Towards high–throughput analyses of fecal samples from wildlife

C. Sarabia, I. Salado, A. Cornellas,
A. Fernández–Gil, C. Vilà, J. A. Leonard

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Abstract

Towards high–throughput analyses of fecal samples from wildlife. High–throughput sequencing offers new possibilities in molecular ecology and conservation studies. However, its potential has not yet become fully exploited for noninvasive studies of free–ranging animals, such as those based on feces. High–throughput sequencing allows sequencing of short DNA fragments and could allow simultaneous genotyping of a very large number of samples and markers at a low cost. The application of high throughput genotyping to fecal samples from wildlife has been hindered by several labor–intensive steps. We evaluate alternative protocols which could allow higher throughput for two of these steps: sample collection and DNA extraction. Two different field sampling and seven different DNA extraction methods are tested here on grey wolf (*Canis lupus*) feces. There was high variation in genotyping success rates. The field sampling method based on surface swabbing performed much worse than the extraction from a fecal fragment. In addition, there is a lot of room for improvement in the DNA extraction step. Optimization of protocols can lead to very much more efficient, cheaper and higher throughput noninvasive monitoring. Selection of appropriate markers is still of paramount importance to increase genotyping success.

Key words: Noninvasive genetic samples, Fecal DNA, Microsatellite genotyping, NGS, Field sampling, Carnivore feces

Resumen

Hacia análisis genéticos de alto rendimiento de muestras fecales de fauna silvestre. La secuenciación de alto rendimiento ofrece nuevas posibilidades en ecología molecular y biología de la conservación. Sin embargo, el potencial de esta técnica no ha sido totalmente explotado para estudios no invasivos, a partir de muestras fecales, de fauna en libertad. La secuenciación de alto rendimiento permite la secuenciación de fragmentos de ADN cortos y podría permitir el genotipado simultáneo de un gran número de muestras y marcadores a un bajo coste. La aplicación de estas técnicas a muestras fecales de fauna silvestre ha sido obstaculizada por la gran cantidad de trabajo requerido en varios pasos, desde la recolección de muestras hasta la secuenciación. Aquí evaluamos protocolos alternativos que podrían permitir un mayor rendimiento en dos de estos pasos: muestreo de campo y extracción de ADN. En este trabajo comparamos dos métodos distintos de conservación de las muestras obtenidas en el campo y siete métodos de extracción de ADN para heces de lobos (*Canis lupus*). Observamos una gran variación en el éxito de genotipado según los protocolos que se sigan. El método de muestreo de campo basado en frotado superficial de los excrementos dio resultados peores que la recolección de un fragmento del excremento. Por otro lado, los protocolos para la extracción de ADN mostraban resultados muy variables y ofrecen mucho margen de optimización y mejora. La optimización de protocolos puede llevar a un monitoreo no invasivo mucho más eficiente, económico y con mayor rendimiento. La selección de marcadores apropiados sigue siendo de importancia vital para incrementar el éxito de genotipado.

Palabras clave: Muestras genéticas no invasivas, ADN fecal, Genotipado de microsatélites, Secuenciación de nueva generación (NGS), Muestreos de campo, Heces de carnívoros

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Carlos Sarabia, Isabel Salado, Anna Cornellas, Alberto Fernández–Gil, Carles Vilà, Jennifer A. Leonard, Estación Biológica de Doñana (EBD–CSIC), Avda. Americo Vespucio 26, 41092 Seville, Spain.

Corresponding author: J. A. Leonard. E–mail: jleonard@ebd.csic.es

ORCID ID: Jennifer A. Leonard: 0000-0003-0291-7819; Carlos Sarabia: 0000-0002-6980-3480; Isabel Salado: 0000-0002-8861-2436; Carles Vila: 0000-0002-4206-5246

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Introduction

Feces are a very useful source of information to study free–ranging animals. They can be used to study many aspects of their ecology such as dietary habits, scent marks, parasite loads, hormonal levels, hunting ranges and distribution, and importantly, yield DNA. Fecal DNA offers a special insight into rare or endangered species and is noninvasive, so it has become increasingly used for ecological studies. Fecal DNA has proved to be an effective method to track animals in the wild (Harrison et al., 2006; Janečka et al., 2011), and it has helped understand complex patterns of population structure (Paetkau et al., 1995; Muñoz–Fuentes et al., 2009; Ruiz–Gonzalez et al., 2015; Norman et al., 2017), estimate population sizes (Taberlet, 1996; Kohn et al., 1999; Harrison et al., 2006; Pérez et al., 2014), detect criptic, endangered or elusive species in the wild (Harrison et al., 2006; Karmacharya et al., 2011; Pérez et al., 2014; Laguardia et al., 2015; Gil–Sánchez et al., 2017), and even has helped to describe complex behavioral patterns (Bischof et al., 2016; Forcina et al., 2019). The results of fecal DNA studies can also help assess and evaluate conservation or management policies (Echegaray and Vilà, 2010; Åkesson et al., 2016). However, feces are a sub–optimal source of DNA for molecular analyses as they are not widely suitable for genomic studies, with few reports of success (Perry et al., 2010).

There are three main challenges for the use of feces as a source of genetic material: presence of polymerase chain reaction (PCR) inhibitors, low amount of endogenous DNA, and DNA degradation and fragmentation. PCR inhibitors can be chemical compounds originating in the food, the digestive track, or the environment after deposition (Wilson, 1997; Monteiro et al., 1997; Rådström et al., 2004; Broquet et al., 2007; Panasci et al., 2011). As a result, the inhibitors vary between species and habitats. There is a low amount of endogenous DNA that comes from the cells swept away by the outer layer of the feces on their transit through the digestive track (Taberlet, 1996), which is normally less than the amount of micro–organismal DNA. Finally, environmental conditions, chemical compounds, and microorganisms can fragment and degrade DNA decreasing the likelihood of amplification through PCR (Deagle et al., 2006; Brinkman et al., 2010; Panasci et al., 2011; Demay et al., 2013; Roques et al., 2014; Agetsuma–Yanagihara et al., 2017). Such processes can lead to PCR failure, allelic dropout (failure to amplify one of two alleles), and/or false alleles (Taberlet, 1996; Miller et al., 2002; Broquet & Petit, 2004; Panasci et al., 2011). To overcome these problems, a number of studies have optimized protocols to sample, extract and amplify DNA from feces (Paetkau et al., 1995; Taberlet, 1996; Frantz et al., 2003; Miquel et al., 2006; Ramón–Laca et al., 2015). However, these methods tend to be labor intensive and are not conducive to high throughput projects.

Today the field of molecular ecology has extensively incorporated high throughput technologies to address a wide variety of research questions. One notable exception is the analysis of microsatellites. Despite the increasing popularity of SNP studies in natural populations, the high polymorphism of microsatellite markers imply that many fewer markers are needed for individual and population assessment, which is of great importance when working with fecal samples of low quality and with small amounts of DNA. However, high throughput technologies have not been fully implemented in microsatellite studies of noninvasive samples, which continues to be very labor–intensive. The potential to simultaneously analyze large numbers of markers from fecal samples could have a dramatic impact on noninvasive studies. There are multiple protocols in field sampling and laboratory analyses that could potentially be optimized to make them more compatible with high throughput analyses. An early step has been taken to optimize field collection of fecal samples for DNA analyses by Ramón–Laca et al. (2015), and their suggestion to field sample feces with swabs could streamline lab analyses by reducing the labor of preparing the samples for digestion. DNA extraction is also a key step to optimize for high throughput, for example by robotization.

We tested field and lab (DNA extraction) methods that could be scaled up to make the analyses of fecal samples for molecular ecology studies more feasible for high throughput studies. We used carnivore feces (from gray wolves, *Canis lupus*) to evaluate the field sampling method proposed by Ramón–Laca et al. (2015) against the more traditional approach of conserving fecal fragments in 70 % ethanol (EtOH). Once established which field sampling method(s) yielded acceptable results, we also compared seven methods for DNA extraction and purification. We evaluated these methods of extraction by assessing the PCR and genotyping success with a multiplex amplification of eight autosomal plus two Y–chromosome microsatellite loci typed using Next Generation Sequencing (NGS) approaches. We report the cost, in time and money, of each method, estimate the number of replicates necessary to overcome uncertainties due to dropout and false alleles, and discuss their potential for high–throughput projects including adaptation for robotization.

Methods

Evaluation of field sampling methods

We first evaluated the relative performance of two field sampling protocols. Eight Iberian gray wolf feces were collected in Asturias, Northern Spain. In the field, we cut each scat in half and preserved one half in a 50–ml Falcon tube with 70 % ethanol, while we swabbed the surface of the other half in situ following Ramón–Laca et al. (2015) and preserved the swabs in ca. 400 µl of Longmire's lysis buffer (Longmire et al., 1997). Samples were kept at –20 °C until DNA extraction. DNA from both swabs and ethanol–conserved fecal fragments was extracted using two different extraction methods: 1) using a QIAamp DNA Stool Kit (Qiagen, Hilden, Germany; 'extraction method 1' or EM1), which performs a silica membrane–based DNA purification of samples with high concentrations of PCR inhibitors;

and 2) an adapted solid phase–reversible immobilization (SPRI) beads–based purification method as in Rohland and Reich (2012), following digestion with proteinase K ('extraction method 2' or EM2). For each scat, we carried out two DNA extractions from the fecal fragment with each method and one from the fecal swab due to low amount of material, resulting in six DNA extractions for each scat.

For the DNA extraction using the QIAamp DNA Stool Kit (EM1) we followed the manufacturer's protocol 'Isolation of DNA from Stool for Human DNA Analysis' for the fecal fragments. Fecal swabs were drained and 150 µl of the lysis buffer was used for DNA extraction. The SPRI–based DNA extraction method (EM2) was carried out as follows. For the ethanol–preserved fecal fragments, 120 mg of dry fecal sample was added to 1200 µl Longmire's lysis buffer in a 1.5 ml Eppendorf tube. These digests were homogeneized by vortex, incubated at room temperature for 10', and centrifuged for 1' at full speed. Supernatant (about 850 µl) was transferred to a new tube. In the case of fecal swabs, 700 µl of Longmire's lysis buffer were added to 150 µl of buffer from the tube with the swab. Proteinase K (500 µg) was added to each digest, and tubes were briefly vortexed and then incubated for 10' at 70 ºC. Digests were purified with MagBeads ® as in Rohland and Reich (2012). Beads were washed twice with EtOH at 80 % and DNA was eluted as in Meyer and Kircher (2010, see 'Reaction Clean–Up Using Solid Phase Reversible Immobilization, SPRI'). All DNA extractions were performed in an isolated lab dedicated to low quality DNA. Before starting lab work, all surfaces were UV–treated for 20' and cleaned with a bleach solution. Filter–tips and isolation suits were also used. All batches of DNA extractions included negative controls.

Four PCRs were done for each DNA extract and negative. Each multiplex PCR reaction contained tailed primers for eight autosomal and two Y–chromosome microsatellites (table 1). Tails on the 5' end were 5'–TCTTTCCCTACACGACGCTCTTCCGATCT for forward and 5'–GAGTTCAGACGTGTGCTCTTCCGATCT for reverse primers. We used Phusion® High–Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA) following manufacturer's specifications: 1X Multiplex Phusion® MasterMix, 10–plex primer mix (0.05 µM per primer), bovine serum albumin (BSA) (200 µM), 2 µl of DNA extract to a final volume of 25 µl. We used the following touchdown PCR program for all multiplex reactions: initial denaturation at 98 ºC for 30''; 10 cycles of 98ºC for 10', 58 ºC for 30'' (decreasing 0.5 ºC per cycle), and 72 ºC for 30''; 20 cycles of 98 ºC for 10'', 53 ºC for 30'' and 72 ºC for 30''; final extension of 10' at 72 ºC and a final heating up step of 95 ºC for 3' to avoid the formation of dimers. Products were checked on a 2 % agarose gel and visualized on a Gel Doc™ EZ Gel transiluminator (Bio Rad, Hercules, CA, USA). PCR products that showed amplification were used for subsequent steps.

PCR products were purified using Sera Mag SPRI beads as in Meyer and Kircher (2010). Purified PCR products were dual indexed by PCR using a Kapa HiFi HotStart ReadyMix (2X) PCR kit for High Throughput Sequencing (Kapa Biosystems, Haufmann–La Roche, Basel, Switzerland). PCR conditions were: 95 ºC for 30''; 25 cycles of 98 ºC for 20'', 60 ºC for 15'', and 72 ºC for 15''; a final extension time of 1' and a heating up step of 95 ºC for 3' before leaving it slowly cool down. PCR products were checked by agarose gel electrophoresis and concentration was estimated against a standard using ImageLab v5.2.1 of BioRad. Products from different reactions were pooled equimolar, cleaned and then sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA).

We defined PCR success as the proportion of those multiplex PCRs that provided suitable amplification bands in agarose for subsequent high–throughput sequencing. Multilocus genotypes were constructed for each PCR product in Geneious v11.0.5 (Kearse et al., 2012). However, not all PCRs with bands could be successfully genotyped for all loci. We estimated the proportion of locus dropout as the proportion of loci that in successful PCR amplifications (with clear band) either failed to be sequenced or provided sequences that did not correspond to the targeted microsatellite (fig. 1, 2). The genotypes obtained were compared to the consensus genotypes obtained for all amplifications (consensus genotypes generated as in the multi–tube approach; Taberlet, 1996). These comparisons allowed the identification of false alleles and allelic dropout. False allele rate was defined as the proportion of genotypes with false alleles and allelic dropout rate was defined as the proportion of genotypes that failed to amplify one of the alleles in heterozygous loci (Taberlet, 1996; Creel et al., 2003; Broquet and Petit, 2004). False allele and allelic dropout rates were calculated using equations 2 and 4 from Broquet and Petit (2004). Finally, we defined genotyping success as the proportion of autosomal genotypes that coincided with the consensus per total number of PCR attempts. We also calculated sexing success just for the Y–chromosome loci as the proportion of successful amplification and correct genotyping of Y–chromosome markers in samples deriving from males.

Evaluating different extraction methods

A high yield of DNA extracted from noninvasive samples has been reported based on chaotropic salts (such as sodium dodecyl sulfate, SDS; Goldenberger et al., 1995; Yu and Morrison 2004), cetyl trimethyl ammonium bromide (CTAB; Zhang et al., 2006), a mixture of phenol–chloroform–isoamyl alcohol (PCI; Sambrook et al., 1989; Goldenberger et al., 1995; Muñoz–Fuentes et al., 2009), and SPRI–beads (DeAngelis et al., 1995; Meyer and Kircher, 2010). We aimed to find a cost–effective DNA extraction method by combining these procedures that could be automated for implementation in DNA extraction robots. In addition to the two methods described above (EM1 and EM2), we evaluated five additional extraction methods using the feces sampled in ethanol (see table 2 for a summary of extraction methods). Two extractions per method per sample were done, until the sample was exhausted.

Table 1. Microsatellite loci and primers: name (Locus), Repeat motif, and Chromosome according to the original publication (reference, Ref: 1, Bannasch et al., 2005; 2, Francisco et al., 1996; 3, Jouquand et al., 2000; 4, Ostrander et al., 1993). For each microsatellite typed, sequences of forward (Fwd_primer) and reverse (Rev_primer) primers are indicated (5'–3'): PIC, polymorphic information content from the original publication.

Tabla 1. Loci y cebadores de los microsatélites: nombre ("Locus"), secuencia repetida ("Repeat motif") y cromosoma, según la publicación original (referencia, Ref: 1, Bannasch et al., 2005; 2, Francisco et al., 1996; 3, Jouquand et al., 2000; 4, Ostrander et al., 1993). Para cada microsatélite genotipado, se indican las secuencias de los cebadores ("Fwd_primer", "Rev_primer") (5'–3'): PIC, contenido de información del polimorfismo según la publicación original.

Locus	Repeat motif	Chromosome	Fwd_primer Rev_primer	PIC	Ref
650–79.3	(CA)n	Y–chromosome	AGTTTCTGCCCAGGAAGGAC AGCTGAGCGGTTTGAAACTT		1
990–35	(GT)n	Y–chromosome	CCATCCGCAGAACAGGTATT GGGCCGCTATTTTAGGTGAT		1
c2096	(GAAT)n	Autosomal	CCGTCTAAGAGCCTCCCAG GACAAGGTTTCCTGGTTCCA	0.37	2
Ren37H09	(GT)n	Autosomal	ATTCCCTTGTATTGCTCAC CCCAAAAAATCCAACCA	0.67	3
Ren49F22	(CA)n	Autosomal	GGGGCTCTGTTATTAGGTG TCATAAGGCAAAGAAAACC	0.66	3
u109	(A)7(T)7(CA)n	Autosomal	AACTTTAAGCCACACTTCTGCA ACTTGCCTCTGGCTTTTAAGC	0.42	4
u173	(TG)n	Autosomal	ATCCAGGTCTGGAATACCCC TCCTTTGAATTAGCACTTGGC	0.78	4
u225	(GT)n	Autosomal	AGCGACTATTATATGCCAGCG CTCATTGGTGTAAAGTGGCG	0.46	4
u250	(AC)nA2(TC)m	Autosomal	TTAGTTAACCCAGCTCCCCCA TCACCCTGTTAGCTGCTCAA	0.75	4
u253	(AC)nAT(AC)m	Autosomal	AATGGCAGGATTTTCTTTTGC ATCTTTGGACGAATGGATAAGG	0.52	4

Extraction method 3 (EM3)

Small samples (100 mg) of feces were sampled from the EtOH–conserved fecal fragments, dried, and digested in 900 µl of CTAB buffer as in Vallet et al. (2008), but adding 750 mg of proteinase K to the original CTAB buffer. Digestions were vortexed briefly and incubated in a shaker at 60 ºC for 1 hour in 2 ml tubes. Supernatant of the digestion was extracted with 1 ml phenol–chloroform–isoamyl alcohol (25:24:1) (Sambrook et al., 1989) and further purified with SPRI beads following DeAngelis et al. (1995), but using the same bead buffer as in EM2. For each volume of supernatant, twice the volume of SPRI bead buffer was used. Particles were washed twice with 1.8 ml of 80 % EtOH and air dried for 4' before elution in 50 µl TLE buffer.

Extraction method 4 (EM4)

This protocol was identical to EM3, but instead of using a CTAB digestion buffer, a SDS digestion buffer was used as in Goldenberger et al. (1995), with the following composition: SDS 1 %, Tris–HCl 100 mM, Ethylenediaminetetraacetic acid (EDTA) 20 mM, NaCl 10 mM, Proteinase K 0.8 mg/µl.

Extraction method 5 (EM5)

This protocol was identical to EM3, but instead of using a CTAB digestion buffer, a CTAB+SDS digestion buffer was used with the following composition: CTAB 10 g/l (1 % w/w), SDS 1 %, Tris–HCl 100 mM, EDTA 20 mM, NaCl 1.4 M, Proteinase K 0.8 mg/µl. These proportions were optimized to ensure homogenization and solubilization of all reactants, avoiding the formation of too much foam.

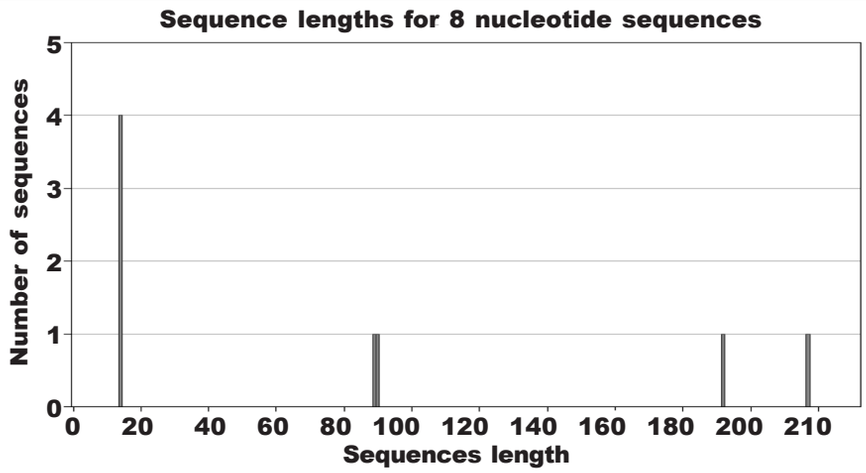

Fig. 1. Example of a locus from a successful multiplex PCR that did not yield an identifiable genotype due to insufficient number of reads, as identified in Geneious v11.0.5 (locus dropout).

Fig. 1. Ejemplo de un locus obtenido a partir de una PCR múltiple exitosa que no produjo ningún genotipo identificable debido al número insuficiente de lecturas, determinado mediante Geneious v11.0.5 (locus nulo).

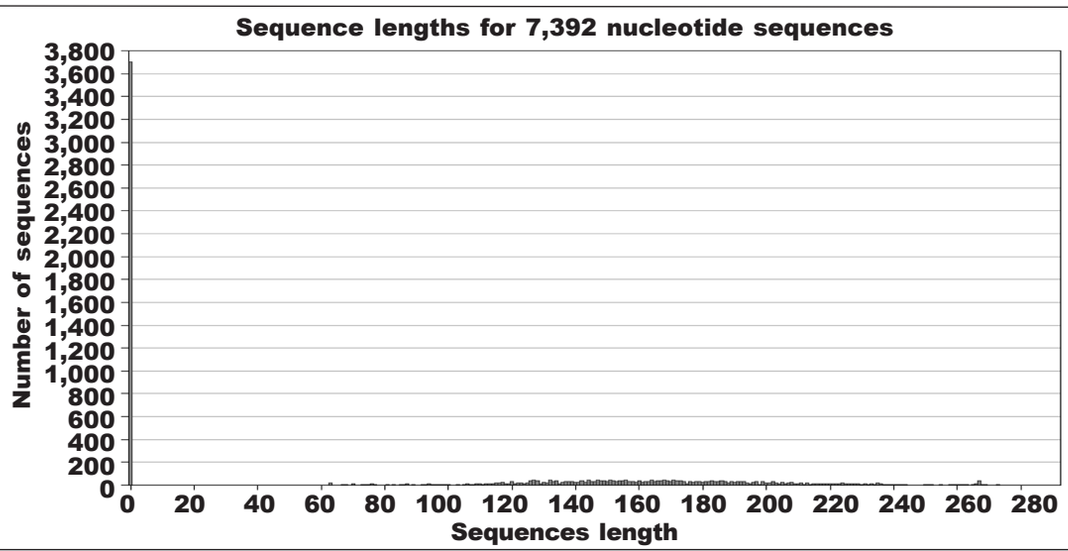

Fig. 2. Example of a locus from a successful multiplex PCR that did not yield an identifiable genotype due to unspecific amplification of spurious fragments and background noise, as identified in Geneious v11.0.5 (locus dropout).

Fig. 2. Ejemplo de un locus obtenido a partir de una PCR múltiple exitosa que no produjo ningún genotipo identificable debido a la amplificación inespecífica de fragmentos mal purificados y ruido de fondo, determinado mediante Geneious v11.0.5 (locus nulo).

Extraction method 6 (EM6)

This protocol was identical to EM3, but after the 60' at 60 ºC in the thermoshaker, an additional centrifugation of 30' at maximum speed (14,000 rpm) was done. The supernatant, approximately 600 µl, was carefully transferred to a new tube, leaving behind the pellet of tissue debris. DNA was subsequently extracted with SPRI beads as in protocol EM3, except DNA was eluted in double–distilled water.

Table 2. Summary of extraction methods: extractions of DNA were performed from fragments of wolf feces preserved in ethanol and air dried. Seven approaches were tested varying digestion buffer, DNA separation and purification. (For abbreviations and protocol details, see text).

Tabla 2. Resumen de los métodos de extracción: la extracción de ADN se realizó a partir de fragmentos de heces de lobo conservadas en etanol y secadas al aire. Se probaron siete métodos cambiando la solución de digestión y los protocolos para la separación y purificación del ADN. (Véase el texto para las abreviaciones y los detalles de los protocolos).

Extraction methods		Sample amount	Digestion	DNA isolation	DNA purification
EM1	QIAmp	180 mg		QIAmp Kit	
EM2	SPRI beads	120 mg	Longmire buffer + + proteinase K		SPRI beads + + double EtOH 80 % wash
EM3	CTAB + PCI + + beads	100–120 mg	CTAB buffer	PCI	SPRI beads + + double EtOH 80 % wash
EM4	SDS+PCI+ Beads	100–120 mg	PCI	PCI	SPRI beads + + double EtOH 80 % wash
EM5	CTAB + SDS + + PCI + beads	100–120 mg	CTAB + SDS + buffer	PCI	SPRI beads + + double EtOH 80 % wash
EM6	CTAB + beads	100–120 mg	CTAB buffer	Centrifugation	SPRI beads + + double EtOH 80 % wash
EM7	CTAB + beads + + reactivation	100–120 mg	CTAB buffer	Centrifugation	SPRI beads + reactivation + + double EtOH 80 % wash

Extraction method 7 (EM7)
This protocol was identical to EM6, but with an additional step of bead reactivation with the same buffer as in protocol EM3 at the beginning of the DNA purification with SPRI beads.

For EM1–EM5, each extraction method was performed twice on each scat. Four PCRs were made for each extract, yielding eight PCR replicates per extraction method and scat. Due to lack of sample at the end of the experiment, methods EM6 and EM7 were only carried out once. EM6 and EM7 did not include a step of phase separation using phenol:chloroform:isoamyl alcohol and therefore had a higher chance to be ineffective at separating DNA from polysaccharides (Zhang et al., 2006), which are likely to act as PCR inhibitors (Monteiro et al., 1997; Schrader et al., 2012). A moderate level of dilution is often recommended to diminish the effect and concentration of PCR inhibitors from environmental samples (Wang et al., 2017), so we hypothesized that a second elution could improve genotyping success. Consequently, for EM6 and EM7 we tested two elutions. We carried out six PCR replicates on each elution.

We estimated amplification success, locus dropout, false alleles, allelic dropout, and sexing success. We assessed the effect of extraction method on the genotyping success through generalized linear mixed model (GLMM) with a binomial distribution and using the function glmer from the lme4 package (Bates et al., 2015) in R (R version 3.5.2). The extraction method, locus and extract were included as fixed effects while feces were considered as random effect. Assumptions for normality and homocedasticity of residuals were checked graphically. We evaluated the model's goodness of fit and the explained variance by fixed and random effects in the model through pseudo–R^2 statistics using the function r.squaredGLMM from the MuMIn package in R (Barton, 2019). The significance of each variable was tested using a Chi–squared test with drop1 function from lme4, comparing the likelihood of the full model without the variable of interest. The model with the highest likelihood value was selected as the most parsimonious model. We also performed pairwise comparisons with Tukey's post hoc test among extraction methods using emmeans package (Lenth, 2018). We estimated the number of amplifications needed to ensure a correct genotype with a probability of 0.99 as in Le Gouar et al. (2009) and Forcina et al. (2019; calculations based on Navidi et al., 1992; Taberlet, 1996; Taberlet and Luikart, 1999), using an Excel spreadsheet kindly provided by Giovanni Forcina and Pascaline Le Gouar.

Results

Evaluation of field sampling methods

The performance of the two field sampling methods was evaluated by PCR amplification success using

Table 3. Evaluation of field sampling techniques: extraction methods (EM) are described in the text; PCR success refers to the proportion of multiplex PCR reactions that produced an amplification band visible in agarose gel from eight different samples; locus dropout (LD), proportion of loci that did not amplify in otherwise successful multiplex amplifications; false alleles (FA), proportion of alleles called that were not true alleles; allelic dropout (AD), proportion of heterozygote alleles that did not amplify; sexing success (SS), rate of successful genotyping of Y–chromosome markers in males; genotyping success (GS), proportion of PCR amplifications that provide the correct genotype.

Tabla 3. Evaluación de las técnicas de muestreo de campo: los métodos de extracción (EM) se describen en el texto; "PCR success" se refiere a la proporción de reacciones de PCR múltiple que produjeron una banda de amplificación visible en gel de agarosa a partir de ocho muestras diferentes; "locus dropout" (LD) es la proporción de loci que no se amplificaron en reacciones de PCR multi–locus exitosas; "false alleles" (FA) es la proporción de alelos identificados que no son reales; "allelic dropout" (AD), proporción de alelos en heterocigosis que no se amplificaron; "sexing success" (SS), tasa de eficacia del genotipado de marcadores del cromosoma Y en machos; "genotyping success" (GS), proporción de reacciones de PCR que generaron el genotipo correcto.

Field sampling techniques	EM	PCR success	LD	FA	AD	SS	GS
Fragment in 70 % EtOH	EM1	80 % (51/64)	0.11	0.06	0.11	0.58	0.61
Fragment in 70 % EtOH	EM2	10 % (6/60)	0.25	0.08	0.07	0.10	0.06
Swab + Longmire's buffer	EM1	31 % (10/32)	0.13	0.06	0.08	0.26	0.24
Swab + Longmire's buffer	EM2	28 % (9/32)	0.14	0.16	0.15	0.23	0.18

two extraction methods. Sampling fragments of feces followed by DNA extraction using the Qiagen kit (EM1) yielded the highest rate of PCR success (80 %, table 3), but the same field sampling method had the lowest rate of PCR success (10 %) when DNA was extracted with beads (EM2).

For those reactions that resulted in multilocus PCR amplification, frequently not all of the loci amplified. For each method, locus dropout ranged from 11–25 % per reaction, and was lowest for the fecal fragment extracted with EM1, and highest when the same sample type was extracted with EM2. Interestingly, false alleles were also lowest when extracting with EM1 for both field sampling methods (6 %). Allelic dropout was highest with swabs extracted with EM2. The overall genotyping success rate, measured as the proportion of loci per PCR replicate that matched the consensus genotype, was highest for fecal fragments extracted with EM1 (61 % for autosomal loci, 58 % for Y–chromosome loci in males). However, the same sampling method had the lowest success rate if extracted with EM2 (6 %).

Overall the best performance was obtained for fecal fragments preserved in ethanol and extracted with EM1. Given the potentially higher performance of sampling fecal fragments instead of swabs, all tests of extraction protocols were carried out on DNA extracts generated from fragments from feces preserved in ethanol.

Evaluation of extraction protocols

To test for replicability, we carried out a glmm with the model 'Genotyping success ~ Extraction method + Locus + Extract + Feces (random effect)' to assess the contribution of the extraction replicate to the total variance in genotyping success, where 'Locus' refers to the identity of the microsatellite loci, 'Feces' refers to the identity of the fecal sample and Extract refers to the extraction replicate. 'Extraction method' (likelihood ratio test LRT = 287.34, $p < 0.001$, N = 536) and 'Locus' (LRT = 391.22, $p < 0.001$) had significant effects whereas 'Extract' did not (LRT = 0.05, $p = 0.831$). In addition, pseudo–R^2 of the different models showed that the extract replicate ('Extract') did not contribute to the total variance of the model (table 4). Thus, the results of the two extraction replicates were pooled for comparisons. The simplest model was 'Genotyping success ~ Extraction method + Locus + Feces (random effect)'. This implies that although all feces were collected at the same time, their individual conditions had an important impact on the final genotyping success and that DNA extraction replicates produced very similar results. Thus, replicates to reduce allelic dropout and false alleles (see below) do not need to be carried out on different DNA extracts but should focus on replicating PCRs. Similarly, different markers showed very large differences in genotyping success, and the genotyping success was clearly different between extraction methods. Post–hoc comparisons showed pairwise differences among extraction methods. EM2, EM6 and EM7 were the worst extraction methods in comparison to EM1, EM3, EM4, EM5.

Although the QIAmp kit (EM1) is widely used (Creel et al., 2003; Panasci et al., 2011; Pérez et al., 2014; Ramón–Laca et al., 2015), it led to slightly worse amplification success rate than some other methods (77 % PCR amplification success for EM1 vs. 100 %

Table 4. Variance explained by the models using pseudo–R^2 statistics of generalized mixed models: $R^2_{GLMM(m)}$, marginal component of R^2 value show variability explained by fixed effects ('Method', 'Locus', 'Extract'); $R^2_{GLMM(c)}$, conditional R^2 value show variability explained by fixed effects plus the random effects ('Feces'); GS, genotyping success. (N = 536, number of observations for all models).

Tabla 4. Varianza explicada por los modelos según estadísticos pseudo–R^2 de modelos mixtos generalizados: $R^2_{GLMM(m)}$, componente marginal de R^2 que muestra la variabilidad explicada por los efectos fijos ("Method", "Locus", "Extract"); $R^2_{GLMM(c)}$, componente condicional de R^2 que muestra la variabilidad explicada por los efectos fijos más los efectos aleatorios ("Feces"); GS, eficacia del genotipado. (N = 536, número de observaciones de todos los modelos).

Models	$R^2_{GLMM(m)}$	$R^2_{GLMM(c)}$
GS ~ Feces	0.00	0.07
GS ~ Method + Feces	0.12	0.20
GS ~ Method+Locus + Feces	0.32	0.40
GS ~ Method + Locus + Extract + Feces	0.32	0.40

PCR amplification success for EM3, EM4, EM5, EM6 and EM7, table 4). However, the lowest locus dropout rate was observed in EM1. Genotyping success was highest for EM4 and EM5 (65 % and 63 %, respectively) although it was not very much higher than for EM1 (60 %). EM4 and EM5 involved the use of SDS in the digestion buffer and a one hour digestion at 60 ºC followed by a phenol–chloroform–isoamyl DNA isolation. EM6 and EM7 had in general lower performance than all other methods (table 5). The performance was clearly worse for the second elutions of EM6 and EM7, but diluting the extract resulted in a reduction of false alleles, although the proportion of allelic dropout became higher.

Replication requirements

We estimated the number of replicates needed to generate reliable consensus in homozygotes with a probability of 0.99. While heterozygote genotypes are usually confirmed with at least two independent replicates of the genotype, homozygotes required several replicates depending on the rate of false alleles and allelic dropout (table 5). A minimum of three replicates were sufficient for EM3, EM4 and EM5. EM2 performed much worse than all other methods, due to the low PCR success rate and low genotyping success rate (table 5). However, these numbers do not take into account the rate of PCR success and locus dropout. If amplifications fail frequently, the number of replicates needs to be increased accordingly. Thus, dividing the number of replicates estimated for homozygous loci by the PCR success rate and by the proportion of amplification (1–locus dropout) for the locus with the 6th lowest locus dropout rate, we estimated the number of replicates needed to obtain reliable genotypes with a probability of 0.99 for 6 of the loci. The results showed a huge variation in the genotyping effort needed with the different methods (table 6), ranging from four (for EM1, EM3, EM4 and EM5) to 40 PCR reactions needed. When a positive amplification of 7 out of 8 loci is required, the number of PCRs required increases in EM6 (to 100 replicates needed), while it remains constant for EM1, EM4 and EM5.

Economic and time–consumption evaluation of extraction protocols

Methods EM3, EM4 and EM5 were more time consuming due to the additional 1 hour digestion step and the phenol–chloroform extraction. Although slightly less time consuming, EM1 was the most expensive method, with all other methods considered being at least three times cheaper (table 6). Methods EM6 and EM7 were reasonably cheap and are more amenable to robotization, although a much higher number of PCR replicates was needed to obtain reliable results.

Discussion

In order to simplify field sampling methods and establish a reasonable protocol for high throughput DNA studies of noninvasive samples from carnivores, we tested both swabbing and feces fragment sampling. The field sampling method of taking a swab instead of a fragment, which could have made lab work much easier, was not successful because the swabs yielded much lower PCR success. The low performance obtained when genotyping swabs contrasts with the general results in Ramón–Laca et al. (2015). These authors observed a big difference in performance between the two approaches when analyzing herbivore feces. The high amount of polysaccharides in herbivore feces from the wall of plant cells acts as a well–characterized PCR inhibitor (Monteiro et al., 1997; Rådström et al., 2004, Ramón–Laca et al., 2015), a less prevalent

Table 5. Evaluation of performance of seven DNA extraction methods: extraction methods (EM) as in the text and summarized in table 2 from seven different samples; second elution indicates DNA extracts that were subject to an additional elution to decrease the concentration of potential inhibitors. PCR success, locus dropout (LD), false alleles (FA), allelic dropout (AD), sexing success (SS) and genotyping success (GS) as described in table 3.

Tabla 5. Evaluación del rendimiento de siete métodos de extracción de ADN: los métodos de extracción (EM) de siete muestras diferentes son los descritos en el texto y resumidos en la tabla 2. La segunda elución indica los extractos de ADN que se sometieron a una elución adicional para reducir la concentración de posibles inhibidores. Eficacia de la PCR ("PCR success"), loci nulos (LD), falsos alelos (FA), alelos nulos (AD), eficacia del sexado (SS) y eficacia del genotipado (GS), descritos en la tabla 3.

Extraction method		Elution	PCR success	LD	FA	AD	SS	GS
EM1	QIAmp	1st	77 % (43/56)	0.08	0.06	0.12	0.58	0.60
EM2	SPRI beads	1st	11 % (6/56)	0.25	0.08	0.07	0.11	0.07
EM3	CTAB + PCI + beads	1st	100 % (52/52)	0.30	0.10	0.12	0.61	0.58
EM4	SDS + PCI + beads	1st	100 % (52/52)	0.25	0.09	0.06	0.67	0.65
EM5	CTAB/SDS + PCI + + beads	1st	100 % (52/52)	0.25	0.09	0.10	0.64	0.63
EM6	CTAB + beads	1st	100 % (42/42)	0.67	0.20	0.18	0.48	0.24
		2nd	71 % (30/42)	0.70	0.14	0.29	0.22	0.14
EM7	CTAB + beads +	1st	100 % (42/42)	0.58	0.16	0.13	0.46	0.30
	+ reactivation	2nd	71 % (30/42)	0.70	0.13	0.24	0.24	0.15

problem in carnivore feces, and this may explain the difference in success between carnivore and herbivore feces. Previous studies have already addressed how dietary habits influence the quality and quantity of DNA extracted with the same method, thereby showing how important it is to define an appropriate field sampling strategy and DNA extraction method for each particular project (Miquel et al., 2006; Ramón–Laca et al., 2015). For carnivores, we recommend to continue using the more traditional field sampling strategy of taking feces fragments. Running a preliminary test on different field sampling methods for fecal DNA extraction in a target species might be advisable before starting a noninvasive monitoring study.

Apart from sampling strategies, different DNA extraction protocols produce very variable results. We saw a higher performance in PCR success, sexing success and genotyping success in SDS–based methods than in all other methods. PCI was generally a good strategy for DNA separation, and although in the literature CTAB –PCI is often referred to as a good method to remove polysaccharides that could inhibit PCR amplification (Zhang et al., 2006; Vallet et al., 2008), we observed a better performance when SDS was present in the digestion buffer. SDS is a key component of the Longmire's 'lysis buffer' (Longmire et al., 1997), works very well for cell lysis (Zhang et al., 2006; Chandra De et al., 2015), and is the basis of a very well–established method for protein purification and molecular weight estimation (Shapiro and Vinuela, 1967; Weber and Osborn, 1969). It seems likely that the higher performance of method EM5 arose from a combination of the polysaccharide elimination activity of CTAB coupled from the cell lysis activity of SDS. However, EM4 performed slightly better than EM5, resulting in a lower allelic dropout.

We also observed that SPRI beads–based methods (EM2, EM6, EM7) could not compete with matrix–based or PCI–based DNA methods for locus dropout, false alleles, allelic dropout or genotyping success. This suggests that a high number of PCR inhibitors cannot be separated from the DNA easily by centrifugation, even after using a well–established digestion buffer such as CTAB. An additional step of DNA isolation or separation remains necessary to ensure that enough inhibitors are removed in carnivore feces. The main reason why a second elution was attempted with EM6 and EM7 was the high probability of finding PCR inhibitors that could not easily be separated from the DNA, inhibitors that could be extracted if a matrix–based or PCI–based method were used. However, using a second elution also dilutes DNA extracts. Although diluting can have a positive effect by reducing the concentration of inhibitors (Monteiro et al., 1997) it also reduces the concentration of available DNA for analysis, increasing allelic dropout (Taberlet, 1996). This effect can be seen in table 3: allelic dropout rates were double for second elutes of EM6 and EM7 than for first elutes, but false alleles were reduced by half. False alleles are normally produced by incomplete addition of extra adenine residues at the 3' end of amplified fragments or slippage in the first steps of PCR (Pompanon et al.,

Table 6. Evaluation of time and economic costs and number of replicates required by the different DNA extraction protocols, extraction methods are described in the text and summarized in table 2: Time, time needed to process one batch of eight samples; Cost, the price per sample in Euros in spring 2019; Replicates (4/8), number of PCR replicates required to establish a correct genotype at four out of eight loci; Replicates (6/8), number of PCR replicates required to obtain a correct genotype at six out of eight loci; and Replicates (7/8), number of PCR replicates required to ensure a correct genotype at seven out of eight loci. Number of replicates needed to achieve a correct consensus genotype with a 99 % probability was calculated for homozygous loci following Le Gouar et al. (2009), taking into account the PCR success rate from table 5: [a] 1 h for reagents of the digestion buffer to dissolve included; [b] 1 h of digestion at 60 ºC included; [c] 1 h of digestion and 30' centrifugation at maximum speed included.

Tabla 6. Evaluación del tiempo, costos económicos y número de repeticiones necesarias para los diferentes protocolos de extracción de ADN descritos en el texto y resumidos en la tabla 2: "Time", tiempo necesario para procesar un lote de ocho muestras; "Cost", precio en euros por muestra, en la primavera de 2019; "Replicates (4/8)", número de repeticiones de la PCR necesarias para obtener un genotipo correcto en cuatro de ocho loci; "Replicates (6/8)", número de repeticiones de la PCR necesarias para obtener un genotipo correcto en seis de ocho loci; y "Replicates (7/8)", número de repeticiones de la PCR necesarias para obtener un genotipo correcto en siete de ocho loci. El número de repeticiones necesarias para obtener un genotipo correcto consensuado con una probabilidad del 99 % se calculó en loci en homocigosis según Le Gouar et al. (2009) y teniendo en cuenta el grado de eficacia de la PCR indicado en la tabla 5: [a] se incluye 1 h para que los reactivos de la solución de digestión se disuelvan; [b] se incluye 1 h de digestión a 60 ºC; [c] se incluye 1 h de digestión y 30 min. de centrifugación a velocidad máxima.

EM	Time	Cost	Replicate (4/8)	Replicate (6/8)	Replicate (7/8)
EM1	3 h	5.78	4	4	4
EM2	3 h	1.39	23	46	46
EM3	5 h 30' [a,b]	1.82	3	4	5
EM4	6 h [a,b]	1.8	3	4	4
EM5	5 h 50' [a,b]	1.82	3	4	4
EM6	3 h 30' [c]	1.64	5	40	100
EM7	3 h 45' [c]	1.64	4	20	20

2005), factors that can be increased by the presence of PCR inhibitors in the extract.

The observation that bead based extraction methods EM2, EM6 and EM7 yielded a much lower genotyping success makes automatized DNA extraction much more difficult. The potential for robotization of DNA extraction has already been described and equipment such as QIAcube (Qiagen, Hilden, Germany) has been suggested for these tasks (Ramón–Laca et al., 2015). Our work shows that for fecal extractions a step of matrix– or PCI–based DNA isolation is still needed, a process that remains time consuming. Both of these methods (PCI and silica) require centrifugation, which is not compatible with standard lab robots. However, protocols that do not require a delicate phase separation (as in PCI) do have the potential to be adapted to lower volumes and done in 96 well plates, which could dramatically increase throughput. In the recent past this would not yield sufficient material to genotype a reasonable number of loci, but if loci are genotyped by NGS, many loci can be multiplexed in few reactions reducing the amount of extract necessary for a study.

Finally, while DNA extraction with the QIAmp kit (EM1) is widely used in literature, methods EM3, EM4 and EM5 require a similar effort in terms of PCR replicates that need to be attempted (table 6) and are three times cheaper. Thus, they are suitable substitutes for the QIAmp kit for labs and projects handling a large number of fecal samples and/or limited budgets. In terms of ecological impact all three methods produce hazardous waste. QIAmp kit includes guanidinium thiocyanate, while EM4 and EM5 (and EM3 although with a slightly lower performance) involve the use of phenol:chloroform:isoamyl alcohol. All these chemicals need to be handed in fume hoods and disposed of carefully. Cleaner and safer extraction methods still need to be developed to ensure low impact on health and environment.

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