DNA bands. These weak PCR products produced 14 good quality sequences (Table 4).

Table 4. Lengths and matching scores of cytochrome c oxidase I (COI) sequences obtained from WaxTags[®] bitten in the wild, when BLASTed on GenBank. The sequences are classified according to the primer used for sequencing.

Primer used for PCR and sequencing	Number of sequences	Sequence length (mean ± SEM)	% MI of sequence to Possum COI (mean ± SEM)	% MI of second-best match (mean ± SEM)
LCO [#]	3	439 ± 2	100 ± 0	83.67 ± 1.67
LCO + HCO	3	580 ± 0	99.67 ± 0.33	82 ± 0
MF1 [#]	14	358 ± 21	98.64 ± 0.39	83.21 ± 0.26

All sequences were the closest match (highest % of Maximum Identity (%MI)) to a COI possum sequence on GenBank. For comparison, the %MI of the second-best match (not possum) is also listed. Fragments BLASTed on GenBank on 13 May 2008. [#]Unidirectional COI sequence. SEM=Standard Error of the Mean.

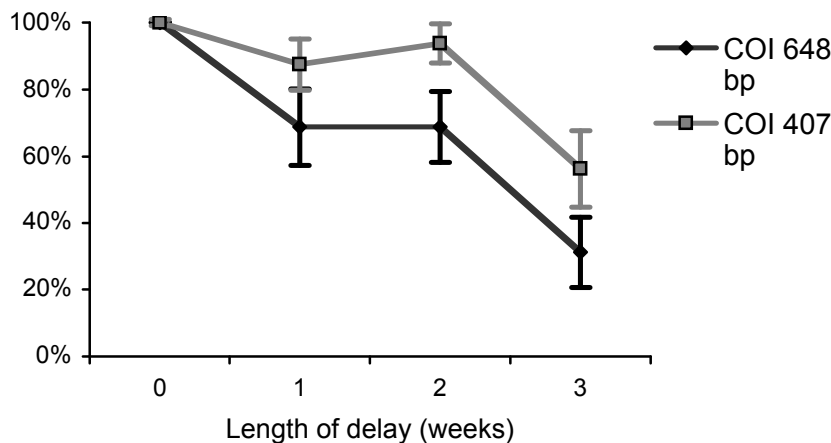
Captive and wild possums combined: The origin of the sample had a significant effect on PCR (+) ($\chi^2_{1,227} = 19.26, P < 0.001$), with field samples having a lower PCR (+) rate for the short and long amplicons (47 and 16% respectively) than captive samples (96 and 47%). An overnight delay had no significant effect on PCR (+) ($\chi^2_{1,227} = 1.45, P = 0.228$), but the length of the amplicon significantly affected PCR (+) ($\chi^2_{1,227} = 65.78, P < 0.001$). The interaction between an overnight

delay and the length of the amplicon on PCR (+) was not significant ($\chi^2_{1,227}=1.12, P=0.291$). There was a significant interaction between the origin of the sample, the length of the amplicon and the overnight delay ($\chi^2_{1,227}= 6.072, P=0.014$); field samples that had an overnight delay had a significantly lower PCR (+) rate (4%) for the long amplicon.

3. Captive possums: effect of a delay of one to three weeks prior to extraction on PCR success according to the length of the amplified region and PCR recipe

The PCR recipe used (standard or improved) had a significant effect on PCR (+) ($\chi^2_{1,111}=9.63, P=0.002$), as did the length of the amplicon ($\chi^2_{1,111}=7.64, P =0.006$). The duration of the delay prior to DNA extraction had a significant effect on PCR (+) ($\chi^2_{3,111}= 11.12, P > 0.001$) and this effect was independent of amplicon length or PCR recipe. PCR (+) rates for both fragment sizes were significantly reduced if they had a one-week delay before extraction, and significantly reduced again when extracted after a three-week delay. However, the short amplicon still had a 94% PCR (+) even after a two week delay (Figure 3).

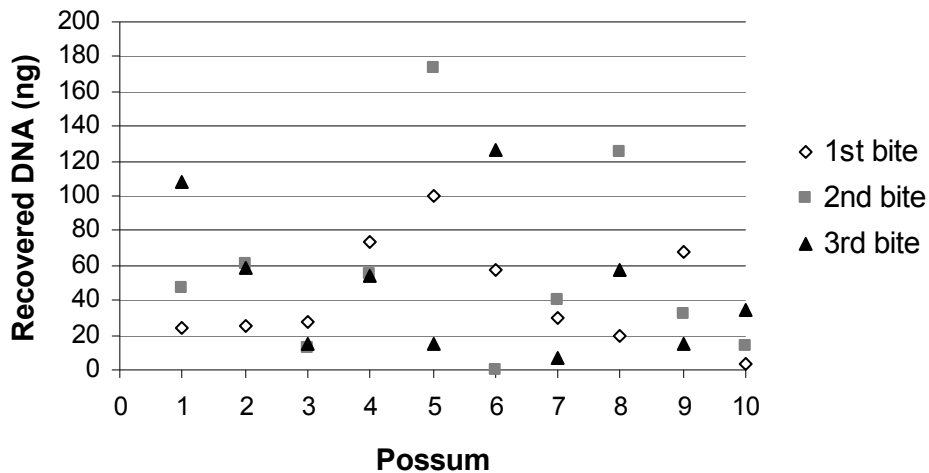
Figure 3. Effect of a one to three week delay prior to extraction on Polymerase Chain Reaction success (PCR (+)%) \pm Standard Error of the Mean according to the length (long, 648 base-pair (bp), and short (407 bp)) of the Cytochrome Oxidase c I (COI) amplicon, with 32 WaxTags[®] bitten by captive possums. The COI region was amplified with both standard and improved PCR recipes.



4. Captive possums: effect of the individual and bite order on PCR success

PCR (+) was not affected by individual DNA-shedding differences ($F_{5,12}=1.03$, $P=0.442$) or bite order ($F_{14,12}=0.88$, $P=0.592$) (Figure 4).

Figure 4. Amount of DNA recovered from 10 different possums. Three WaxTags[®] were bitten by each animal, with half hour intervals between bites.



5. Captive possums: effect of the magnitude of the bite on PCR success according to the length of amplicon and PCR recipe

The estimator of the magnitude of the bite (amount of wax remaining on the bitten WT), had no effect on the amount of DNA recovered (ng) ($F_{1, 159}: 0.02$, $P=0.88$). However, the length of the COI amplicon had a significant effect on PCR (+), with the short fragment being amplified in significantly higher amounts than the long amplicon ($F_{2, 159}: 7.95$, $P<0.001$). The improved PCR recipe also produced significantly higher PCR (+) of the long COI fragment ($F_{1, 159}: 15.84$, $P<0.001$) than the standard PCR recipe.

6. Minimum length of COI sequence required for possum barcoding

All of the 50 bp portions of the COI sequence were identified as belonging to the possum species with a 100% maximum identity score (none of the next-closest matches had scores over 96% maximum identity), and thus were unequivocally matched to possum COI (Table 5).

Table 5. Maximum identity scores of 50 or 100 bp sections of the long COI fragment as matches to the possum sequence.

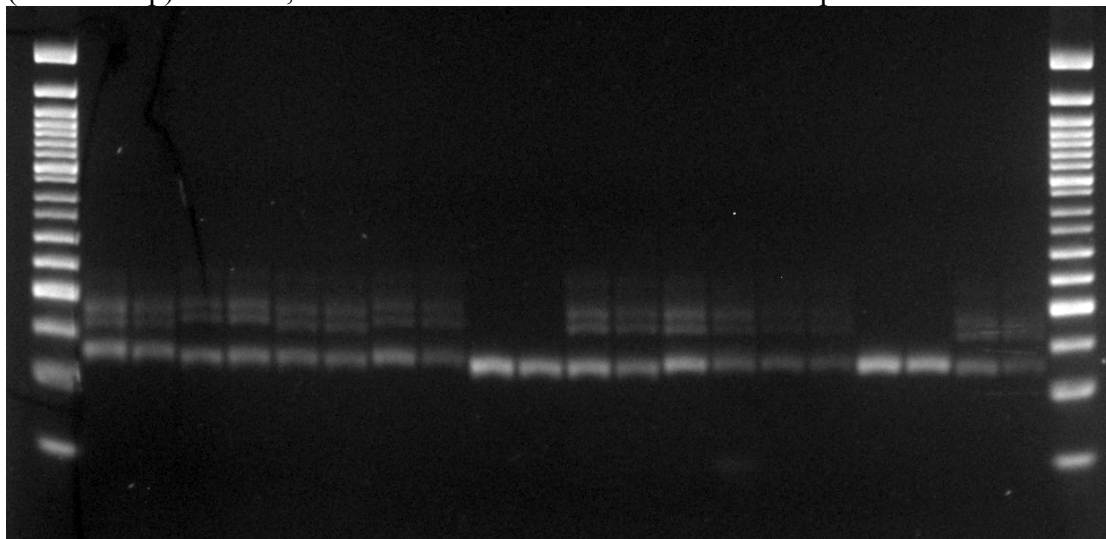
Length of section of COI 600 bp fragment	% Maximum identity matching Possum (mean \pm SEM)	% Maximum identity of second-best match (mean \pm SEM)
50 bp (12 sections)	100 \pm 0	94.42 \pm 0.56
100 bp (6 sections)	100 \pm 0	88.67 \pm 0.95

All sequences were the closest match (highest % of Maximum Identity (%MI)) to a cytochrome c oxidase I possum sequence on GenBank. For comparison, the %MI of the second-best match (not possum) is also listed. Fragments BLASTed on GenBank on 13 May 2008. # Unidirectional COI sequence.

7. Amplification of possum microsatellites

Amplification of TV53 and TV54 had 94% PCR (+) from captive WT samples ($n= 31$) and 67% PCR (+) for wild WT samples ($n=6$). TV58 had PCR (+) of 81% ($n= 27$). TV5.64 was amplified successfully (100% PCR (+)) from the 10 WT and respective matching tissue samples. Allelic size variations for TV5.64 were observed between individuals, seven of which were heterozygotes with both alleles within 100-200 bp long. Tissue and WT samples had TV5.64 similarly amplified in intensity, and allelic sizes were consistent for each animal (Figure 5).

Figure 5. High resolution agarose gel showing allelic variability in TV5.64 for an ear and WT DNA sample for each of 10 captive possums, respectively. Lanes on two sides are 50 bp ladder (1350-50 bp) markers, the lowest band of each ladder is of 50 bp.



DISCUSSION

Our experiments show that DNA can be recovered and amplified from traces of saliva on WaxTag[®] bait interference devices. The quality of the sequences obtained from WT bitten by captive or wild possums is comparable to those obtained from blood samples (Trial 1 and 2). Even though neither of the extraction protocols trialled are specifically designed for trace DNA, both the Tissue (ATL) and Blood or Cultured Cells (AL) protocols of the DNeasy Kit (Qiagen) recovered DNA from the bitten WT in a sufficient amount to produce positive PCR products of the 648 bp long COI fragment with 56% of the samples (Trial 1). The COI sequences obtained from WT PCR product had similar quality and length to those obtained from blood samples, indicating that the quality and quantity of recovered DNA is useful for the detection of the species with COI sequences, and maybe for other molecular purposes. Future users of the method may choose, for practical reasons only, between a DNA extraction method that is completed over two days, and a method that can be completed on the day of the collection of WT, depending on the number of samples that can be handled at one time. The method would also allow for an overnight delay between collection from the field and processing of samples, if this was necessary. An indoor overnight delay at room temperature prior to DNA extraction of captive or field WT had no significant effect on amplification success rates of either size of the COI amplicon of this study (Trial 2). However, longer delays showed that PCR rates are significantly reduced after one week at room temperature before extraction (Trial 3), especially for the longer COI fragment. The possible advantages of using other storage conditions, such as refrigerating or freezing, could be investigated, as they may allow for extension of delay times prior to extraction when necessary, but for practical reasons we prefer a simple method which does not require carrying heavy cooling

equipment in the field.

Amplification of a short fragment is likely to be more successful when working with degraded DNA samples, as even after an indoor delay of two weeks at RT, amplification of the 407 bp COI fragment was unaffected (Trial 3), while PCR (+) of the 648 bp fragment was significantly reduced. This can be attributed to DNA degradation over time, which reduces the probability of recovering and amplifying long DNA fragments more than short ones (Hajibabaei *et al.*, 2006b). According to our results we suggest that WT should be collected as soon as possible from the outdoors (ideally by 10 AM on the morning after they have been bitten), as DNA degradation on the WT is probably due to environmental conditions. The precise effect of daily temperatures, UV light or relative humidity could be investigated if more precision was required to determine WT collection times for different outdoor environments. However, when working with degraded DNA, PCR (+) can be optimized in the laboratory. For example, the improved PCR recipe, with 0.5 μ M more dNTPs, and 1 mM more $MgCl_2$, an important cofactor in enzymatic catalysis of the synthesis reaction, gave a significant improvement on amplification success rates for both COI fragments (Palumbi, 1996). Further optimisation and refinement of the PCR protocol may be possible.

As shown in Trial 4, there was no significant effect of the individual on PCR (+). This was confirmed in Trial 3, in which the PCR (+) rate of the short amplicon of fresh WT was 100%, with every animal ($n=16$) shedding enough DNA for amplification. Neither does the amount of biting of the WT relate to DNA amplification success rate, as seen in Trial 5. According to these results, there would be no reason to suppose that some WT in the field would be more worthy of processing than others, as WT with only a few bite scratches, or WT with bites which could have been second or third WT bites of an individual in that night have an equal chance of success of amplification of DNA for analysis as any other bitten WT.

It is encouraging that most PCR products of the short COI amplicon that had weak bands still generated DNA sequences that allowed the reliable identification of species. However, for the purpose of identifying possum DNA, only a COI sequence as short as 50 bp is necessary (Trial 6), so shorter COI amplicons could be used if DNA degradation of field WT is high. Taking into consideration both the 50 bp minimum length of the COI fragment needed to identify the species with certainty, and the higher PCR (+) of short amplicons in degraded samples, we recommend amplifying a short COI fragment (407 bp or less) when using WT in the wild for detecting possum presence. A short COI region (85 bp) which is possum specific has been selected from within the COI amplicon to replace amplification the 407 and 648 bp COI fragments for a much shorter COI fragment, whose amplification would not only be more resistant to DNA degradation but also possum-specific. This COI region could be used in future microsatellite studies for screening samples by quantitative PCR prior to genotyping, in order to quantify the amount of genomic DNA recovered from the bitten WT, and filter-out any non-possum DNA extractions.

Since COI alone does not allow genotyping of individuals, the conditions of amplification of possum microsatellites from DNA recovered with WT are currently being optimized. Eight microsatellites (Taylor and Cooper, 1998) which are currently being tested with DNA from tissue and bitten WT have been previously used for genotyping possums from pellets and ear tissue (Morgan *et al.*, 2007). Another two loci are TVM1 (Lam *et al.*, 2000) and TV5.64 (Nicola Aitken, pers comm.). The successful amplification of four microsatellites from DNA recovered from WT which were PCR (+) for the short COI amplicon, and the detection of variability between individuals for TV5.64 (Trial 7) is promising. Depending on the variability of the population in question, six or seven microsatellites could be enough for reliably genotyping possums in NZ (Morgan *et al.*, 2007).

Currently, a larger field trial is being carried out in NZ native bush to more accurately estimate the number of WT from which DNA can be reliably recovered and later used for genotyping. If field WT samples can be used for genetic tagging, then this technique would be an important and novel tool for monitoring surviving possums after poisoning operations, as well as for studying home-range changes and rates of repopulation over time. Such studies could help quantify any “sink-effect” (i.e. possum immigration) from neighbouring populations after reduction of numbers in a control area (Cowan and Clout, 2000). The long-term goal is to use this DNA technique to help plan future possum control operations, to increase their efficiency, and to reduce their cost by better targeting patches of survivors.

From an international perspective, the conservation of endangered marsupials such as those found in Australia and Chile, as well as monitoring of other animals that may be inclined to interfere with WT in the wild, could benefit from non-invasive DNA sampling using saliva as the DNA source, replacing or complementing other methods used to study the dynamics of small populations, as these are currently limited to traditional live trapping and direct handling of the animals, or genotyping faeces.

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