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A simple and effective method for obtaining mammal DNA from faeces

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The use of faecal DNA, although a promising tool for the population monitoring of mammals, has not yet become a fully exploited and standard practice, mainly because low target DNA concentration, DNA degradation, and co-purification of inhibitors demand extra laboratory procedures to improve success and reliability. Here we evaluate a simple method that enables sampling of DNA in the field through the collection of the intestinal cells present on the surface of a scat using a swab. The swab is immediately placed in a vial containing a lysis buffer that preserves the DNA for its later extraction. DNA extracts of three species of herbivores (goat, fallow deer and white-tailed deer), two carnivores (Iberian lynx and domestic dog) and one omnivore species (brushtail possum) were characterised in terms of target and total DNA quantity, PCR inhibition and genotyping success. Direct comparison was carried out with duplicate samples preserved in 96% ethanol and extracted via a commonly used commercial DNA extraction kit for faecal material. Results from these comparisons show that swabbing the samples in situ not only simplifies field collection and sample handling in the laboratory, but generally optimises target DNA recovery, minimises co-purification of PCR inhibitors and provides good quality DNA for the species tested, especially for herbivores. This method is also less time-consuming and more cost-effective, thus providing a more convenient and efficient alternative for non-invasive genetic studies.

Non-invasive samples, and particularly faeces, provide potential sources of genetic information for ecological and demographic surveys. Faecal material can yield information of presence, abundance, genetic diversity, relatedness, phylogeography, sex and dispersal (Schwartz and Monfort 2008, see Beja-Pereira et al. 2009 for a comprehensive review of non-invasive genetic sampling). Faecal material as a DNA source has many advantages, such as 1) non-invasiveness: meaning there is no need for handling or even locating the animals; 2) availability: faeces are constantly generated by all individuals; and 3) relatively easy detection: faeces are normally the most obvious remnants from scarce or elusive animals and can be detected by trained dogs (McKay et al. 2008).

However, there are significant limitations to the routine use of faecal DNA. These include scarcity and degradation of the DNA and the co-purification of PCR inhibitors. These factors dramatically reduce the genotyping success of faecal samples and impose the need for pilot optimisation analyses and multiple genotyping replicates, which overall increase the cost of such studies (Fernando et al. 2003). These limitations have historically made faecal DNA either impracticable or unaffordable. Despite an increase in non-invasive DNA usage in general (Taberlet et al. 1999), and faecal DNA in particular since the 1990s (Frantz et al. 2003, Rutledge et al. 2009, Caryl et al. 2012, Ebert et al. 2012), prospects for its extensive application to population monitoring have remained unfulfilled. Since all the above limitations are ultimately intrinsic to the sample, finding more efficient methods of collection, preservation and DNA extraction that are viable in the field and laboratory should widen the use of faecal DNA for population monitoring and genetic studies, and eventually facilitate its use in the expanding field of genomics (Perry et al. 2010).

Faeces usually carry PCR inhibitors from the soil, diet contents or the digestive system. Problems caused by co-extracted inhibitors, together with DNA degradation and low template DNA concentrations, range from amplification failure to allelic dropout and peak imbalance (Opel et al. 2010). Removal of inhibitors normally involves an extra step of incubation with materials such as InhibitEX tablets (Qiagen, Hilden, Germany) or starch (Zhang et al. 2006, Kawamoto et al. 2013). Alternatively, partial inhibition can be circumvented by diluting the extract and increasing the number of cycles in the PCR, although this can result in higher rates of amplification failure and genotyping errors (Fernando et al. 2003). Inhibition is thus a major source of genotyping failure and can substantially increase genotyping costs. Consequently, methods that minimize inhibitor carryover should be favoured in surveys based on faecal DNA.

Most of the stool DNA is from an exogenous origin, i.e. non-target DNA from microbes, diet or coprophagous

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animals (Bradley and Vigilant 2002); whereas the endogenous DNA (target DNA in this study), originated from sloughed epithelial cells of the intestine wall (Fernando et al. 2003), constitutes a minor proportion of the total faecal DNA (Perry et al. 2010). Since epithelial cells are supposed to accumulate in the outer layer of the scat, several sampling strategies have targeted the enrichment of these cells by peeling off, scraping or washing the outer layer of the scat before DNA extraction. However, whole or large portions of scats are still commonly sampled in the field (Ball et al. 2007, Cullingham et al. 2010, Reddy et al. 2012). (See Eggert et al. 2005 and Tende et al. 2014 for reviews on faecal preservation methods). Unfortunately, molecular ecology sampling campaigns often take place in remote areas under severe conditions that do complicate these collection procedures, and impose transport and storage limitations (Camacho-Sanchez et al. 2013). Alternatively, a swab can be used directly in the field to recover the outer layer of faeces enriched for sloughed intestinal cells, thereby avoiding the need for storing and transporting bulky pieces of faeces in ethanol, silica beads or as frozen samples (Rutledge et al. 2009). Although the swabbing sampling protocol, ex situ or in situ, has proven effective for a few species (Ball et al. 2007, Rutledge et al. 2009, Cullingham et al. 2010, Renan et al. 2012), no cross-species studies have formally validated whether the added simplicity impacts on DNA recovery, inhibition rates and subsequent PCR amplification efficiency.

The aim of this study is to validate a standardised simple field collection and automated high-throughput extraction protocol for faecal DNA that has potential for widespread application. The simple method of swabbing the faecal samples directly in the field, i.e. swabbing in situ, immediate preservation in lysis buffer and DNA extraction with standard protocols is evaluated across a selection of mammal species with different diet and digestive systems. We study three herbivorous ungulate species: white-tailed deer *Odoicoleus virginianus*, fallow deer *Dama dama* and goat *Capra hircus*; two carnivorous species: dog *Canis lupus familiaris* and Iberian lynx *Lynx pardinus*; and an omnivorous marsupial, although is mainly herbivorous: the common brushtail possum *Trichosurus vulpecula*. DNA extracts are characterised in terms of PCR inhibition, DNA quantity (total and target DNA yield), and final genotyping success. Results are compared against the most common, nevertheless more laborious and costly method of preserving the sample in 96% ethanol, collecting the outer layer, and extracting it with the QIAamp DNA Stool Mini Kit (Qiagen).

Material and methods

Sample collection and storage

A total of 10 goats *Capra hircus*, 12 fallow deer *Dama dama*, 9 white-tailed deer *Odocoileus virginianus*, 20 dogs *Canis lupus familiaris*, 20 Iberian lynx *Lynx pardinus* and 21 brushtail possum *Trichosurus vulpecula* samples were subjected to the collection/extraction validation test.

Six to 10 pellets or one-two droppings were taken from each individual (depending on the size and type of scat). Half the samples were kept in ≥ 96% ethanol. The other subset was wiped with a wet sterile plastic-applicator rayon-tip swab (COPAN, Brescia, Italy) or a wooden-applicator cotton-swab (lynx). Handle and applicator were removed with the aid of the lid of the tube so only the head was preserved in 1.5 ml vials containing 0.5 ml of Longmire lysis buffer (Longmire et al. 1997). All faecal samples were fresh (no more than 24 h old). Sterile gloves and forceps were used to avoid contamination.

Herbivore faecal samples were collected as part of a larger sampling campaign (Ramón-Laca et al. 2014). Ear clips from most of the individuals were also taken and preserved in Longmire buffer to be used as standards in the quantitative and qualitative analyses. Dog samples were collected at the kennels of the Society for the Prevention of Cruelty to Animals Auckland Incorporated (SPCA). Samples were divided so that a dropping was kept in ethanol and its counterpart from the same pile was wiped to fully coat a swab. A sample of dog muscle was obtained from the Wildlife Post-Mortem Service, Massey Univ. (Palmerston North, New Zealand) and kept frozen. Iberian lynx faeces were taken from captive animals at El Acebuche breeding centre (Doñana National Park). All lynx individuals and their genotypes were known from ongoing studies (Casas-Marce et al. 2013). Brushtail possum samples from wild animals were collected at Orongorongo Valley, (Wellington, New Zealand) during a routine trapping programme, in which three pellets were kept in ethanol and another three pellets were swabbed and the swab kept in Longmire buffer. One possum ear clip stored frozen from another project was also used in this study.

Lynx procedures were carried out at Estación Biológica de Doñana (EBD) (Seville, Spain) while the remainder were processed at Landcare Research (LCR), (Auckland, New Zealand). DNA extractions, PCR reagents set-up and post-amplification steps were carried out in three separate rooms in both laboratories. Extra measures to prevent contamination were carried out at EBD where DNA extractions were performed in a laboratory specifically dedicated to low copy DNA.

DNA extraction

Peeled-off ethanol-preserved samples (SK)

Three pellets of possum and herbivore samples kept in ethanol were processed in the laboratory with a sterile scalpel to remove the outer layer, and 0.64 − 3.47 g of faecal material were scraped from dog and lynx stools. These scrapings were used as starting material for subsequent DNA extractions. All DNA extractions of peeled material were performed using the QIAamp DNA Stool Mini Kit (Qiagen) following manufacturer's 'Protocol for Isolation of DNA from Stool for Human DNA analysis' (and 'Isolation of DNA from Larger Volumes of Stool' for dog and lynx), with the only variation of a 56°C overnight incubation step. Extra care was taken when extracting these samples to avoid cross-contamination by using new disposable utensils (forceps, petri dishes, gloves, scalpels and bench covers) for each sample.

Samples swabbed in situ and preserved in Longmire buffer (SW)

Sample digestion was performed directly in the same tube by adding 500 µl of DXT (Qiagen) tissue digest buffer and

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5 µl of enzyme DX (Qiagen) or by only adding 20 µl of proteinase K (20 mg ml^{-1}) (lynx samples) followed by overnight incubation at 56°C. Each sample was then extracted by taking 220 µl of the lysate (110 µl for lynx samples), so that extraction replicates could be carried out if needed. Extractions were conducted in an automated extraction instrument (QIAxtractor and QIAxtractor DNA reagents, Qiagen) or manually using DNeasy Blood and Tissue kit (Qiagen) for lynx samples, following manufacturer's instructions. In both methods, DNA was eluted in 70 µl of DXE (Qiagen) elution buffer for herbivore samples, 100 µl of DXE for dog and possum and 100 µl of AE (Qiagen) solution for lynx.

Tissue samples

DNA extractions were performed by taking 200 µl of the Longmire buffer or using ca 3 mm of muscle or ear clips and adding it to 500 µl of DXT (Qiagen) buffer and 5 µl of proteinase DX (Qiagen). This was followed by overnight incubation and DNA extraction as per swabbed-samples above with final DNA eluted in 100 µl of DXE (Qiagen).

DNA quantification and PCR inhibition assessment

Target and total DNA were quantified and their ratio was compared across the two procedures (SK and SW). Total DNA was quantified by fluorometry using a Picogreen dsDNA dye kit (Quant-iT, Invitrogen, by Life Technologies, CA, USA) as in Ball et al. (2007). Five to seven serial dilutions of the λ DNA standard ranging from 100 to 1.5 ng μl^{-1} were processed in duplicate and used to construct the standard curve. Fluorometric measurements were performed in an EnSpire Multimode Plate Reader for herbivore samples, in a VICTOR Multilabel Plate Reader (MA, USA) for lynx, and in a QuantiFluor-ST Handheld Fluorometer (Promega, WI, USA) for dog and possum.

Different nuclear loci (Table 1), depending on the species, were amplified in a quantitative PCR (qPCR) approach to estimate the target DNA concentration. From four to nine serial dilutions, ranging at least four orders of magnitude, were included in duplicate for herbivore species and in triplicate for dog, lynx, and possum, to be used as standards. Faecal DNA samples were run in duplicate with 1 µl of the DNA extract added to 5 µl of LightCycler 480 SYBR Green I mix (Roche, Basel, Switzerland), 0.5 µM of forward and reverse primers (0.2 µM for possum and dog) in a 10 µl reaction. Quantitative-PCR runs were carried out on a Rotor-Gene 6000 (Corbett Research Pty Ltd, Sydney, Australia) at LCR and a Stratagene Mx3000P-QPCR System (Agilent Technologies, CA, USA) instrument at EBD, with a first holding step of 5 min at 95°C, followed by 45 cycles of 95°C for 10 s, 60°C for 30 s, 72°C for 30 s, and a final melt cycle ramping from 60 to 95°C to check the specificity of the products, with the only variants being 65°C of annealing temperature for dog and 40 cycles for the herbivores. A third replicate of the samples was included in the run in which 1 µl of the second greatest concentration standard was spiked as an internal inhibitor PCR control (IPC) to assess the level of inhibition in the faecal extract.

Optimal baseline for each individual sample in the qPCR, correcting for differences in efficiency, was estimated using LinRegPCR ver. 2012.1 (Ruijter et al. 2009). Output values of the standards from LinRegPCR were used to build a calibration curve and estimate target DNA concentrations, corrected by the dilution factor where applicable. Inhibition was characterised by the difference between the average C_q of the second greatest concentration standard and the C_q value of the replicate that had the IPC included (ΔC_q). A sample was considered inhibited when $\Delta C_q > +0.5$. Inhibited samples were subjected to further tests by diluting out the inhibitory compound while retaining sufficient DNA concentration to be detected by the qPCR assay. Non-inhibited samples were expected to display $\Delta C_q < 0$ because of additional template in the reaction.

DNA quality assessment

Sample DNA quality was evaluated using the genotype Quality Index (QI) (Miquel et al. 2006) obtained in a multi-tube PCR approach (four replicates) for six to 12 microsatellite loci (STR) depending on the species (Table 2). A single replicate of their matching tissue samples was also run for the herbivore species. The STR markers were amplified in a multiplex-PCR using QIAGEN Multiplex PCR kit (and QIAGEN Type-it Microsatellite PCR kit for lynx) in a final volume of 10 µl containing 1 × Master Mix, 0.15 − 0.2 µM of each primer and 1 or 2 µl of template for tissue or faecal samples, respectively. Qiagen recommended cycling conditions were used (40 cycles and annealing temperature of 57°C, with the only exception of possum samples that were performed at 60°C). Locus SRCRSP15 for the goats and loci HUJ1177 and BM1706 for white-tailed deer were amplified individually and then combined with the multiplex-PCR product for capillary electrophoresis. Amplified fragments were analysed in a ABI 3130xl Genetic Analyzer and scored using Genemapper ver. 4.1 (Applied Biosystems). For possums and dogs a consensus genotype was scored as homozygote when the genotype was observed three times; both alleles had to be observed at least twice for a

Table 1. Loci used to quantify target DNA by quantitative PCR.

Species	Locus	Fragment size (bp)	Primer name	Primer sequence (5′-3′)	Reference
C. lupus familiaris	MC1R	209	MC1R-dog&catF MC1R-dog&catR	CGCCCATGTATTACTTCATCTGTTGCC CACGGCGATGGCGCCCAGGAA	Kanthaswamy et al. 2012
L. pardinus	ZFX/Y	225	ZF-3f ZF-2r	ACATAATTTCAGTCCATACG TACATCTCTTACACCGAAAC	Casas-Marce et al. 2010
T. vulpecula	GnRH	330	GnRHf GnRHr	ATGGCAAACAGAGCCTACCTTGAGCAG AGCGTACCACTGCACGGTCACATTCCA	Eckery et al. 2002
O. virginianus, C. hircus, D. dama	BMC1009	270–304	BMC1009f BMC1009r	GCACCAGCAGAGAGGACATT ACCGGCTATTGTCCATCTT	Talbot et al. 1996

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Table 2. Microsatellite loci used in this study. †Modified from Menotti-Raymond et al. (1999); ‡Modified from Faircloth et al. (2005).

Species	Loci	Reference
O. virginianus	BMC1009	Talbot et al. 1996
	BM1706, BM757	Bishop et al. 1994
	RT1, RT13, RT7	Wilson et al. 1997
	RM188	Barendse et al. 1994
	OarFCB304	Buchanan et al. 1994
C. hircus	MAF65	Crawford et al. 1995
	SRCRSP-25, SRCRSP-23	Yeb et al. 1997
	ILST029	Maudet et al. 2001
	RT7	Wilson et al. 1997
	ILST030	Kemp et al. 1995
	SRCRSP-8	Bhebbe et al. 1994
	SRCRSP-15	Kogi et al. 1995
	HUJ1177	Shalom et al. 1993
	BMC1009	Talbot et al. 1996
	OarFCB304	Buchanan et al. 1994
	BM757	Bishop et al. 1994
D. dama	BMC1009	Talbot et al. 1996
	BM1706, BM757	Bishop et al. 1994
	RT1, RT7	Wilson et al. 1997
	RM188	Barendse et al. 1994
	OarFCB304	Buchanan et al. 1994
	HUJ1177	Shalom et al. 1993
	T156	Jones et al. 2002
L. pardinus	Fca082b, Fca453b, Fca547b, Fca161b	Casas-Marce et al. 2013†
	Bc1ATb	Casas-Marce et al. 2013‡
	Fca571, Fca115, Fca043, Fca045	Menotti-Raymond et al. 1999
C. lupus familiaris	CATA1, PEZ03, PEZ05, PEZ06, PEZ08, PEZ12, PEZ20. FHC2010, FHC2054, FHC2079	Halverson and Basten 2005
T. vulpecula	Tv16, Tv19, Tv53, Tv54, Tv58	Taylor and Cooper 1998
	TvM1	Lam et al. 2000

heterozygote call. Otherwise genotype replicates were compared with their reference profile derived from a corresponding tissue sample to detect genotyping errors. Allelic dropout and false alleles occurrence (Table 4) was calculated using GIMLET (ver. 307) (Valière 2002). Since no reference profiles were available for dog and possum, errors rates were calculated only for each replica as the proportion of discrepancies to consensus.

Target DNA concentration and ratio of target to total DNA values were log-transformed, and QI values were arcos-transformed, in order to meet the assumption of normal distribution of the differences between pairs. The overall effect of species and method and their possible interaction was tested in a two-way ANOVA, introducing sample as a random effect. Subsequently, the statistical significance of differences between treatments (methods) for each variable and species were assessed with paired t-tests. All the above statistical analyses were performed using JMP statistical software (ver. 9) (SAS Inst.). In addition, a Pearson correlation test was used to assess the correlation between the QI and: 1) target DNA, 2) target to total DNA %, and 3) ΔC_q. These correlations were analysed considering the overall dataset, SK values only, SW values only, and for each group of animals: herbivores, carnivores and possum (Supplementary material File A1).

Results

Total DNA and target DNA concentrations, inhibition rate and genotype quality (QI) varied widely between samples, extracts, and species (Supplementary material File A1). Samples absorbed 21.2%, 37.8%, 5.2% and 36.7% of the global variation in each of these variables, respectively. Both factors, species and extraction method, had a significant effect on these the variables in a two-way ANOVA, with the only exception of method having no effect on total DNA yield. SW method yielded significantly higher target DNA and QI than the SK method across the study. The interaction between species and method was also significant for all variables, indicating that the effect of the method was variable among species (Supplementary material File A1). Most of the total DNA concentration values fell within the 1−100 ng μl^{-1} range (Table 3, Supplementary material File A1). The only exceptions were some dog and lynx samples whose yield was lower, particularly in the dog SW samples. None of the non-template controls of the qPCR reached the threshold before cycle 38 or before the lowest concentration standard. qPCR efficiency ranged between 0.8 and 1.2, and correlation coefficients r^2 were above 0.94 among replicates. Average target DNA concentration of SW samples was between 3- (lynx) and 20-fold (goat) higher than their SK counterparts for all species except dog (Table 3). A significantly higher concentration of target DNA in SW samples was obtained for goat ($t = 3.865$, $DF = 9$, $p = 0.0019$), white-tailed deer ($t = 6.285$, $DF = 9$, $p < 0.001$), lynx ($t = 3.372$, $DF = 19$, $p = 0.0016$) and with a greater effect for possum ($t = 11.835$, $DF = 19$, $p < 0.001$). Higher target DNA concentration was also obtained for the fallow deer SW samples ($t = 1.957$, $DF = 11$, $p = 0.0381$). In contrast, for dog the average target DNA concentration of SK samples was over two-fold the concentration of the swabbed samples ($t = -2.317$, $DF = 19$, $p = 0.984$). The target-to-total DNA ratio in the SK extracted samples ranged from 0.779 % for possum to 7.57 % for fallow deer. This ratio was significantly higher in SW samples for all species (Supplementary material File A1) except for Iberian lynx ($t = 1.045$, $DF = 19$, $p = 0.155$), and ranged from 3.417% in dog to 56.43% in fallow deer.

The PCR inhibition assay revealed the occurrence of inhibition in 20.65% of the SK samples and 16.3% of the SW samples, where 9 out of the 15 inhibited SW samples originated from lynx. ΔC_q average values for SW samples are lower than the corresponding SK ΔC_q values for possum, and significantly lower for fallow deer ($t = -3.573$, $DF = 11$, $p = 0.002$), white-tailed deer ($t = -2.273$, $DF = 9$, $p = 0.024$) and goat ($t = -2.372$, $DF = 9$, $p = 0.02$. In contrast, average ΔC_q values of SW samples were higher than those of SK samples for lynx and dog, but the difference did not reach statistical significance and ΔC_q was still below zero for dog. Average ΔC_q values of the three herbivores SW samples and dog SW and SK samples were negative, reflecting the additional template added.

All species and sample types analysed produced a QI over 0.5 (Fig. 2, Supplementary material File A1).

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Table 3. Results of the evaluation of paired faecal extractions performed with the swabbing/lysis buffer (SW) and the standard QIAamp Stool Mini Kit protocol (SK). Values reported are the mean of all the samples analysed for that particular test and species (± standard deviation).*QI of 19 samples. **QI of 21 samples. Dog, Iberian lynx and brushtail possum DNA extractions were eluted in 100 µl of elution buffer. All ungulate species DNA extractions were eluted in 70 µl.

Species (n)	Method	Mean total DNA (ng µl^{-1})	Mean target DNA (ng µl^{-1})	Target-to-total DNA (%)	Mean ΔC_q	QI
Goat (10)	SK	14.984 (± 19.021)	0.237 (± 0.235)	2.270	1.636 (± 4.572)	0.984 (± 0.019)
	SW	11.960 (± 19.347)	4.871 (± 4.761)	49.536	−1.87 (± 1.740)	0.998 (± 0.007)
White-tailed (10)	SK	7.776 (± 5.610)	0.321 (± 0.656)	5.936	2.468 (± 5.375)	0.722 (± 0.128)
	SW	40.68 (± 27.611)	2.894 (± 2.195)	6.632	−1.568 (± 0.928)	0.969 (± 0.053)
Fallow deer (12)	SK	31.903 (± 42.356)	2.388 (± 4.467)	7.570	0.071 (± 2.490)	0.958 (± 0.044)
	SW	21.453 (± 22.615)	12.378 (± 12.885)	56.43	−1.568 (± 2.487)	0.949 (± 0.101)
Iberian lynx (20)	SK	10.044 (± 9.197)	0.371 (± 0.897)	6.419	0.170 (± 0.373)	0.904 (± 0.191)
	SW	24.083 (± 10.774)	1.274 (± 1.764)	6.175	0.459 (± 0.981)	0.979 (± 0.057)
Dog (20)	SK	12.793 (± 7.903)	0.103 (± 0.118)	1.137	− 1.020 (± 0.717)	0.936 (± 0.095) *
	SW	1.242 (± 0.579)	0.045 (± 0.071)	3.417	−0.884 (± 0.832)	0.929 (± 0.094) *
Possum (20)	SK	2.704 (± 2.736)	0.012 (± 0.013)	0.779	0.790 (± 3.243)	0.742 (± 0.206)**
	SW	2.248 (± 1.395)	0.102 (± 0.051)	5.044	0.165 (± 2.028)	0.942 (± 0.087)**

Genotype quality was higher for SW in all species tested except for dog, again not reaching statistical significance. Low QI for Iberian lynx, white-tailed deer, and possum genotypes obtained from SK extracts can mostly be attributed to one single problematic STR locus with remarkably high rates of amplification failure (Fca082b, BM757 and Tv53 respectively). Both amplification failure and genotype error rates tended to be lower for SW samples (Table 4).

Results from the Pearson correlation test (Supplementary material File A1) showed QI to be more correlated with ΔC_q ($r = -0.187$) and target to total DNA ($r = 0.181$) than with the target DNA concentration ($r = 0.08$). In every correlation test performed, the possum SK samples showed the highest *r*-values.

Discussion

The simple sampling practice of swabbing the surface of the scat in situ, immediately storing the swab in lysis buffer, and extracting DNA by standard silica-based protocols (SW method), generally yielded target DNA in greater quantity and quality than preserving the faeces in 96% ethanol, collecting the outer layer, and extracting with the QIAamp DNA stool mini kit (Qiagen) (SK method). This result was consistent for all species analysed, except dog, and was more evident for the three herbivores. Even for species where the gain is not that significant in terms of DNA yield or quality, the added advantages of the SW method regarding sampling, transport, and storage logistics still justifies its application over more standard SK method.

Sample collection

The method proposed here requires less and lighter sampling equipment, both great advantages for sampling campaigns in remote or isolated places. It is also transport convenient (IATA dangerous goods v. October 2012 compliant) and does not require further manipulation steps or treatments for long periods of time. Furthermore, the samples

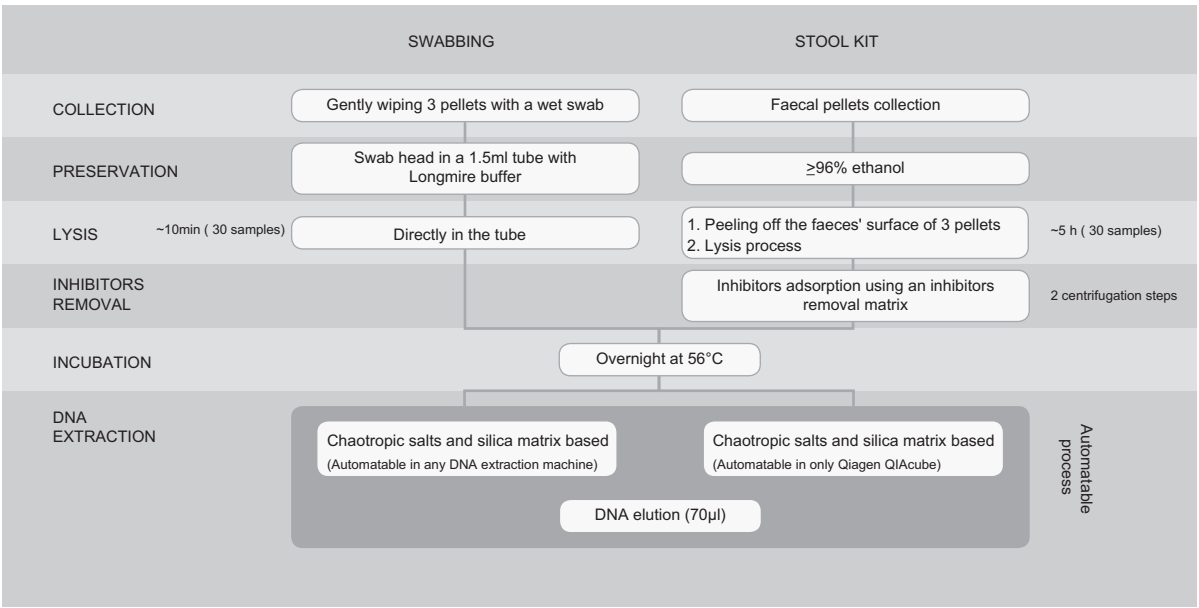


Figure 1. Diagram of the steps carried out in each of the two methods.

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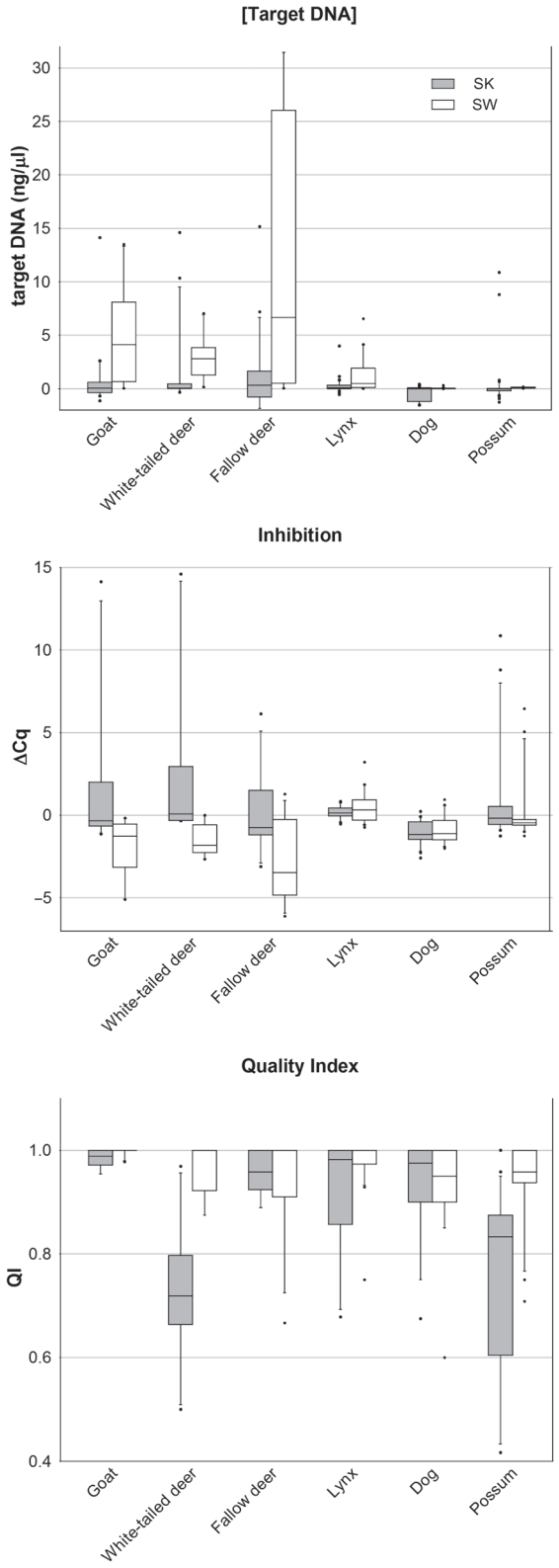


Figure 2. Target DNA concentration, inhibition and quality index of stool-kit (SK) samples versus field swabbed (SW) samples. Mean values of $\Delta Cq < 0.5$ are not considered inhibited.

are transferred directly in situ to a lysis buffer in which the DNA accumulates with time (Kilpatrick 2002). Such a practice is likely to maximise the gastrointestinal epithelial cells recovery compared to other preservation procedures, since there is no friction or disturbance of the samples that could cause loss of epithelial cells. Gut cells can be washed or rubbed off when using ethanol or silica gel, or can remain attached to the plastic bag used to freeze the samples in as observed in Rutledge et al. (2009). Importantly, this swabbing technique is a simple method that field technicians can follow, as demonstrated here, where the majority of samples were collected by non-laboratory specialists. Simple written instructions were provided and an example video of the sampling technique has been made available (<http://youtu.be/zniEFYLSgOI>).

DNA extraction methods

Both procedures tested in this assay isolate DNA under the same silica-based principle. Nevertheless, the stool-kit procedure requires a significant time investment, (~4,5 h to process only 20 samples preserved in ethanol, for peeling off the outer layer and removing the inhibitors, before the actual extraction steps). Nonetheless, this extraction time could be somewhat reduced by breaking off a small quantity of sample instead or washing the faecal material in a buffer solution (e.g. PBS) and use the sediment of this wash as starting material for DNA extraction (Palomares et al. 2002, Luikart et al. 2008). The aforementioned commercial kit method also involves more expenses and human resources than the swabbing technique. It entails a total of 10 centrifugation steps, numerous labelling and pipetting steps, and also requires a large amount of plastic-ware. All these factors are potential sources of error, contamination and loss of sample. In our laboratory the stool kit extracted samples cost over NZ$ 11, labour costs excluded, and the extraction cost of the swabbed samples was approximately NZ$ 5.7. Zhang et al. (2006) found the stool-kit to fail for some herbivorous animals, in agreement with our finding of the three species of herbivore showing the greatest difference in terms of target DNA concentration and PCR inhibition. Although the stool-kit procedure could be partially automated by performing the second half of the extraction in a QIAcube (Qiagen), the first half (sample processing and inhibitor removal) had to be done manually. The swabbing/Longmire technique is a shorter procedure that requires less handling, labelling and pipetting, consequently leading to less sample loss, laboratory errors, and contamination, and thus saving time and money. This new approach enables high-throughput DNA isolation from faecal material within most adequately equipped molecular laboratories, as high-throughput extractions can also be carried out in other DNA extraction machines or in 96-well lysis blocks and collection plates using a centrifuge. Shorter periods of incubation could be taken into consideration, as ours were just found convenient when used in combination with the stool kit because of its protracted labour. Only 25% of the swabbing method lysate was used in the DNA extraction, so replicates could be done if necessary.

Total and target DNA yield

Total DNA yield seemed not to be related with target DNA concentration, nor followed any evident pattern. Total DNA yield varied more across species and among samples than with

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Table 4. Amplification failure, allelic dropout and false allele occurrence (%).

	Amplification failure		Allelic dropout across loci		Allelic dropout across samples		False alleles across loci		False alleles across samples	
Species	SK	SW	SK	SW	SK	SW	SK	SW	SK	SW
Goat	0.62	0	1.3	0.3	1.3	0.4	0	0	0	0
White tailed deer	15	0	9	1	15.7	1	0.3	0	4.8	0
Fallow deer	4.16	4.16	0	0	0	0	2.2	3.1	1.9	2.8
Iberian lynx	6.25	3.03	7.9	0.9	8.1	1.1	0.6	0.3	1	1
Dog	1.97	3.28	7.5	6.5	6.1	5.9	2.6	3.2	5.9	7.3
Possum	11.5	3.57	12.3	1.7	15	2.1	0.3	0	0.4	0
Mean	6.58	2.34	6.33	1.73	7.7	1.75	1	1.1	2.33	1.85

extraction method. In our experiment total DNA amount was generally higher for the herbivore species, in agreement with Ball et al. (2007), but not for possum, and was very variable within species, suggesting individual conditions are also affecting total DNA yield as suggested by Fernando et al. (2003). The noticeable lower total DNA concentration in dog SW samples could be due to physical interference of the mucus layer when swabbing the samples (Kupchella and Steggerda 1972), although further studies would need to be carried out to confirm this.

Target DNA yield tended to be higher for the SW than for the SK extracts. The usual picogram range of DNA concentration from faecal DNA (Morin et al. 2001, Reddy et al. 2012) is increased here to the nanogram range when using the swabbing technique for all species, except possum and dog. All species showed higher target DNA concentration from SW samples with the exception of dog, yet the swabbed dog samples yielded a three-fold higher proportion of target to total DNA when compared with SK samples (Table 3). The higher target DNA concentration obtained when performing the swabbing technique coupled with the target DNA being in a greater proportion for most species (Table 3, Fig. 2), indicates the swabbing technique is recovering more sloughed cells and less diet or microbial material from the faeces than the stool-kit approach. Dog and possum DNA yield might be increased by collecting more intestinal cells by wiping the entire surface of the dog scat, as only one side was gently rubbed for this experiment, or by swabbing more than three possum pellets.

Inhibition

In this study SW extracts showed higher inhibition rates than SK extracts for carnivores. SW extracts showed lower inhibition rates for herbivores and possum (Fig. 2), despite the use of an extra inhibitor-removal step in SK extractions. Increased inhibition of carnivore SW extracts could be due to the wooden-stick and cotton head swabs used to collect the lynx samples, or to endogenous inhibitors present only in carnivores, like polysaccharides from the anal scent glands or melanin from the prey.

Genotyping success and quality

A multi-tube approach was used in this study to compare the amplification success between the two sampling methods. While the average genotype quality index (QI) of all animals extracted with the stool kit was 0.87 (median: 0.94), the average QI of the swabbed samples was 0.96 (median: 1). Furthermore, the variance of QI within species tended to be higher for SK than SW samples. Amplification failure was recurrent, being remarkably high for some loci with the SK procedure, which nearly consistently failed in each of three species (lynx, white-tailed deer and fallow deer). This phenomenon could have been misinterpreted as a null allele if reference tissue samples or other collection/extraction methods and genotyping amplifications were not undertaken. It is known that the amplification success of different primers and loci can be affected by extraction and collection interactions in different ways (Renan et al. 2012), but it must be emphasised that amplification was in general more robust with the SW method.

PCR inhibition can be identified as the main cause of amplification failure and low QI in SK herbivore extracts, while low concentrations of target DNA could be the main cause of low genotyping success in possum and dog. Morin et al. (2001) discarded samples with less than 2 pg μl^{-1} of target DNA and found it unlikely to get allelic dropouts from samples of more than 0.2 ng of target DNA. On average we obtained less than 0.2 ng μl^{-1} DNA both for the SK possum samples and also on both dog treatments. Allelic dropouts were detected, especially for possum samples, although they were not carried over to the consensus. On the other hand, higher concentrations of target DNA overall contributed to the higher genotype quality for the SW approach. The number of replicates required for a high quality genotype could be therefore reduced for species with target DNA concentration within the nanogram per microlitre range, thereby saving considerable time and money. Alternatively, the freely available software Pedant (Johnson and Haydon 2007) or the model developed by He et al. (2011) could help determine how many replicates would be necessary to reach a chosen level of confidence. The correlation coefficients observed between QI and other variables suggest inhibition and the target to total DNA ratio may be more of a concern when using fresh samples for which the amount of starting DNA is usually not limiting, as in this study.

In this experiment all STR amplifications were successfully undertaken in one multiplex reaction, contrary to the often used two-step PCR consisting in a multiplex pre-amplification of a few cycles followed by singleplexes or microsatellite multiplex amplifications (Piggott et al. 2004, Arandjelovic et al. 2009, Reddy et al. 2012). The higher target DNA yield obtained with the SW procedure should in any case reduce the

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need for the two-step PCR procedure and contribute to further reducing cost and work load when genotyping faeces. Supporting our results, Renan et al. (2012) obtained considerably better results for Asiatic wild ass swabbed samples than for two-step (ethanol/silica) preserved samples. While they had slightly better results for frozen swabs rather than for swabs preserved in lysis buffer, freezing the swabs is not always a viable option when samples are taken directly in the field. Since our study used fresh samples, results are expected to be poorer with aged or degraded samples for both sampling methods. We recommend gently wiping the samples to fully coat the swab head, and to swab the upper surface of one scat (or at least three− four for pellet-like stools), since it should be the less degraded part as it is the first area to get dried (Fernando et al. 2003) and is not in contact with the possible moisture and humic acids of the ground. We also recommend using plastic-handle swabs (non-hollow) because these are easier to split than wooden swabs, and particularly because they do not absorb the preservative buffer, what could reduce the DNA yield (Rutledge et al. 2009).

In summary, collecting the samples using a swab and preserving them directly in the field optimises the epithelial cells recovery, especially in herbivores, most likely by reducing the loss of the outer layer cells that can happen while handling or preserving the samples. We demonstrated that faecal DNA can be extracted in bulk, by swabbing the samples and storing them in lysis buffer directly in the field, and using standard DNA extraction protocols that circumvent the need for extra sample manipulation or inhibitor-removal steps. The simplified protocol performed equally well than more elaborate and expensive protocols, and in most species tested, which cover a range of mammals with different digestive systems, even resulted in improvements on target DNA yield, PCR inhibition, and genotype quality. Furthermore, it is less time consuming than current procedures, reduces potential problems with cross contamination, minimises laboratory errors, is storage and transport convenient and most importantly, is cost-effective and tenable for automated laboratory procedures. The streamlined and improved method proposed here should greatly facilitate the use of faecal DNA for routine non-invasive genetic monitoring of wild mammal populations, and also foster genomic scale analyses based on faeces (Perry et al. 2010).

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Supplementary material (available as Appendix wlb.00096 at <www.wildlifebiology.org/readers/appendix>). File A1: Results summary. File A2: Genotyping errors and STR loci PCR conditions.

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