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A Widely Applicable Protocol for DNA Isolation from Fecal Samples

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Feces are increasingly used as sources of DNA for genetic and ecological research. This paper describes a new method for isolation of DNA from animal feces. This method combines multiple purification steps, including pretreatment with ethanol and TE, an inhibitor-absorber made of starch, the CTAB method, the phenol–chloroform extraction method, and the guanidinium thiocyanate-silica method. The new method is efficient according to PCR results of 585 fecal samples from 23 species and costs much less than the commercial kits. The protocol can be tailored to the specific purpose of examining different diets of animals and can be performed with routine laboratory reagents.

KEY WORDS: fecal sample; DNA isolation; protocol.

INTRODUCTION

Fecal samples have long served as readily accessible resources for genetic and ecological studies of wild animals (Constable *et al*., 1995; Höss *et al*., 1992; Kohn and Wayne, 1997). From the day researchers recognized that abundant information could be found in the feces of their elusive study animals, efforts have been made to promote appropriate laboratory techniques. Several methods have been proposed to extract DNA from feces, such as the guanidinium thiocyanate-silica method (Eggert *et al*., 2003; Frantz *et al*., 2003; Höss and Pääbo, 1993; Reed *et al*., 1997; Taberlet *et al*., 1996), an aqueous two-phase system method (Lantz *et al*., 1996), a phenol–chloroform method (Ernest *et al*., 2000), the Chelex

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100 method (Walsh *et al*., 1991; Reed *et al*., 1997), and a washing technique (Machiels *et al*., 2000). The application of these methods is limited, however, especially for herbivorous species. The present methods for DNA isolation from feces have various limitations, such as the presence of inhibitors, the amount of feces used, and the requirement of preservation fluids or the use of fresh feces (Machiels *et al*., 2000). The most popular choice is a commercial kit produced by Qiagen (QIAamp DNA Stool Mini Kit), which actually employs a shallow lysis by a proprietary buffer, an inhibitor–absorber, and a silica gel membrane to remove inhibitors from the DNA solution (Li *et al*., 2003). The kit method is efficient and convenient but is comparatively expensive, and it sometimes fails in samples from herbivorous animals in our study. Here, we introduce a new method that can be applied with routine laboratory components and instruments and can be tailored to animals with different feeding habits.

MATERIALS AND METHODS

DNA Isolation of Fecal Samples

The 585 fecal samples, representing 23 species, were obtained from the wild, zoos, and hospitals (Table I). We collected approximately 20 g of fecal pellet from every sample, with disposable plastic gloves, and stored this mass in 95% ethanol using a polypropylene bottle at room temperature. The storage time ranged from 1 day to 23 months.

Fecal DNA was extracted using the following reagents and protocol, beginning with 1–1.5 g of feces weighed into a 15 mL centrifuge tube. The feces were vortexed and washed using 5 mL ethanol, then centrifuged (4000 $\times$ g, 2 min) to pellet the fecal particle, and the supernatant was discarded. The washing step was repeated once using 5 mL TE (10 mM Tris, 1 mM EDTA, pH 8). Three milliliter TNE (10 m mol/L Tris-Cl, 0.5% SDS, 1 m mol/L $CaCl_2$) and 50 μL Proteinase K (20 mg/mL) were added to the centrifuge tube, and the whole was incubated at 55°C for 1–2 h. Subsequently, the lysate was centrifuged (4000 $\times$ g, 1 min) to pellet the fecal particle. The supernatant was then transferred into a new 15 mL centrifuge tube containing 3 g potato starch (Sigma). The tube was continuously vortexed for 1 min to suspend the starch completely, and the suspension was incubated for 1 min at room temperature. The starch tube was centrifuged (8000 $\times$ g, 3 min) to pellet the starch. Then 600 μL supernatant was pipetted into a new 2 mL centrifuge tube, to which 150 μL NaCl solution (3.5 mol/L) and 250 μL CTAB solution (0.7 M NaCl, 10% cetyl trimethyl-ammonium bromide, CTAB) were added, followed by incubation at 70°C for 10 min. The mixture was extracted twice using an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), then the supernatant was transferred to a new tube. An equal volume of binding buffer (4 M guanidine hydrochloride, 1 M potassium acetate, pH 5.5)

Table I. Species Sampled in This Study

Species	No. samples	Successful amplifications[a]
Giant panda, *Ailuropoda melanoleuca*	120	120 (100)
Brown bear, *Ursus arctos*	2	2
Red panda, *Aliurus fulgens*	72	70 (97.2)
Leopard cat, *Prionailurus bengalensis*	1	1
Gray snub-nosed monkey, *Rhinopithecus bieti*	153	129 (84.3)
Golden snub-nosed monkey, *Rhinopithecus roxellana*	4	4
David's macaque, *Macaca thibetana*	2	2
Hainan black crested gibbon, *Hytobates hainanus*	21	21 (100)
Human, *Homo sapiens*	20	20 (100)
Asian elephant, *Elephas maximus*	134	120 (89.6)
Wild camel, *Camelus ferus*	1	1
Sika deer, *Cervus nippon*	2	2
Red deer, *Cervus elaphus*	1	1
Fallow deer, *Dama dama*	1	1
Tufted deer, *Elaphodus cephalophus*	1	1
Elk deer, *Elapurus davidianus*	25	25 (100)
Takin, *Budorcas taxicolor*	5	5
Bharal, *Pseudois nayaur*	1	1
Chinese goral, *Naemorhedus caudatus*	1	1
Tibetan gazelle, *Procpra picticaudata*	11	11 (100)
Przewalski's gazelle, *Procapra przewalskii*	5	5
Przewalski's wild horse, *Equus caballus*	1	1
Porcupine, *Hystrix brachyura*	1	1
Total	585	545 (92.9)

[a]Amplification in PCR of mtDNA cytochrome *b* with pairs L14724 and H15149. The values in parentheses are percentages.

was added to the tube, mixed gently, and applied to a spin column (from EZNA Cycle-pure kit, Omega; loading Ultrafree-MC 30000 filter membrane, Millipore), then loaded in a 2 mL microcentrifuge tube and centrifuged at 6000 $\times$ g for 30 s. Subsequently, the filter membrane was washed twice by centrifuge (8000 $\times$ g, 1 min) using 750 μL 75% ethanol. The DNA was eluted with 200 μL TE, and 50 μg/mL RNase was added.

PCR and Sequencing Analysis

Ten microliters of the extracted product was run on agarose gel, stained with ethidium bromide, and examined under UV light. In the PCR reactions, partial sequences (425 bp) of mitochondrial DNA cytochrome *b* were amplified with primers L14724 (5′-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3′) and H15149 (5′-AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3′) (Irwin *et al*., 1991). Polymerase chain reaction (PCR) was performed in 50 μL reactions using 1–5 μL template DNA, 1 μM of each primer, each dNTP at 1 mM, 2.5 mM $MgCl_2$, 1 $\times$ PCR buffer, and 1 U *Taq* DNA polymerase (Takara). In all

reactions, BSA (bovine serum albumin 0.5 μg/μL) was added to the reaction mix. DNA was amplified for 35 cycles of 30 s at 95°C, 40 s at optimum annealing temperature, and 1 min at 72°C, preceded by 5 min at 95°C followed by 10 min at 72°C. The amplified products were detected on a 2% EB-agarose gel and examined under UV light to confirm the success of the amplification procedure. The fecal DNA with positive bands could be used to amplify various gene fragments of mtDNA, including the 12S rRNA gene fragment (405 bp) with universal primers L1091 (5′-AAA CTG GGA TTA GAT ACC CCA CTA T-3′) and H1478 (5′-GAG GGT GAC GGG CGG TGT GT-3′) (Kocher *et al*., 1989), and control region fragments (420–550 bp in various species, primers and sequences not provided). To confirm the reliability of the DNA isolation method, we analyzed the sequences of the amplification products. The PCR products were purified by a Cycle-pure kit (Omega) and sequenced using an ABI Prism 377 Genetic analyzer. The authenticity of the sequences was confirmed by Blast in GenBank or by comparison with published homologous sequences of target animals.

Also, the multiple microsatellite loci were amplified using the fecal DNA of giant panda and Asian elephant that could amplify the 425 bp cytochrome gene *b*. Twelve microsatellite loci of giant panda (Ame-μ005, Ame-μ010, Ame-μ010, Ame-μ011, Ame-μ013, Ame-μ015, Ame-μ016, Ame-μ019, Ame-μ024, Ame-μ025, Ame-μ026, Ame-μ027; Lu *et al*., 2001) and five loci of Asian elephant (EMX-1, EMX-2, EMX-3, EMX-4, EMX-5; Fernando *et al*., 2001) were amplified using fluorescently labeled primers (FAM, HEX, or TET). Amplifications were performed in 20 μL reaction volumes using HotStar *Taq* Master Mix Kit (Qiagen) according to the manufacturer's manual. The PCR products were visualized on a polyacrylamide gel and sequenced using an ABI Prism 377 DNA sequencer with GS350 Tamara marker. All gels were analyzed using Genescan 2.0 software.

To detect the background DNA from the extracted product, such as microbial DNA, amplifications were executed for the 16S rRNA gene of eubacteria using primers pA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and pH (5′-TAC CTT GTT ACG ACT T-3′) (Edwards *et al*., 1989) with the same reaction system of mtDNA gene.

During the experiments, contamination was monitored by a negative extraction control (mock extraction submitted to PCR) per extraction and a negative PCR control (PCR without extract).

RESULTS

Although there were failures, most fecal DNA showed an obvious band that indicated the existence of high quality DNA (Fig. 1). In almost all samples, amplifications of mtDNA sequences were successful (Table I, Fig. 2). Among the various species, the percentage of positive amplification with primer L14724/H15149 ranged from 84.3 to 100% (Table I) in a multisample group ($n > 10$), which

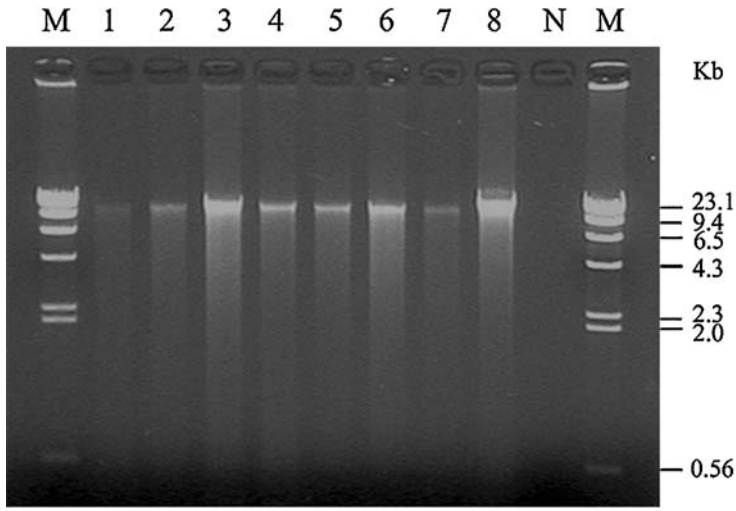

Fig. 1. Fecal DNA from eight Asian elephant (*Elephas maximus*) fecal samples detected on a 1.0% EB-agarose gel. Lane N, negative control. Lane M, λ-*Hin*dIII digested DNA ladder.

may result from the inconsistent quality of fecal samples collected in the wild. When fecal DNA samples were amplified for 12S rRNA gene or control region, the samples with positive cytochrome *b* band all provided positive PCR products (data not provided). In each fecal DNA sample with positive mtDNA amplification

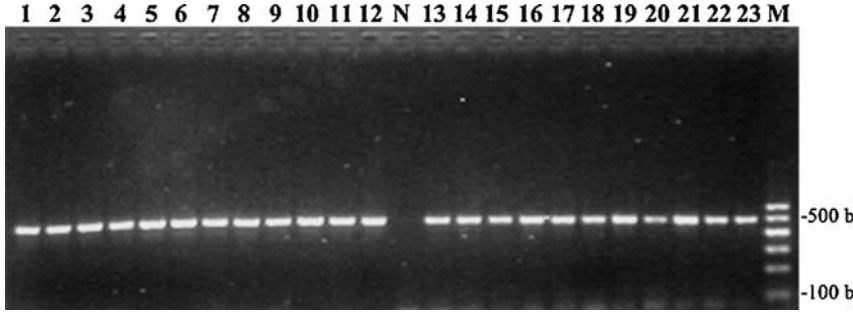

Fig. 2. Amplified products of mtDNA cytochrome *b* gene from fecal DNA of different animals using primers L14724 and H15149, detected on a 2.0% EB-agarose gel. Lanes: 1, giant panda; 2, brown bear; 3, red panda; 4, leopard cat; 5, gray snub-nosed monkey; 6, golden snub-nosed monkey; 7, David's macaque; 8, Hainan black crested gibbon; 9, human; 10, Asian elephant; 11, wild camel; 12, Sika deer; 13, red deer; 14, fallow deer; 15, tufted deer; 16, elk deer; 17, takin; 18, bharal; 19, Chinese goral; 20, Tibetan gazelle; 21, Przewalski's gazelle; 22, Przewalski's wild horse; 23, porcupine; M, 100 bp DNA ladder; N, negative control.

Table II. Success of Microsatellite Amplification in Two Species

Species	No. samples (zoo/wild)	Success (successful PCR/total PCR)		
		All	Zoo	Wild
Giant panda, *Ailuropoda melanoleuca*	96 (32/64)	72.5% (835/1152)	97.1% (373/384)	60.2% (462/768)
Asian elephant, *Elephas maximus*	55 (7/48)	79.6% (219/275)	94.3% (33/35)	77.5% (186/240)

product, at least one PCR product (mtDNA control region, 12S rRNA gene, or cytochrome *b* gene) was sequenced. The sequences of all amplification products ($\geq$ 372 bp) testified that they all accorded with a homologous fragment from the target animal. In the present study, partial sequences have been submitted to GenBank (GenBank accession nos. AY390359-AY390369, AY397657-AY397669, and AY465747).

PCR performance in microsatellite loci varied among species and samples. The 96 giant panda samples (zoo 32, wild 64) and 55 Asian elephant samples (zoo 7, wild 48) were amplified. The total percentage of positive amplification is 72.5% in giant panda and 79.6% in Asian elephant (Table II). There was significant difference, however, between PCR performance of microsatellite in fresh samples (from zoos) and old samples (from wild). The fresh fecal samples showed a high percentage of positive amplification (97.1% in giant panda, 94.3% in Asian elephant) (Table II).

PCR reactions on the 16s rRNA gene of eubacteria with primers pA and pH were all successful in 92 random selected fecal samples from seven species (giant panda 37, red panda 9, Asian elephant 32, gray snub-nosed monkey 4, sika deer 2, takin 3, elk deer 5) (Fig. 3), reflecting that microbial DNA existed widely in fecal DNA and acted as background DNA.

DISCUSSION

Generally, it is quite difficult to assess the availability of DNA extracted by quantifying it using conventional methods (e.g., fluorometry and EB-agarose gel) because (1) the quantity of DNA from the target animal is too low, (2) it could be too degraded for amplification even if large amounts were extracted, and (3) much of the DNA could be from microbes when using scats (Taberlet and Luikart, 1999). In the present study, bands of fecal DNA indicated that a large quantity of DNA had been isolated, which we ascribe to the use of large amounts of feces in the protocol. Therefore, we think the new protocol can isolate enough DNA for

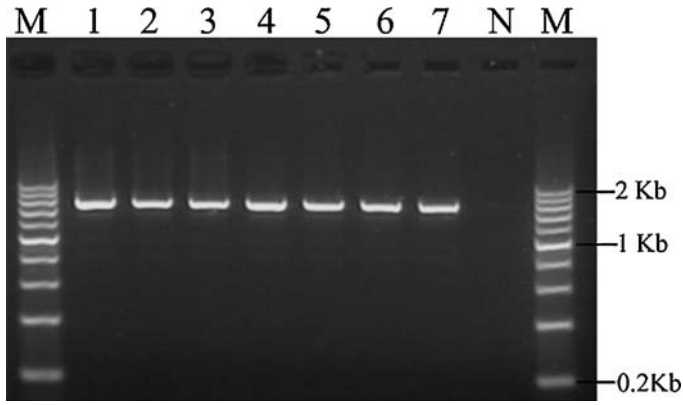

Fig. 3. PCR products of the 16S rRNA gene of eubacteria detected on a 2.0% EB-agarose gel, amplified from fecal DNA of seven species using primers pA and pH. Lanes: 1, giant panda; 2, red panda; 3, Asian elephant; 4, golden snub-nosed monkey; 5, Sika deer; 6, takin; 7, elk deer; N, negative control; M, 200 bp DNA ladder (200 bp ~2 KB).

extensive genetic analysis. To test the validity of the fecal DNA, we employed a published empirical method (Murphy *et al*., 2000) to adapt the polymerase chain reaction to evaluate the availability of fecal DNA. The high-amplification percentage of mitochondrial genes in fecal DNA and subsequent sequencing analysis for PCR products all confirmed that the new method is an efficient and reliable protocol for fecal DNA isolation. Furthermore, our method also obtained a good amplification percentage with microsatellites, especially in the fresh sample (Table II). There was a significant difference, however, between zoo samples and wild samples during the amplification of microsatellites, which indicated that the age of the sample significantly affected the quality of fecal DNA (Table II).

Combining as it does several purification steps, this new method works efficiently with fecal samples from animals with various diets, such as herbivores, carnivores, and omnivorous animals. For samples from some carpophagous animals, an additional extraction after the phenol/chloroform/isoamyl step with chloroform can effectively remove phenolic contaminants. Because polysaccharides have been proven to be strong PCR inhibitors (Monteiro *et al*., 1997), this was an important step to remove them effectively in the DNA isolation process. In the present study, we used a CTAB—phenol/chloroform/isoamyl—extraction step to remove polysaccharides. The addition of the CTAB buffer can be neglected for samples containing less polysaccharide. Reducing the lysis time can also decrease the content of polysaccharides. For a big dollop of feces, it is better to store the

sample in ethanol and vortex strongly to remove small debris, which can be easily transferred by pipets and thoroughly lysed. In the wash step using water and ethanol, soluble substances (e.g., some inhibitors) are removed. In the following step, a rapid lysis with SDS solution is performed, so that only cells from the gut are lysed. In the adsorption step, the potato starches absorb bile salts, bilirubin, and other inhibitors (Bianchini *et al*., 1989; Cheah and Bernstein, 1990, Deuter *et al*., 1995). At the same time, the potato starches reduce the volume of the lysate and facilitate subsequent extraction. To remove the polysaccharide components in the lysate, a CTAB extraction method frequently used for plants has been modified. In the salinizing lysate, NaCl suppressed coprecipitation of polysaccharides and DNA (Fang *et al*., 1992). Polysaccharide was taken up by CTAB rather than DNA and was removed in the phenol:chloroform:isoamyl alcohol (25:24:1) extraction (Jones and Walker, 1963). In the later steps of DNA isolation, we used the spin column (based on a silica gel membrane) to purify and recover the DNA rapidly.

It has been shown that the inhibitor and low DNA amounts blocked genetic study based on fecal DNA (Taberlet and Luikart, 1999). To improve the amplification, endeavors were often focused on these targets in the fecal DNA isolation methods mentioned earlier. The two factors, however, do not compromise each other, so few methods could deal with them effectively at the same time. In the present study, we overcame these barriers by lysing a comparatively large quantity of fecal material (1–1.5 g) and using multiple purification steps.

Compared with previous fecal DNA isolation methods, the new method has several advantages. It accepts the feces preserved in ethanol, even for quite a long time at room temperature (close to 2 years). So field sampling can become more convenient, using ethanol rather than the conventional storage methods such as silica beads and DMSO solution (Taberlet and Luikart, 1999). The method uses crude feces, and no special pretreatment of the sample is necessary. Although the new method takes about 20 min longer than the QIAamp DNA Stool Mini Kit (Qiagen) to extract a single sample, the cost is approximately one-fifth. Also, it is usually hard to achieve satisfactory results using the QIAamp DNA Stool Mini Kit for herbivorous animals. Hence, our method is a better option to conduct molecular scatology research on omnivorous and herbivorous animals, especially when plenty of fecal material is available.

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