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SHORT COMMUNICATION

Single-tube HotSHOT technique for the collection, preservation and PCR-ready DNA preparation of faecal samples: the threatened Cabrera's vole as a model

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Abstract A cost-effective, reliable and efficient method of obtaining DNA samples is essential in large-scale genetic analyses. This study examines the possibility of using a threatened vole species, *Microtus cabrerae*, as a model for the collection and preservation of faecal samples for subsequent DNA extraction with a protocol based on the HotSHOT technique. Through the examination of the probability of multi-copies (mitochondrial) and single copy (microsatellite) loci amplification (including the genotype error) and of the DNA yield (estimated by real-time qPCR), the new protocol was compared with both the frequently employed methods that successfully use ethanol to preserve faecal samples and with commercial kit-based DNA extraction. The single-tube HotSHOT-based protocol is a user-friendly, non-polluting, time-saving and inexpensive method of faeces sample collection, preservation and PCR-quality gDNA preparation. This technique therefore provides researchers with a new approach that can be employed in high-throughput, noninvasive genetic analyses of wild animal populations.

Keywords Noninvasive method · Sample collection · Sample preservation · DNA extraction · HotSHOT technique · Kit-based protocol · Real-time qPCR · Mitochondrial · Microsatellite

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Introduction

Genetic sampling and molecular research are important elements in the study of wild animal populations. Moreover, the use of genetic data has become essential in surveys and conservation plans since genotyping enables species to be accurately identified. Additionally, sex ratios, individual membership, population size, inbreeding levels and the genetic erosion of populations can be assessed. However, the collection of tissue and/or blood samples from free-ranging animals can be difficult or impractical. Faeces are one of the best noninvasive animal samples for analysis because they are easy to find in the wild and provide more information (i.e. relating to diet, stress hormone status, parasite infection and animal DNA) than other sample types (Goymann 2005; Luikart et al. 2008; Schwartz and Monfort 2008; Pauli et al. 2010). It is easier to extract DNA from compact pellets than from non-pelleted faeces given that in the former case pellets can be easily washed to obtain mucosal intestinal cells from their surfaces (Flagstad et al. 1999). The liquid used to wash the surface contains relatively few PCR inhibitors, and therefore, it does not necessarily require the use of expensive, time-consuming DNA extraction kits and additional steps designed to remove inhibitors. Faeces surface washing

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yields a high amplification success, low genotyping error rates and large quantities of DNA (Beja-Pereira et al. 2009). Nevertheless, there are still a number of factors such as faecal collection, preservation and DNA extraction methodologies that will affect the success of the gDNA extraction, and therefore, these points must be taken into account (Nsubuga et al. 2004). The use of commercial kits for these purposes can be expensive, laborious and time-consuming.

Since its first application, the hot sodium hydroxide and Tris (HotSHOT) technique (Truett et al. 2000) has proved to be a rapid and inexpensive method for obtaining good quality DNA from different tissue types (e.g. Jørgensen et al. 2005; Klover and Hennighausen 2007; Reif et al. 2008; Alcaide et al. 2010). Successful modifications of this technique have been used to extract DNA from zebrafish tissues (Meeker et al. 2007) from continental aquatic invertebrates cysts (Montero-Pau et al. 2008) and from small, hard-bodied parasites (the so-called HotSHOT Plus ThermalSHOCK) (Alasaad et al. 2008). The savings in time and reagents as compared to traditional DNA preparation methods are substantial, and the quality of the DNA obtained is as good as—or even slightly better than—that obtained by traditional methods.

The aim of this study was to optimize a user-friendly, inexpensive, time-saving and non-polluting protocol for the collection, preservation and PCR-ready DNA preparation of faecal samples based on the HotSHOT technique. The results in terms of the probability of gene amplification (including the frequency of genotyping errors) and the DNA yield using this new methodology were compared with those from protocols using ethanol (ETOH) preservation and kit-based DNA extraction.

In this study, we used faecal samples from the threatened Cabrera's vole *Microtus cabrerae* as a model (Mitchell-Jones et al. 1999). This vole is endemic to Portugal and Spain (Blanco and González 1992; Cabral et al. 2005) and is found in small colonies in a highly fragmented distribution that is subject to strong annual fluctuations. It is listed in the European Community Habitats Directive (92/43/EEC), the Berne Convention (82/72/CEE) and the IUCN Red List. The use of indirect noninvasive approaches is potentially of great interest and, in particular, the extraction of DNA from faeces represents an extremely valuable and powerful tool for the study of this rare and elusive species.

Materials and methods

Sample collection and preservation

Faecal samples from Cabrera's voles were collected from caged animals in Jerez and Granada Zoos (30 *M. cabrerae*) (Andalucía, Spain) and from droppings of trapped free-ranging *M. cabrerae* (30 animals) from various locations in Andalucía (Spain). From each animal's faeces, six pellets were collected: (1) three faecal pellets (~0.04 g) were placed in a 1.5-mL microcentrifuge tube with approximately 1 mL of absolute ETOH, while (2) three other pellets were deposited in a 1.5-mL microcentrifuge tube with 150 μL of alkaline lysis reagent (25 mM NaOH and 0.2 mM disodium EDTA; this reagent is prepared by dissolving the salts in water without adjusting the pH). The lysis buffer was added to wash faecal pellets for 5 min by gently hand-shaking the microcentrifuge tube and then one of two procedures was employed: either (1) the pellets were removed with sterile toothpicks or, alternatively, (2) the lysis buffer was recovered using Pasteur pipettes, the non-dissolved pellets were discarded and the lysis buffer returned to the same microcentrifuge tubes; in all cases, ~100 μL of lysis buffer was recovered. During the sample collection, disposable latex gloves and sterile toothpicks were used. All samples were kept at environmental temperature in the field and then kept at −20°C in the laboratory. Tissue samples were collected from six adult *M. cabrerae* from Jerez and Granada Zoos (Andalucía, Spain) for positive controls. Each tissue sample was conserved in 1.5 mL microcentrifuge tube with approximately 1 mL of absolute ETOH.

DNA extraction

1. In the case of the faecal pellets preserved in ETOH, DNA was extracted using DNeasy Blood & Tissue Kits (Qiagen), with an initial 5-min washing of faecal pellets in 150 μL of lysis buffer, from which 100 μL were used directly in the extraction protocol as if the sample was blood. This protocol is widely used and is highly successful for DNA extraction and the conservation of faecal samples (Maudet et al. 2004; Luikart et al. 2008; Alasaad et al. 2010, 2011).
2. For the HotSHOT protocol, the microcentrifuge tubes with 100 μL lysis buffer were heated to 95°C for 30 min; then, samples were cooled to 4°C and 100 μL neutralizing reagent (40 mM Tris–HCl; this reagent is prepared by dissolving Tris–HCl, not Tris base, in water without adjusting the pH) were added.

The DNA was extracted from tissue samples following the standard HotSHOT protocol as described by Truett et al. (2000). For all protocols, the final buffer volume was 200 μL. DNA extracts were kept at −20°C until analysis. All extractions and reagent preparations were conducted in a low-copy number DNA laboratory, physically isolated from modern DNA laboratories and post-PCR laboratories. Possible contamination was monitored by using a number of extraction blanks at all extraction steps.

PCR amplification, sequencing and genotyping

Each 30 μL PCR mixture consisted of 2 μL of gDNA (from tissue or faecal samples), together with the PCR mixture containing primer pairs (1) MiKa1 (5′-ATTACTCCTT-TAAACCATGG-3′) and MiKa2 (5′-CTAATAGACAAAA-TAGGGATGGGG-3′) (Alasaad et al. 2011) for mitochondrial control region DNA amplification; or (2) Moe1F (5′-TGGTTGTTCTGTGGTGAATACAG-3′; labelled with the fluorescent 6-FAM) and Moe1R (5′-ACAGTAAGCAGTTTATCCACAAACC-3′) (Van De Zande et al. 2000) for Moe1 microsatellite locus amplification (0.25 μM of each primer), 0.12 mM dNTP, 3 μL of 1× kit-supplied PCR buffer, 1.5 mM $MgCl_2$, 0.4% BSA, 1.5 μL DMSO and 0.2 μL (0.2 U/reaction) Taq polymerase (Bioline). Samples were subjected to the following thermal profile for amplification in a 2720 thermal cycler PTC-0200 DNA Engine thermal cycler (Bio-Rad): 4 min at 94°C (initial denaturation), followed by 40 cycles of three steps of 1 min at 94°C (denaturation), 1 min at 55°C (annealing for Mika1 and MiKa2 primers) or at 60°C (annealing for Moe1F and Moe1R primers), and 50 s at 72°C (extension), before a final elongation of 5 min at 72°C. PCR blanks (reagents only) were included with each PCR experiment.

Sequencing reactions were carried out using the BigDye Terminator v1.1 Cycle Sequencing Kit technology following the manufacturer's instructions (Applied Biosystems). Fragments were resolved on an automated DNA sequencer (Applied Biosystems 3130xl Genetic Analyzer), and DNA sequences were aligned and edited using the software BioEdit (Hall 1999).

Aliquots of 20 μL of formamide with LIZ Size Standard and 2 μL of microsatellite PCR product were analysed on the same automated DNA sequencer. Allele sizes and genotypes were determined using GeneMapper 3.7 (Applied Biosystems) followed by manual proofreading. Allelic dropout and false allele rates were calculated with a maximum likelihood-based method implemented in PEDANT (Johnson and Haydon 2007).

Real-time quantitative PCR

Approximately 100-bp fragment of the Moe1 microsatellite was used for assessing gDNA yield by real-time quantitative PCR (RT qPCR). Standards for RT qPCR were taken from high-quality tissue extracts from Cabrera's voles. Initial extract concentrations were measured with a spectrophotometer (Nanodrop®, ND-1000 Spectrophotometer) and dilutions were made as necessary to obtain the desired DNA concentration ranges (between 50 ng/μL and 0.003 ng/μL, four-fold dilutions). Standard dilution and extracts were replicated twice during each assay; PCR blanks were added in all assays.

Amplification reactions contained 0.3 μM of each primer, 12.5 μL of 1× GoTaq® qPCR Master Mix (Promega), 2 μL of DNA extract and Nuclease-free water in a final volume of 25 μL. Cycling conditions consisted of a 2-min pre-denaturation step at 95°C, followed by 45 cycles of amplification for 15 s at 95°C and 1 min at 60°C. In order to check for primer dimer and other PCR artefacts, a final dissociation curve was performed: 1 min at 95°C followed by 30 s at 55°C and increasing up to 95°C for 30 s. RT qPCR was carried out in an Mx-3005P cycler (Stratagene). Data were analysed in MxPro v4.00 (Stratagene).

Results and discussion

Noninvasive sampling is a potentially cost-effective and efficient means of monitoring wild animals that precludes the need for captures and avoids undue disturbance.

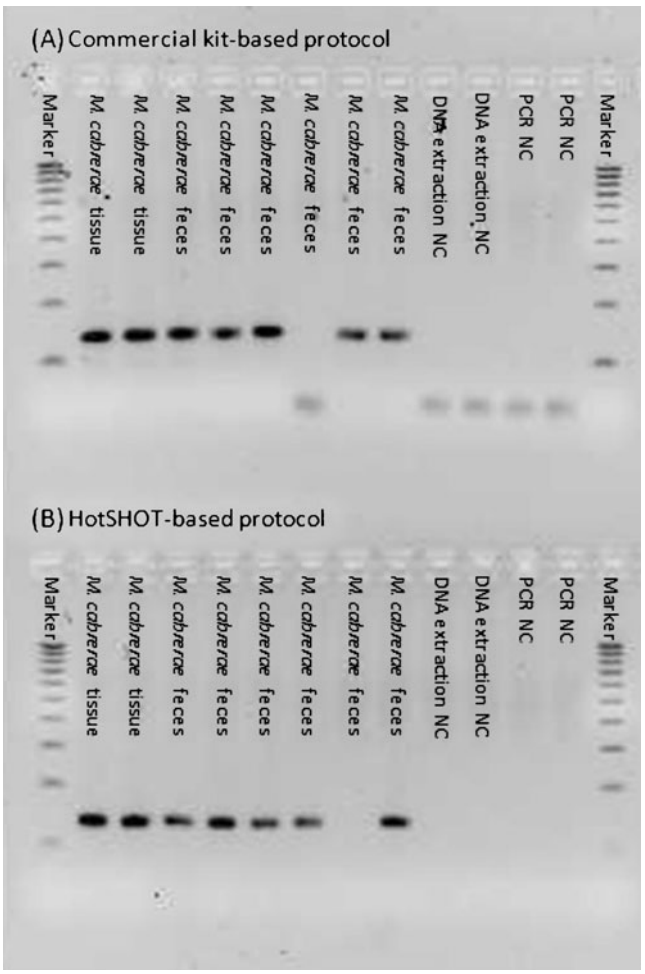

Fig. 1 PCR amplifications of the mitochondrial control region using primers MiKa1 and MiKa2 in 2% agarose gel from representative samples from *M. cabrerae*. **a** PCR amplifications using commercial kit-based protocol. **b** PCR amplifications using HotSHOT-based protocol. *NC*: negative control

Nonetheless, the increasing number of different protocols for the collection, preservation and DNA extraction of faecal samples in use makes it difficult for researchers to know which protocol is most reliable or which requires further testing and optimization (Beja-Pereira et al. 2009). Furthermore, there are inconsistencies between some studies and interaction between preservation techniques and extraction methods exist (Piggott and Taylor 2003; Beja-Pereira et al. 2009).

As a control protocol, we used an ETOH preservation and commercial kit DNA extraction methodology. The criteria of success of our novel technique were the probability of gene amplification (including the frequency of genotyping errors) and the DNA yield.

ETOH was used as a preservation reagent in large volume (1 mL) and in high concentrations. ETOH is widely used to conserve faecal samples, since it preserves the external mucous layer containing cells packed against the faecal material (Beja-Pereira et al. 2009). In our protocol, the first solution of the HotSHOT technique preserves DNA, since it contains EDTA, which serves as a chelator inhibiting the enzymes that degrade DNA.

Because of the small size of Cabrera's vole pellets, we used three pellets (~0.04 g) in each extract to increase the probability of DNA extraction success. Hence, the number of the pellets and the reagents volume should be adjusted specifically to each species studied.

We used a small fragment of the mtDNA control region of Cabrera's vole (multi-copies gene) and one microsatellite marker (single-copy) to evaluate our new protocol using *M. cabrerae* faecal samples. Sequencing analysis demonstrated that the 140-bp amplicons of the mtDNA control region amplified in our study were identical to those reported by Alasaad et al. (2011) (GenBank accession numbers FR695397-FR695403). Four alleles were detected from Moe1 microsatellite ranging between 93 and 101 bp, and

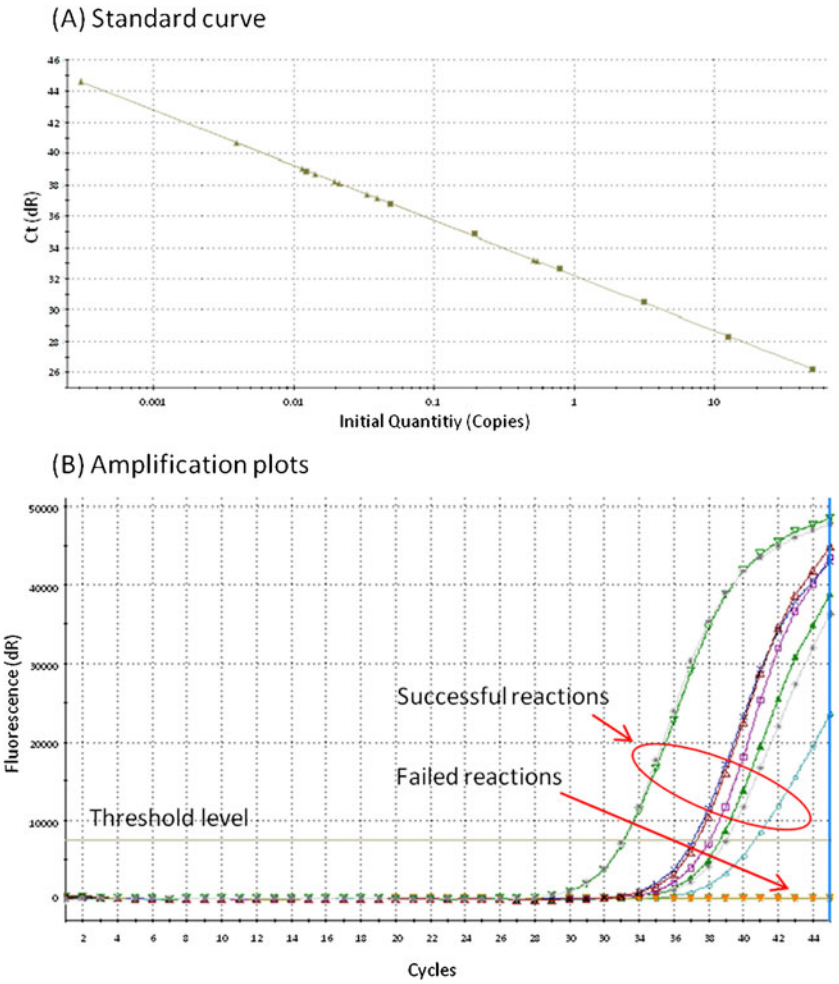

Fig. 2 **a** Representative standard curve from *M. cabrerae* tissue samples: *X*=initial DNA quantity (copies) vs. *Y*=C_T. Dilutions of DNA concentration ranged between 50 ng/μL and 0.003 ng/μL (−4-fold dilutions). **b** Representative amplification plots of Moe1 microsatellite from *M. cabrerae* faecal samples, using HotSHOT- and Kit-based protocols: *X*=PCR cycle number vs. *Y*=fluorescence signal. C_T is the cycle at which the fluorescence exceeds a predetermined threshold level. The threshold level is an arbitrary level of fluorescence chosen on the basis of the baseline variability

no deviation from Hardy–Weinberg equilibrium was detected using the software package Cervus v.3.0 (Marshall et al. 1998).

Two hundred forty locus-specific reactions were carried out (mtDNA and microsatellite). The PCR amplification ratio was 85% for the control protocol (102 mtDNA and STR reactions out of 120: N=60) and 83.34% for the single-tube HotSHOT technique (100 mtDNA and STR reactions out of 120: N=60). Evidence shows that no statistical difference (Fisher's exact test: $p<0.01$) exists between the two methods regarding the success of the PCR reactions (Fig. 1). For microsatellite data, allelic dropout and false allele rates (considering the resulted genotypes from HotSHOT technique and the control protocol as duplicate genotypes) were below 0.01 (N=60). Hence, our genotyping yielded very robust results using both protocols.

Rather than simply considering the percentage of positive PCR amplifications (only an approximate measure of success), the success of our procedure was measured in terms of the concentration of the amplifiable DNA recovered (Morin et al. 2001). Using conventional methods (e.g. spectrophotometer), it is difficult to quantify the amount of DNA extracted from faecal samples, since this type of apparatus is inefficient for calculating trace quantities of DNA; neither are these methods able to estimate DNA degradation or differentiate between DNA from the target species and from the microbes that are often present in faecal DNA extractions (Morin et al. 2001). To cope with these limitations, we used RT qPCR. The DNA yield was 0.201±0.201 ng/μL for the control protocol (N=30) and 0.197±0.264 ng/μL for our novel single-tube HotSHOT technique (N=30) (Fig. 2). No statistical differences were detected between the protocols regarding the yield of the soluble DNA measured by RT qPCR ($p<0.01$).

In this study, we used compact pellets of faecal samples from Cabrera's vole as a model, although in other cases, results will possibly be influenced by the species in question, its diet, the sample age and the environmental conditions in which faeces samples are collected (Murphy et al. 2003; Nsubuga et al. 2004; Piggott 2004).

The results of the present study unambiguously show that our new protocol for faecal sample collection, preservation and DNA extraction based on HotSHOT technique is as accurate as techniques using ETOH preservation and gDNA-prepared using commercial kits. The HotSHOT-based protocol probably reduces the chance of human error and/or risks of cross-contamination given that its single-tube protocol minimizes manipulation time. Our methodology is user-friendly, time-saving, economical and non-polluting and thus provides investigators with a new approach that is a noninvasive alternative to current protocols in use in the study of wild animals.

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