Genotyping Brushtail Possum Fecal Pellets and Ear Tissue to Identify Bias in Trap-Catch Monitoring

David R. Morgan, Dianne M. Gleeson, Robyn L. J. Howitt, and **Graham Nugent**
Landcare Research, New Zealand

ABSTRACT: Strategic management of brushtail possum populations in New Zealand is presently dependent on the use of a standardized trap-catch procedure for monitoring population trends. Where this has been used in the first few months after control, calculated rates of increase often far exceed known reproductive and dispersal rates, suggesting that trapping-based population indices immediately following control are biased low. We are investigating the problem by genotyping DNA extracted from possum fecal pellets and using matching genotypes in the ear tissue of trapped possums as a measure of trappability. We have used quantitative polymerase chain reaction (PCR) to determine the threshold of possum DNA required from fecal samples in order to obtain an accurate genotype (>99%) from field samples. This has enabled the removal of "allelic dropout" as a source of error in obtaining accurate genotypes from such noninvasive DNA samples. Validation tests were conducted on fecal samples collected from caged (i.e., identifiable) possums, and fecal pellets and ear tissue from trapped possums in the field. The tests confirmed that the sample collection and preservation procedures used resulted in accurate identification of possums from both types of sample material, although fecal pellets older than about 7 days were unlikely to yield sufficient DNA for amplification and genotyping. Genotyping of a large quantity of sample material collected before control, and at 1, 4, and 9 months after control, in 2 replicated field trials is proceeding and revealing information on the trappability of possums that survive control, and the contribution of immigration to the populations after control.

KEY WORDS: brushtail possum, DNA amplification, genotyping errors, genotyping wildlife, microsatellites, New Zealand, population indices, possum, trap catch, *Trichosurus vulpecula*

INTRODUCTION

Assessment of the relative density of brushtail possum (*Trichosurus vulpecula*) populations is a key component in the ongoing management of New Zealand's pre-eminent vertebrate pest, whether for the eradication of bovine Tb or for the protection of conservation values. Presently, the effectiveness of possum control is usually assessed using a standard trapping protocol known as the Residual Trap Catch Index (RTCI) following control (NPCA 2004). The index is the percentage of trap-nights resulting in a possum capture or escape, and this is compared to an operational target (usually between 1 and 5% catch rate) to evaluate operational effectiveness. However, there is growing evidence that the RTCI may underestimate residual possum populations at low-density. Pest managers in some regions have recorded low or even zero RTCIs immediately following control, but when the population was retrapped several months later, capture rates implied rates of recovery that far exceeded biological reality. An initial field investigation of the problem found that the bias could be only partly explained by immigration (G. Nugent, unpubl. data), which was limited to a relatively narrow 1-km-wide boundary strip. Since operational areas are typically 5,000-20,000 ha, immigration is an unlikely cause of the high rates of increase.

We postulate that this bias arises from an underestimation of the surviving population where trap-monitoring is done soon after control, rather than overestimation later.   Several possible reasons why possums may be less trappable soon after control compared with 4-9 months later were summarized by

Arthur *et al*. (2002) in reviewing literature on the behavior of possums at reduced population density. Survival may occur if possums fail to encounter poison baits or traps when home ranges are smaller than the spacing of bait stations or traps, or, if some possums are predominantly canopy-dwelling at the time of control. Little is known about the possible effects of control on the movement behavior of possums: disruptions to social groupings may lead to more restricted, cautious behavior immediately after control, and hence lower trappability. Possums that survive a sublethal dose of acute poison develop a strong, long-lived bait-shyness (Morgan *et al*. 2002), and this suggests that possums released from traps could also develop a temporary trap-shyness. Whatever the reason for the bias, its importance is that pest managers using such data may be misled into accepting that Tb-control or conservation objectives have been met when they have not, and consequently wrongly paying pest control contractors for having reduced the possum population below a target RTCI threshold.   To assess possible bias in trapping, we are comparing the genotypes of ear tissue from trapped possums with those obtained from DNA fingerprinting of fecal pellets collected as a nonbiased population sampling method. The trappability of the population will be estimated by the proportion of fecal DNA genotypes that are matched by genotypes of trapped possums.

The use of microsatellite markers has become the method of choice for animal identification based on DNA genotyping (Sunnucks 2000). Microsatellites are non-functional, repeating sequences of 2-20 pairs of alleles of the 4 nucleic acids comprising DNA and arise due to

strand slippage during replication. These regions of DNA accumulate mutations rapidly, and as such, the number of repeating sequences in some loci can vary sufficiently to distinguish between individuals. A number of microsatellite loci have been isolated from possums that have sufficient allelic diversity to identify individuals within a wild population (Taylor and Cooper 1998).

While collecting fecal pellets may be expected to provide unbiased samples, genotyping errors occur due to either low DNA quantity, low DNA quality, the presence of PCR inhibitors, or difficulties in amplifying excessively long sequences that are prone to fragmenting, as reviewed by Waits and Paetkau (2005). This leads to failure to amplify DNA (e.g., DNA recovery was unsuccessful in up to 80% samples in 5 molecular scatology studies [mean = 31%] [Frantzen *et al.* 1998]), and consequently to "missing" alleles (i.e., allelic dropout) or false alleles (i.e., incorrect genotyping). Another source of error, the "shadow effect", occurs where there are not enough loci/allele combinations available to separate individuals. Measures recommended to address these difficulties include: pilot studies to assess the quality and quantity of DNA likely to be encountered in feces of the species being studied (Piggott 2004), increasing the quantity of fecal material collected to 3-6 samples (Frantzen *et al.* 1998), and use of at least 5 loci to overcome the shadow effect when genotyping fecal samples (Mills *et al.* 2000). Overcoming PCR inhibition in fecal samples can be difficult due to high bacterial concentrations, and requires estimation of the maximum volume of DNA extract that doesn't inhibit the PCR reaction, yet provides sufficient DNA to return an accurate genotype.

This paper focuses on the development and validation of the genotyping method we are using for assessing trap-catch bias, and presents some preliminary results.

METHODS
DNA Extraction
Genomic DNA was extracted from the surface mucous layer of possum fecal pellets, which contains cells sloughed from the intestinal lining. Approximately 100-300 mg was collected using a sterile scalpel blade. This was placed in a 1.5-ml locking tube and a protocol modified from Fernando *et al.* (2003) was followed involving enzymic digestion, centrifuging, extraction of the DNA in phenol/alcohol, and purification with a QIAquick Gel Extraction Kit. The final elution volume of 40 µl containing DNA was stored at 4°C.

Samples of ear tissue (~3 × 3 mm square) were collected from the outer extremity of the ear with a biopsy punch. Genomic DNA was extracted using the BioRad Quantum Prep Aquapure Genomic DNA Tissue Kit following the manufacturer's protocol. All quantities were halved, to provide additional sample material for later amplification if required. The final elution volume of 50 µl containing DNA was stored at 4°C.

Microsatellite Amplification
The low DNA content of noninvasive samples, such as fecal pellets, makes DNA amplification difficult, and there is the potential for erroneous results when one of 2 alleles at heterozygous microsatellite loci fails to be amplified (i.e., "allelic dropout"). To address this problem, a TaqMan® assay was designed to measure the amount of amplifiable nuclear DNA present in possum fecal samples, enabling screening of samples for likely amplification failure. We followed the calibration procedure given by Morin *et al.* (2001), in which the quantity of sample DNA and success of genotyping was used to determine the number of replications per sample required to provide >99% accuracy of returning the correct genotype using 2 PCR replicate samples. A single possum microsatellite sequence (comprising 222 base pairs) was used as the target sequence as appropriate primers were able to be designed for the TaqMan® assay. Both the primers and probe used were designed by Applied Biosystems (Assays-by-Design®, Foster City, CA).

Analysis was performed using the Rotor-Gene Analysis software v. 5.0. Duplicate sets of standards of known amounts of DNA were included with each set of samples. A standard curve was rejected if the correlation coefficient of the trendline was <0.95. The standard DNA curves were derived from 5 dilutions of phenol/chloroform-extracted possum ear DNA quantified using a BioSpec-mini spectrophotometer (Shimadzu). DNA amounts in the standard were: 10 ng, 2.5 ng, 1.25 ng, 312 pg, and 156 pg. A "no-template control" (NTC) was included with each standard curve.

Samples that yielded sufficient DNA were then genotyped using methods described by Taylor and Cooper (1998) for 7 microsatellite loci they identified as providing a high degree of variability. Alleles were sized using GeneMapper v. 3.5 software (Applied Biosystems).

Matching Genotypes
Matching was achieved by concatenating the 3-digit numbers identifying the location of each of the paired alleles at each of the 7 microsatellite loci. This resulted in a 42-digit number for each genotype which, when ordered, revealed matching samples. Close similarities were revealed where a pair of alleles failed to amplify. In such cases, matching was based on the 6 remaining loci. The ordered, corrected matches were then sequentially numbered to provide a genotype reference for all other analyses.

Validation of Sample Collection and Genotyping Procedures
Two validation tests were conducted to ensure that the procedures used for collection and preservation of fecal pellets and ear tissue resulted in accurate identification of individual possums. In the first test, "fresh" fecal pellets (i.e., <12 h since deposited) from 30 uniquely identified caged possums held at the Landcare Research animal facility were collected as single pellets (*n* = 10) or groups of 3 pellets (*n* = 20), thereby providing differing amounts of sample DNA. Pellets were placed in 25-ml plastic sample tubes and occasionally shaken over a period of 5 h (to simulate movement after collection in the field) before preserving either by freezing (*n* = 10) or immersion in 95% alcohol added to the tube (*n* = 10). One week later, further fecal pellets were collected from 10 possums previously sampled and from 10 "new" possums.

A second validation test was conducted to test the reliability of genotyping fecal pellets and possum ear tissue collected in the field in a forested area adjacent to the proposed study site. The surface mucous layer of fecal pellets containing DNA may be expected to be readily degraded by rainfall and bacterial action. Since Nugent (unpubl. data) recovered sufficient DNA for successful genotyping from pellets up to 27 days old with no rain and from fresh pellets exposed to up to 180 mm rainfall, we firstly calibrated the changing appearance of fresh fecal pellets as a basis for prioritizing samples for assay. A sample of 3-10 pellets was obtained from 6 freshly killed possums and marked at several known locations within the proposed study site 3 weeks before the first trapping survey started. Daily inspection enabled subjective classification of pellet age in 3 categories: fresh, aged, and old, as described in Table 1. Six samples, each of 3 pellets, were then collected in each of the 3 age classes. These were transferred to 95% ethanol within 5 h of collection, and one pellet from each sample was selected for genotyping, the remaining pellets being retained in case insufficient DNA was extracted. Six possums were trapped and small notches (see above) of ear tissue were taken from each ear. Within 5 h, tissue samples were placed individually in 95% ethanol. A stainless steel ear tag was also introduced to one of the pairs of samples collected from each possum to assess the possible influence of this method of sample identification on DNA integrity. All samples were submitted for genotyping with sample numbers that were independent of sample treatment to ensure "blind" assays.

Preliminary Assessment of Trappability

Fieldwork is not yet complete. The experimental design comprises 2 replications of a sequence of possum population surveys: 1 month before control, and 1, 4, and 9 months after control. During each survey, population size is independently estimated by trapping and by fecal DNA mark-recapture methods. For logistical reasons, the 2 replications of this procedure have been initiated 12 months apart with the second replicate due for completion in May 2006. Control was conducted using ground-based application of cyanide poison bait in the first replicate and aerial application of 1080 (sodium monofluoroacetate) bait in the second.

Study sites were located in 2 areas of mature native forest: Replicate 1 was conducted on 90 ha in the Catlins Forest near the southwest coastline of the South Island, and Replicate 2 is being conducted on 100 ha of the Pureora Forest to the west of Lake Taupo in the North Island. The main criterion considered in the selection of these sites was the density of the possum population, which we required to be 1-2 possums per hectare to balance the cost of genotyping a large proportion of the population before control with the need for sufficient survivors after control for adequate sampling. Thus, we aimed at being able to identify 100-200 individual possums in each study site before control and 10-20 after control. Sites were also chosen because they comprised mature forest typical of sites where trap-catch underestimation following control has been reported. At each site, a marked sampling grid was established with lines spaced approximately 75 m apart, and sample plots ($n = 246$ at Catlins, $n = 267$ at Pureora) located at 25-m intervals along lines. The coordinates of all plots were logged by GPS. Possums were trapped for 7 nights during each survey using Victor #1 coil spring traps set at each sample plot. Each possum captured had a triangular notch of ear tissue removed (see above) for genotyping, and then a small stainless steel, uniquely numbered ear tag was fitted to enable rapid identification at subsequent capture. Possums were then released at their capture site.

Fecal pellets, in 3 age categories as described above, were collected throughout each site by searching a radius of around 5 m at each trap site and on bare ground visible from transects located midway between trap lines. All fecal pellets were placed in 50-ml sample tubes, immersed in 95% ethanol within 5 h, and stored at room temperature.

RESULTS AND DISCUSSION
Estimating Genotyping Accuracy

Genotyping of samples derived from both fecal pellets and ear tissue of individual possums revealed that samples that contained greater than 200 pg/reaction were required to provide >99% accuracy in obtaining the correct genotype with duplicate reactions (Table 2). Samples containing less than 40 pg/reaction require an impractical number of replicates for >99% accuracy.

Validation of Sample Processing and Genotyping

Genotypes were obtained from all 30 of the initial samples collected from caged possums confirming that our collection and preservation procedures did not denature the DNA, and that sufficient DNA could be extracted from at least 1 pellet. Of the 20 possums providing fecal pellets 1 week later, the 10 previously sampled were correctly matched while the 10 not previously sampled gave unique genotypes. This confirmed the reliability of genotyping from at least 1

Table 1. Criteria to guide assessment of possum pellet age, based on the monitoring of fecal pellets aging under field conditions.

Criterion	Fresh (1 - 2 days old)	Aged (3 - 6 days)	Old (>7 days)
Colour	Light yellowy brown or brown	Brown	Brown - dark brown
Sheen	Shiny	Semi-glossy	Matt - semi-gloss
Mucous Layer	Moist with mucous (apart from any dew/light rainfall)	Mucous layer absorbed but may be moist with dew/rain	Mucous layer absorbed but may be moist with dew/rain
Shape	Often with some well-defined flattish areas on the pellet surface	No flat areas	No flat areas, and some erosion of the surface is likely
Ends	Often with a point at one end	Both ends rounded	Both ends rounded
Fungi	None	None	Fungal growth may be present

Table 2. Rate of allelic dropout associated with varying quantities of samples of possum DNA, and predicted number of reactions needed to provide near certainty of accurate genotyping.

Pg DNA / Reaction	Allelic Dropout Average across All Loci (%)	Replicates Required to Return >99% Accuracy of Genotype
<40	72	Too many for practical purposes
40 - 150	41	8
150 - 200	22	4
> 201	4	2

fecal pellet regardless of preservation method.

Unique genotoypes were obtained from all fresh and aged single pellets collected in the field. However, none of the old pellets were successfully genotyped, indicating that they should be accorded lower priority for genotyping. All paired ear-tissue samples were correctly and uniquely matched, confirming the suitability of ethanol-preserved ear tissue for genotyping and the lack of any influence of accompanying identification tags in sample tubes.

Preliminary Field Results

Trap catches at the Catlins site demonstrated the pattern noticed elsewhere, and which provoked this study. Low numbers of possums were trapped immediately after control (Post 1) followed by an increase of 170% over the next 8 months, clearly exceeding the highest published rate of annual increase (38%) (Keber 1987) (Figure 1). However, these data do not necessarily indicate a reduced level of trappability immediately after control, because immigration into the small study area may at least partly explain the increased trap-catch. Nevertheless, of the possums tagged before control that survived (estimated from the numbers recovered during control), the percentage trapped increased by 49% over 8 months, suggesting increasing trappability, albeit in a small sample.

Genotyping fecal samples from both replicates is providing poorer success compared with that of ear tissue and is presently constraining our genetic analysis of trappability (Table 3). Large amounts of DNA were extracted from ear tissue samples, ensuring that all samples were genotyped. By comparison, only 46% of the fecal pellets yielded sufficient DNA (i.e., >200 pg/µl)

to permit amplification for successful genotyping, and this is probably the main reason why only 30% of the fecal pellet genotypes (i.e., 69/233) match those of ear tissue from trapped possums. It is also possible that the population sampling intensity given by the fecal pellet searching was lower than that achieved by trapping. Pellet age was found to be even more critical than in the preliminary study (G. Nugent, unpubl. data), with the higher rainfall experienced at the Catlins site assumed to result in a low recovery of DNA from pellets estimated to be older than 7 days (Figure 2). In addition, the relatively high recovery rate of DNA in fresh pellets was reduced in those stored longer than 264 days.
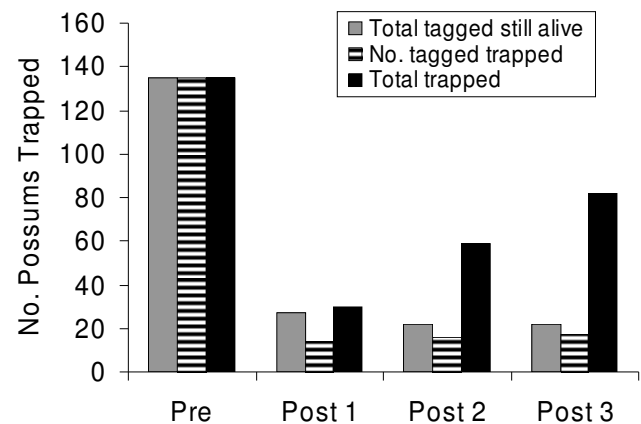

Figure 1. Numbers of possums trapped in each survey at the Catlins study site (equal trap effort). All possums trapped were ear-tagged and released. Number alive after control was estimated from number found dead by contractors working intensively throughout the area during the control operation with the aid of dogs.

Table 3. Effectiveness of genotyping fecal pellet and ear tissue samples based on amount of DNA recovered, and number of genotypes consequently identified from the two types of sample material. Data are for both replicates combined, and approximately 80% completed. As initial ear tissue samples produced ample (>50,000 pg/µl) DNA for genotyping, most samples were not quantified but assumed to contain similarly large concentrations.

|	Fecal Pellet	Ear Tissue
No. Samples Processed	450	360
Mean Quantity of DNA Extracted (pg/µl) Range (pg/µl)	770 0 - 22,076	>50,000 Not quantified
No. Samples with > 25 pg/µl DNA (and %) Mean Quantity of DNA (and SEM) (pg/µl)	206 (45.7) 1,691 (154)	360 (100) >50,000
% Samples with < 25 pg/µl DNA Mean Quantity of DNA (and SEM) (pg/µl)	244 (54.3) 19.6 (3.8)	0 -
% Amplified Samples Successfully Genotyped	100	100
No. Unique Genotypes Identified	127	345
Mean No. Matching Samples per Genotype	3.5	1.04
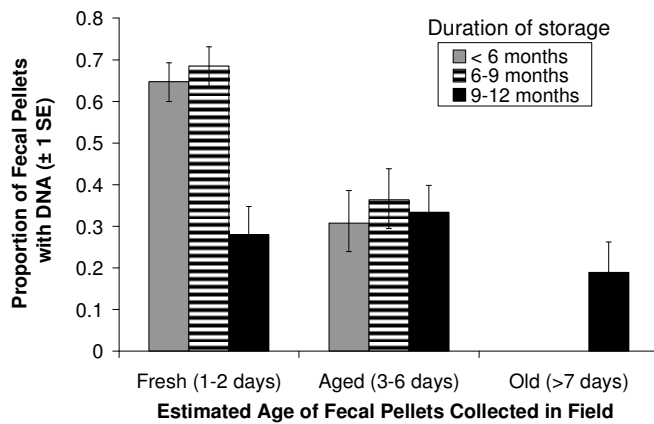
Figure 2. Proportion of fecal pellets (±SEM) of different age and storage time in which genotyping was successful.

Despite an average of 3.5 fecal pellets (from different defecations) showing the same genotype, a smaller (but presently unknown) proportion of the population was genotyped by fecal samples compared with ear tissue. The low recovery rate of DNA from fecal pellets resulted in 70% of the genotypes of trapped possums not being used in the estimation of trappability due to an absence of matches with fecal DNA. This inefficiency in experimental procedure warrants further research aimed at establishing protocols that maximize efficiency of survey effort and accuracy of genotyping. Sampling intensity for fecal pellets could be increased considerably before the cost of doing so outweighs the cost of detecting new genotypes. Sample quality may be better preserved by use of alternative preserving and storage (e.g., Frantzen *et al*. 1998). Differences in the efficiency of genotyping the 2 sample types were compounded by the inability to avoid collecting and assaying multiple fecal samples, while this was avoided for ear tissue samples once possums were ear-tagged. Out of 360 ear tissue samples, the 15 duplicates collected probably indicate possums having lost ear tags over the course of the study, suggesting that tagging both ears may overcome this problem.

The greater number of genotypes identified to date in ear tissue is exemplified by the greater range of values for each allele locus compared with those for fecal pellets. Values for all 7 loci were determined for all ear tissue samples, but of the 206 fecal pellet samples amplified, 55

had amplification failure at one locus, most likely due to the presence of PCR inhibitors. In 43 cases, differences between at least 2 other alleles compared with other samples indicated that the genotypes were dissimilar. In the remaining 12 cases, similarity was based on 6 loci. However, a review of 10 microsatellite studies of wild birds and mammals (Mills *et al*. 2000) concluded that 5 or more loci should be sufficient to ensure accurate matching.

Despite these present limitations, a trend in the data (Table 4) is consistent with the inferences made from trapping data. In the first post-control survey conducted 1 month after control, 10 surviving genotypes were identified from fecal DNA but only one was matched by ear tissue from trapped possums, indicating a low trapping probability of 10%. While few fecal genotypes have yet been found from samples collected 4 months after control for meaningful assessment, the third survey, 9 months after control, produced matches with ear tissue suggesting an increase to 28% trappability.

Clearly the low number of fecal genotypes relative to ear-tissue genotypes is presently limiting our ability to determine patterns of trappability over time. Allelic dropout was removed as a source of error from our study as we determined the quantity of DNA that would ensure >99% probability of returning correct genotypes. However, this precluded a significant number of fecal samples that returned lesser quantities of DNA, and this could partly account for the low number of fecal pellets matching trapped possums. In particular, the 75 "trapped" genotypes present before control that have not yet been detected in pre-control fecal samples pre-empts the possibility of using their occurrence in post-control assessments of trappability of survivors. We are therefore increasing the population samples identified by fecal pellets by: 1) repeating the assay of some samples using the stored duplicate samples of extracted DNA, thereby gaining improved probability of accurate sample genotyping; 2) reducing the threshold for sample amplification from 200 to 25 pg/µl DNA resulting in a further 46 samples being available for assay, albeit with a lower probability of successful genotyping; 3) processing an additional 145 samples collected on intermediate lines; and 4) using the ethanol in which pellets were stored for assay as initial results suggest that, at least for "old" pellets, at least as many genotypes can be detected in the ethanol compared with fecal surface layer.

Table 4. Trappability of the population based on matching of the two sample types before and at 1, 4, and 9 months after control. Data are incomplete and for the first replicate of the study only.

Sample Type		One Month before Control	Months after Control			Total
			1	4	9	
Fecal Pellets	No. collected	239	63	38	73	**413**
	No. successfully genotyped	150	15	6	23	**194**
	No. unique genotypes	96	10	3	18	**125**
Ear Tissue	No. collected	135	18	44	36	**233**
	No. successfully genotyped	135	18	44	36	**233**
	No. unique genotypes	135	18	44	36	**233**
Matched Samples	No. fecal pellet genotypes matched by ear tissue genotypes	60	1	3	5	**69**
% trappability of the population		63	10	100	28	**54**

LITERATURE CITED

ARTHUR, T., D. RAMSEY, AND M. EFFORD. 2002. Changes in possum behaviour at reduced density – a review. Landcare Research Contract Report LC0102/102, Landcare Research, Lincoln, New Zealand. 28 pp.

FERNANDO, P., T. N. C. VIDYA, C. RAJAPASKE, A. DANGOLLA, AND D. J. MELNICK. 2003. Reliable noninvasive genotyping: fantasy or reality. J. Hered. 94:115-123.

FRANTZEN, M. A. J., J. B. SILK, J. W. H. FERGUSSON, R. K. WAYNE, AND M.H. KOHN. 1998. Empirical evaluation of preservation methods for faecal DNA. Mol. Ecol. 7:1423-1428.

KEBER, A. W. 1987. An enquiry into the economic significance of possum damage in an exotic forest near Taupo. Ph.D. dissert., Lincoln University, Lincoln, New Zealand. 212 pp.

MILLS, L. S., J. J. CITTA, K. P. LAIR, M. K. SCHWARTZ, AND D. A. TALLMON. 2000. Estimating animal abundance using non-invasive DNA sampling: promise and pitfalls. Ecol. Applic. 10:283-294.

MORGAN, D. R., L. MILNE, AND C. O'CONNOR. 2002. Learned bait-shyness by possums (*Trichosurus vulpecula*) towards baits containing cyanide, 1080, cholecalciferol, or brodifacoum. Proc. Vertebr. Pest Conf. 20:282-289.

MORIN, P. A., K. E. CHAMBERS, C. BOESCH, AND T. L. VIGILANT. 2001. Quantitative PCR analysis of DNA from noninvasive samples for accurate microsatellite genotyping of wild chimpanzees (*Pan troglodytes*). Mol. Ecol. 10: 1835-1844.

NPCA 2004. Protocol for possum population monitoring using the trap-catch method. National Possum Control Agencies, Wellington, New Zealand. 32 pp.

PIGGOTT, M. P. 2004. Effect of sample age and season of collection on the reliability of microsatellite genotyping of faecal DNA. Wildl. Res. 31:485-493.

SUNNUCKS, P. 2000. Efficient genetic markers for population biology. Trends Ecol. Evol. 14:323-327.

TAYLOR, A. C., AND D. W. COOPER. 1998. Microsatellite markers for the Phalangerid marsupial, the common brushtail possum (*Trichosurus vulpecula*). Mol. Ecol. 7: 1780-1782.

WAITS, L. P., AND D. PAETKAU. 2005. Noninvasive genetic sampling tools for wildlife biologists: a review of applications and recommendations for accurate data collection. J. Wildl. Manage. 69:1419-1433.